Fc receptors: regulatory mechanisms and function in antibody therapy

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Fc receptors: regulatory mechanisms and function in antibody therapy

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**"A healthy person has a thousand wishes,
but a sick person has only one."**

– Indian Proverb

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CHAPTER 1

General introduction

Immunoglobulins, better known as antibodies, are an essential part of the immune system by specifically binding foreign antigens and eliminating them. Antibodies consist of two identical Fab (antigen binding) domains with hypervariable regions at the N-terminus which determine the specificity to an antigen and an constant Fc (crystallizable) domain which mediates the effector functions of antibodies (Fig. 1A). B cells can express antibodies on the surface as antigen receptors (BCR) or excrete them to circulate through the body. In humans, five antibody isotypes are known: IgG, IgA, IgE, IgM, and IgD. These isotypes are distinguished by the C-terminus of the heavy chains, which is not variable. The isotypes differ in function, biological properties, and location within the body. The exposure to antigen, the (inflammatory) environment, and interaction of B cells with T helper cells determines the type of humoral (adaptive) immune response. Antibody functions include direct neutralization of pathogens, recruitment of the complement system to directly lyse pathogens or infected cells, and cellular effector functions like phagocytosis and antibody-dependent cellular cytotoxicity (ADCC) of infected cells (Fig. 2A). As the name suggests, Fc receptors (FcR) bind to the Fc domain of antibodies. Fc receptors are mainly expressed on cells of the innate immune system, and thereby provide a crucial bridge between the adaptive and innate immune system.

Fc receptor family

The human FcR family comprises receptors for all five antibody isotypes: FcγR (IgG), FcαR (IgA), FcεR (IgE), FcμR (IgM), and FcδR (IgD). Most leukocyte Fc receptors are hetero-oligomeric complexes with a unique ligand binding α-chain and one or more accessory subunits (β-, γ-, or ζ-chains). The immunoreceptor tyrosine-based activation motif (ITAM) is essential in triggering biological functions of activating FcR. In addition, some FcR induce inhibitory signals via the immunoreceptor tyrosine-based inhibitory motif (ITIM). For multi-chain FcR (FcγRI, FcγRIIIa), the signaling motif is present in the non-covalently associated FcR γ-chain, whereas for single-chain FcR (FcγRIIa, FcγRIIb), this motif is present in its own cytoplasmic domain[1,2]. The activation of cells via Fc receptor crosslinking is regulated by the amount of antibodies bound to FcR and the ratio of activating and inhibitory Fc receptors on immune cells.

The FcγR family is the most extensively studied. It comprises the four activating receptors FcγRI, FcγRIIa, FcγRIIc, and FcγRIIIa, as well as one inhibitory receptor, FcγRIIb. In addition, FcγRIIIb is a glycophosphatidylinositol (GPI)-linked receptor without any known intrinsic signaling capacity. These receptors are expressed on most myeloid cells, including monocytes, macrophages, and neutrophils (Table 1). FcγRI is the only receptor with a high affinity for monomeric IgG (K_D of 10^{-8}-10^{-9} M), while the other FcγR have a low affinity for monomeric IgG. The different FcγRs bind the IgG subclasses (IgG1, IgG2, IgG3, and IgG4) with different specificities and avidities (Fig. 1B). FcαRI is the human receptor for IgA1 and IgA2 that poorly binds monomeric IgA but binds multimeric IgA avidly. FcγRs, as well as FcαRI, will be further discussed in **chapter 2.**

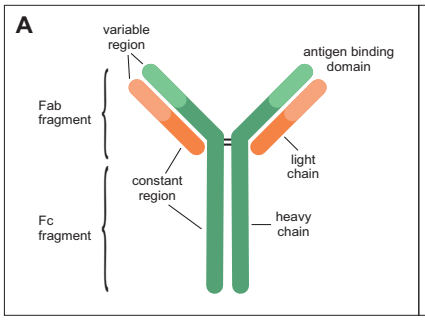
After crosslinking of activating FcR by immune complexes, kinases of the SRC family phosphorylate the tyrosine residues of the ITAM. These phosphotyrosines now provide a

docking site for kinases of the SYK family that bind via their SH2 domains, which in turn become activated[3]. Via the activation of several downstream targets, SYK activation results in activation of the RAS-MAPK pathway, increased intracellular calcium levels, and ultimately activation of NF-κB transcription factors. These signals can activate the immune cell and lead to *e.g.* phagocytosis, oxidative burst, and cytokine release. Crosslinking of the inhibitory FcγRIIb leads to phosphorylation of the ITIM by LYN and this recruits and activates the SH2-containing phosphatases SHIP and SHP1. These phosphatases will then dephosphorylate the kinases downstream of ITAM signaling[4,5]. This counterbalances the activating signaling cascades and can abrogate activating FcR effector functions.

Antibody therapy

Cancer is a group of malignant diseases characterized by abnormal cell growth after cells acquire multiple genetic mutations. Standard care for cancer patients includes surgical removal of the tumor, radiation therapy, and chemotherapy. However, these treatments are not very specific for tumor cells and often healthy tissue is damaged as well. Antibodies are very specific for their target and can make use of the patients' own immune system to eliminate tumor cells. This makes antibodies promising agents for targeted therapy of cancer. Several decades ago, rituximab was the first monoclonal antibody (mAb) to be FDA-approved for the treatment of cancer patients. Since then, many more mAbs have been developed for cancer therapy. Besides targeting cancer cells, mAbs are also used as prophylaxis/treatment of autoimmune and infectious diseases. All clinically approved mAbs are of the IgG isotype, mostly IgG1. This has also boosted the attention for FcγR, as the activation of FcγR on immune cells can trigger eradication of target cells.

The mechanism of action of mAbs involve direct Fab-mediated and indirect Fc-mediated effects (Fig. 2B). Binding of mAbs to FcγR on immune cells exploits the normal cellular effector functions of antibodies. Examples of Fab-mediated anti-tumor effects include blocking the ligand binding site of growth factor receptors (*e.g.* anti-EGFR mAb



B

	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2
FcγRI	+++	-	+++	+++	-	-
FcγRIIa	+++	++/+*	+++	++	-	-
FcγRIIb	+	-	++	+	-	-
FcγRIIIa	++	-/+#	+++	-/++#	-	-
FcγRIIIb	+++	-	+++	-	-	-
FcαRI	-	-	-	-	+++	+++

Figure 1. General antibody structure and IgG and IgA subclass binding to FcγRs and FcαRI. (A) General antibody structure depicting the Fab and Fc fragments and highlighting the variable and constant regions of antibodies. (B) Binding avidity of the different IgG and IgA subclasses to the human FcγRs and FcαRI. Multivalent binding is depicted. * indicates binding for the FcγRIIa-131H/R polymorphism, respectively. # indicates binding for the FcγRIIIa-158F/V polymorphism, respectively. Adapted from Bruhns *et al.*[46] and Wines *et al.*[47].

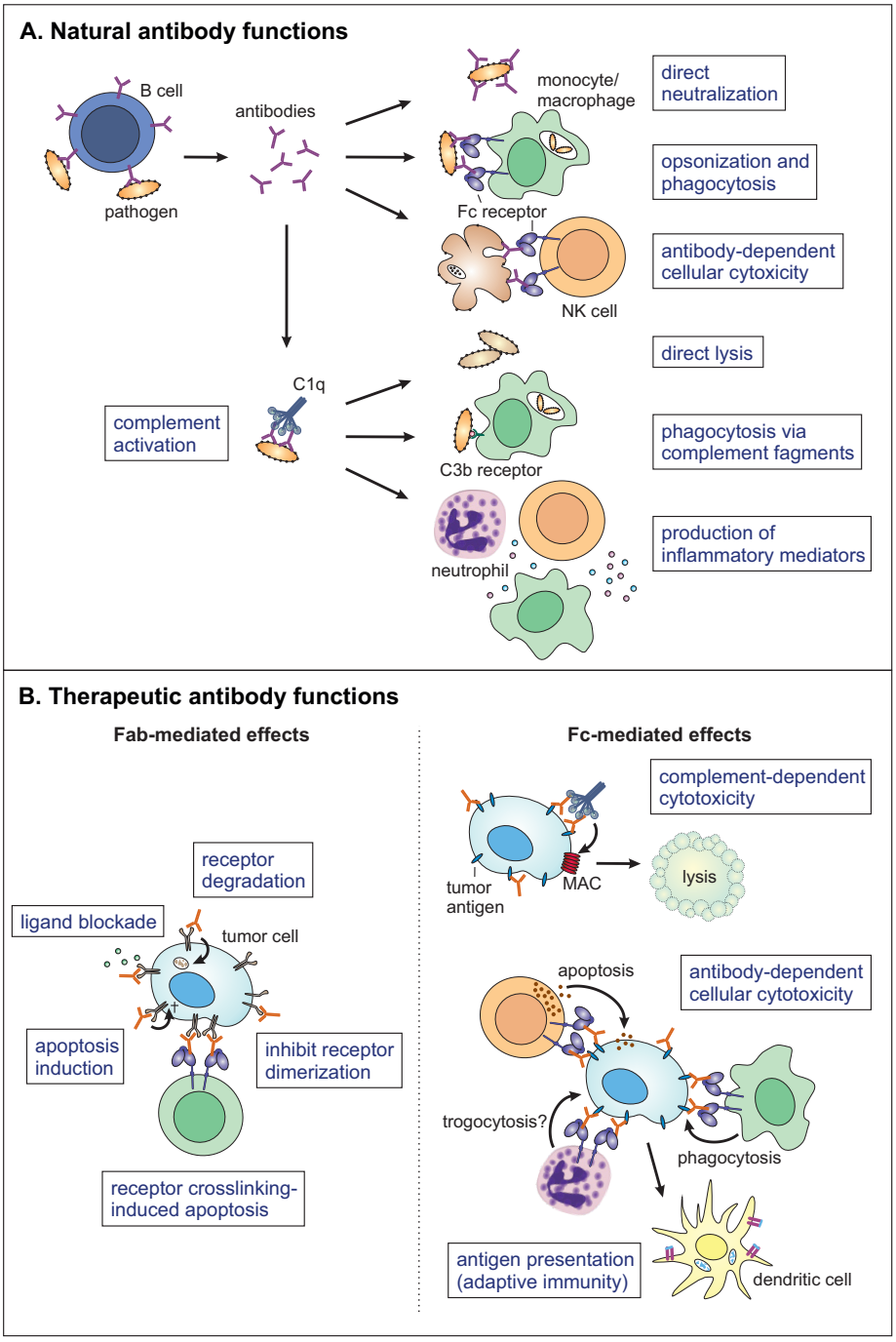

A. Natural antibody functions
B cell
pathogen
antibodies
monocyte/
macrophage
direct
neutralization
opsonization and
phagocytosis
Fc receptor
antibody-dependent
cellular cytoxicity
NK cell
direct lysis
C1q
complement
activation
phagocytosis via
complement fagments
C3b receptor
production of
inflammatory mediators
neutrophil
B. Therapeutic antibody functions
Fab-mediated effects
Fc-mediated effects
complement-dependent
cytotoxicity
tumor
antigen
MAC
lysis
receptor
degradation
ligand blockade
tumor cell
apoptosis
antibody-dependent
cellular cytotoxicity
apoptosis
induction
inhibit receptor
dimerization
trogocytosis?
phagocytosis
receptor crosslinking-
induced apoptosis
antigen presentation
(adaptive immunity)
dendritic cell

Figure 2. Natural and therapeutic antibody effector functions. (A) B cells express antibodies as antigen receptors. Specific B cells can bind to pathogens (such as bacteria, viruses, and fungi) and in response differentiate into antibody-producing plasma cells. These antibodies can opsonize the pathogen and then elicit several effector functions, including direct neutralization, Fc receptor (FcR)-mediated responses like phagocytosis or antibody-dependent cellular cytotoxicity (ADCC). The complement system can also be activated via the classical pathway and lead to elimination of the pathogen. The production of inflammatory mediators like chemokines and cytokines by innate immune cells is stimulated after complement activation but also after phagocytosis or ADCC. (B) Therapeutic monoclonal antibodies (mAb) that bind to antigens expressed on tumor cells mediate anti-tumor effects via various mechanisms of action. Direct effects include blocking the binding of ligands like growth factors, inducing receptor internalization and subsequent degradation, inhibiting the dimerization of a receptor, and inducing apoptotic signals either directly or by crosslinking receptors via FcR-expressing cells. Indirect effects include complement-dependent cytotoxicity (CDC) and ADCC. CDC can directly result in tumor cell lysis by the formation of a membrane attack complex (MAC). mAbs mediate ADCC via different FcR-expressing cells: NK cells induce apoptosis of the tumor cell by release of cytotoxic granules containing perforin and granzymes, monocytes/macrophages phagocytose tumor cells, and neutrophils most likely kill tumor cells by extensive trogocytosis. Dendritic cells can take up dying tumor cells and present tumor antigens to T cells. This can initiate an adaptive immune response against the tumor.

cetuximab), inducing receptor internalization and degradation (*e.g.* anti-HER2 mAb trastuzumab), and directly inducing pro-apoptotic signals (*e.g.* anti-CD20 mAb obinutuzumab)[6-8]. Indirect effects include the activation of the classical complement pathway by IgG leading to complement-dependent cytotoxicity (CDC) and engagement of FcγR leading to ADCC of tumor cells[9-11]. ADCC is mediated by recruiting cytotoxic effector cells like natural killer (NK) cells, monocytes/macrophages, and polymorphonuclear cells (PMNs, mainly consisting of neutrophils).

The relative contribution of these mechanisms of action during mAb therapy remain unclear, although the potency of ADCC and direct Fab-mediated effects is generally accepted. Mouse models have indicated the important role that FcγR play during mAb therapy. For example, in mice lacking the FcR γ-chain or mice deficient in ITAM signaling mAb therapy for CD20-expressing tumors is abrogated[12,13]. In humans, the role of FcγR has been further demonstrated by genetic polymorphisms of FcγR that influence clinical outcome of mAb therapy. For FcγRIIA, FcγRIIIA, and FcγRIIIB single nucleotide polymorphisms (SNPs) have been described and extensively characterized that alter binding towards IgG. One example is the FcγRIIIA-158F/V SNP, of which FcγRIIIA-158V has increased IgG binding compared to FcγRIIIA-158F. Indeed, patients with FcγRIIIA-158VV have better responses and progression-free survival after treatment with mAbs[12,14-16].

Improving antibody therapy

Although mAb therapy can be effective in treating malignancies, patients are rarely cured by mAb therapy alone. Indeed, considerable efforts are made to enhance anti-tumor effects of mAbs. One approach to improve the effectiveness of mAb therapy is using other antibody subclasses or isotypes than IgG1[17]. The other IgG subclasses, IgG2, IgG3, and IgG4, have all been tested for antibody therapy (IgG subclasses reviewed elsewere[18]). IgG3

mAbs induce more efficient lysis of tumor cells compared to IgG1[19], although the half-life is shorter and IgG3 is highly susceptible to cleavage by proteolytic enzymes. IgG2 and IgG4 mAbs induce less Fc-mediated effector functions, but can therefore be efficiently used as blocking or neutralizing mAbs[20,21]. The use of IgE antibodies has been investigated especially for treating solid tumors, since IgE has a long tissue half-life and FcεRI-expressing effector cells are found in the tumor microenvironment[22,23].

IgA is the most studied isotype as alternative for IgG mAb therapy[24-26]. IgA antibodies engage FcαRI and thereby different effector cells are recruited compared to IgG (Table 1). In unfractionated human leukocytes, IgA mAbs induce more tumor cell killing than IgG mAbs[27]. This is mainly mediated by engaging FcαRI on PMNs, since purified PMNs can very efficiently induce cytotoxicity of tumor cells with IgA but hardly with IgG antibodies[28]. Therefore, with IgA mAbs high numbers of effector cells are available for inducing tumor cell killing. Furthermore, there is no inhibitory receptor for IgA and no polymorphisms have been described for FcαRI that might reduce binding of IgA. Together, this has led to extensive preclinical testing of IgA mAbs. Both *in vitro* and *in vivo* effective anti-tumor therapy of IgA mAbs against different tumor antigens has been demonstrated[3]. These data support the introduction of IgA mAbs as alternative into clinical development.

FcR activation is tightly regulated to prevent immune responses by monomeric antibodies. In part, this is accomplished by the low affinity of most of the FcR during homeostasis. Furthermore, several FcR require a danger signal, like chemokines and cytokines, to become activated (primed) at the site of infection/inflammation. This process of receptor activation is termed inside-out signaling and refers to a rapid increase in ligand binding capacity in response to extracellular signals without changing receptor expression. Inside-out signaling has been described for FcγRI, FcγRIIa, and FcαRI and is also well-known for integrins[31-34]. This process might be exploited to improve the clinical outcome of mAb therapy by increasing FcR avidity through cytokine stimulation. Many studies have investigated the combination of mAb therapy with cytokine treatment and cytokine stimulation can indeed enhance mAb therapy[35-39]. However, cytokines induce

Table 1. Human IgG and IgA Fc receptor expression patterns

	IgG receptors					IgA receptor
Name	**FcγRI**	**FcγRIIa**	**FcγRIIb**	**FcγRIIIa**	**FcγRIIIb**	**FcαRI**
CD	CD64	CD32a	CD32b	CD16a	CD16b	CD89
Mono/macro	+	+	+/-	+	-	+
NK cells	-	-	-	+	-	-
Neutrophils	(+)	+	+/-	-	+	+
DC	+	+	+	-	-	*
Basophils	-	+	+	-	+/-	-
Eosinophils	-	+	-	-	-	+
Mast cells	(+)	+	-	-	-	-
B cells	-	-	+	-	-	-
Platelets	-	+	-	-	-	-

* Controversial. + indicates expression; (+), inducable expression; +/-, very low percentages or rare subsets express the receptor; -, no expression. Mono/macro, monocytes and/or macrophages.

many immunological responses, making it difficult to dissect the role of FcR inside-out signaling here. **Chapter 2** further discusses FcR inside-out signaling and the influence on mAb therapy.

Many other approaches to improve mAb therapy are being explored, like increasing C1q binding by altering the mAb glycosylation to enhance CDC, protein engineering to enhance Fc-FcγR interactions, designing antibody-drug conjugates, or generating bispecific antibodies[40]. Further increasing our understanding of mAbs and their receptors will improve mAb therapy efficacy.

Scope of thesis

The research described in this thesis focuses on the regulation and activation of Fc receptors and how this can be used in antibody therapy for cancer. **Chapter 2** serves as a general introduction into the field of Fc receptor inside-out signaling and reviews FcR activation in the context of mAb therapy.

The high affinity receptor for IgG, FcγRI, is saturated with monomeric IgG *in vivo*, but also after isolation or extravasation of immune cells. Because of this, FcγRI has been far less studied than the low affinity FcγR. However, an important role for FcγRI during inflammation, autoimmune responses, and mAb immunotherapy in tumor models has been established[41-44]. It was demonstrated that FcγRI, saturated with prebound IgG, was capable of effective IC binding after cytokine stimulation[31]. This phenomenon was termed inside-out signaling because ligand binding of the receptor is rapidly enhanced after intracellular signaling without altering its expression levels. In **chapter 3**, we study the mechanisms of FcγRI inside-out signaling. The mobility and nanoscale organization of FcγRI in the plasma membrane are measured using high resolution imaging techniques and the role of phosphorylation and actin cytoskeleton investigated. Another mechanism by which FcγRI might increase its IC binding could be a conformational change after cytokine stimulation. This has also been described for integrins, where inside-out signaling is characterized by a large conformational change of the integrin after activation. Several antibodies have been generated that recognize this activated state of integrins[45]. **Chapter 4** describes the generation of novel anti-FcγRI antibodies that are tested for recognizing an active or inactive state of FcγRI. Furthermore, the characteristics of currently available antibodies against FcγRI are investigated.

SNPs of FcγR have been described and extensively characterized for several members of the FcγR family. These SNPs can alter ligand binding and influence the outcome antibody therapy. In **chapter 5** the functional consequences of three nonsynonymous SNPs of FcγRI are studied.

Antibody therapy with IgG mAbs is part of the standard treatment of several forms of cancer. IgG mAbs engage FcγRs on effector cells to initiate ADCC. However, IgG mAb therapy is rarely curative and this has in part been attributed to exhaustion of cellular effector mechanisms. Co-engagement of activating FcγRs with the inhibitory FcγRIIb on monocytes or the non-signaling FcγRIIIb on neutrophils also limits the cytotoxic activity of IgG mAbs. Engaging FcαRI with IgA anti-tumor mAbs results in more efficient activa-

tion of neutrophils compared to IgG and induces different effector functions. In **chapter 6**, we investigate the combination of IgG and IgA mAbs in inducing enhanced cytotoxicity of tumor cells both *in vitro* and *in vivo*.

Many FcR associate with the FcR γ-chain that contains the signaling motifs and stabilizes FcR surface expression. The FcR γ-chain is often referred to as 'common gamma chain', while this name is more often used for the common cytokine receptor gamma chain, leading to confusion in the literature. **Chapter 7** clarifies this confusion by reviewing the difference between the common cytokine receptor gamma chain and the FcR γ-chain. Finally, in **chapter 8** the results and insights obtained during these research projects are discussed and future perspectives outlined.

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CHAPTER 2

Fc receptor inside-out signaling and possible impact on antibody therapy

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Fc receptors (FcR) are expressed on immune cells and bind to the Fc tail of antibodies. This interaction is essential for FcR-mediated signaling and triggering of cellular effector functions. FcR activation is tightly regulated to prevent immune responses by non-antigen-bound antibodies or in the absence of 'danger signals'. FcR activity may be modulated at the plasma membrane via cross-talk with integrins. Additionally, cytokines at the site of infection/inflammation can increase FcR avidity, a process referred to as inside-out signaling. This regulatory mechanism has been described for FcγRI (CD64), FcγRIIa (CD32a), and FcαRI (CD89) and is also well-known for integrins. Key cellular events during inside-out signaling are (de)phosphorylation, clustering, cytoskeleton rearrangements, and conformational changes. The latter can be studied with antibodies that specifically recognize epitopes exposed by the active (high affinity) or inactive (low affinity) state of the FcR. These antibodies are important tools to investigate the role of FcR activation in disease settings. Research on FcR has gained momentum with the rise of monoclonal antibodies (mAb) entering the clinic for the treatment of cancer and other diseases. The clinical outcome of mAb therapy may be improved by increasing FcR avidity by cytokine stimulation.

INTRODUCTION

Fc receptors (FcR) are transmembrane proteins that bind to the Fc tail of antibodies and are widely expressed on leukocytes. Antibody-opsonized pathogens can cross-link FcR, leading to phagocytosis and pathogen killing by phagocytic leukocytes. In the case of antibody-opsonized cells (*e.g.* virus-infected cells, malignant cells), processes termed antibody-dependent cellular cytotoxicity/phagocytosis (ADCC/ADCP) are triggered. Other cellular processes elicited by the interaction of FcR with antibodies include cytokine release, reactive oxygen species (ROS) production, and antigen presentation. These processes emphasize the central role of FcR in bridging the humoral and cellular responses of the immune system. FcR-mediated effector functions are exploited by therapeutic antibodies, including those approved for the treatment of cancer. The importance of FcR in antibody therapy was demonstrated in mouse tumor models with FcR-deficient mice and in patients with genetic variations of FcR[1-3].

This review focusses on the human FcR family that comprises receptors for all five antibody classes: FcγR (IgG), FcαR (IgA), FcεR (IgE), FcμR (IgM), and FcδR (IgD). Most FcR are hetero-oligomeric complexes with a unique ligand-binding α-chain that can associate with accessory subunits (β-, γ-, or ζ-chains). FcR are further classified into activating and inhibitory receptors. Activating FcR require an immunoreceptor tyrosine-based activation motif (ITAM) for their effector functions, whereas inhibitory FcR depend on an immunoreceptor tyrosine-based inhibitory motif (ITIM) for signaling. For multi-chain FcR (FcγRI, FcγRIIIa), the signaling motif is present in the non-covalently associated FcR γ-chain, whereas for single-chain FcR (FcγRIIa, FcγRIIb), this motif is present in its own cytoplasmic domain (Fig. 1A)[4,5]. Receptor cross-linking induces phosphorylation of the ITAM/ITIM, which initiates the signaling cascade leading to activating/inhibitory responses.

Imbalanced immune responses might lead to autoimmune diseases like arthritis and systemic lupus erythematosus (SLE), in which healthy tissues are targeted by the immune system or damaged by inflammation responses[6]. Indeed, neutrophils are very effective in killing opsonized bacteria but also have the greatest capacity to induce tissue damage (*e.g.* by releasing ROS and proteases). Therefore, it is important that FcR activation and signaling are tightly regulated and that FcR are not triggered by non-antigen-bound antibodies, especially since antibodies are abundantly present in the serum (700–1600 mg/dl IgG, 70–400 mg/dl IgA, and 40–230 mg/dl IgM in healthy adults)[7]. In part, FcR regulation is accomplished by the low affinity of most of the FcR during homeostasis. This ensures that only multiple immobilized antibodies on the target surface provide sufficient valency for low affinity FcR to bind the Fc domains and form a stable interaction. In addition, several FcR need a danger signal to become activated, *e.g.* by locally produced cytokines and chemokines. The latter way of receptor activation (or priming) is termed inside-out signaling and is a well-established concept for integrins. Inside-out signaling refers to the rapid increase of ligand binding capacity (or avidity) in response to extracellular signals, without changing receptor expression levels. In this review, we use the term affinity for the strength of individual receptor-ligand bonds (modulated by conformational changes),

valency for the number of these bonds (modulated by receptor mobility and clustering), and avidity for the overall strength of the interaction (combination of affinity and valency).

Integrins are a family of 18 α- and 8 β-subunits that form αβ-heterodimers. For integrins, inside-out signaling induces conformational changes and clustering of integrins in the plasma membrane which lead to avidity modulation. Integrins on circulating leukocytes are generally maintained in an inactive, non-adhesive state. They are activated by cytokines and chemokines at the site of infection/inflammation to facilitate adhesion and transmigration of leukocytes. Integrins in an inactive state adopt a low affinity, bent or 'closed' conformation. Binding of adaptor proteins, like Talin-1, to the intracellular tail of integrins destabilizes the association between the α- and β-subunit. This leads to the separation of the α- and β-subunit cytoplasmic and transmembrane domains, causing the integrin to extend in a switchblade-like motion towards the outside. In this way, an 'extended closed' conformation with an intermediate affinity is obtained. The final step in integrin activation is the opening of the headpiece, leading to an 'extended open' conformation with high affinity[8].

Several antibodies have been identified that recognize the active (extended open), but not the inactive, conformation of specific integrins, *e.g.* CBRM1/5 that recognizes active Mac-1 (CD11b/CD18, αMβ2)[9]. This indicates that neo-epitopes are exposed on integrins after inside-out signaling and these antibodies are extremely helpful to study inside-out signaling of integrins. Besides conformational changes and binding of intracellular proteins, several other mechanisms have been described to be important for the inside-out activation of integrins: (de)phosphorylation of intracellular domains, clustering, and cytoskeleton rearrangements. Even though inside-out activation of integrins is widely studied, many details and differences between types of integrins are still unclear and require further research.

Like for integrins, inside-out control of FcR has been described for FcγRI (CD64), FcγRIIa (CD32a), and FcαRI (CD89), indicating that this is an important regulatory process for FcR. Also for FcR, changes in receptor conformation, clustering, and phosphorylation state have been reported, indicating that integrin and FcR inside-out signaling involve common mechanisms. Here, we will review the mechanisms of inside-out signaling for FcR and discuss how this might be applicable to antibody therapy.

FC GAMMA RECEPTOR INSIDE-OUT SIGNALING

In humans, the FcγR family comprises the four activating receptors FcγRI, FcγRIIa, FcγRIIc, and FcγRIIIa, as well as one inhibitory receptor, FcγRIIb. In addition, the FcγR family includes FcγRIIIb, which is a glycophosphatidylinositol (GPI)-linked receptor without any known intrinsic signaling capacity (Fig. 1A), although it can induce effector functions via *e.g.* FcγRIIa or integrins (discussed below)[10-13]. FcγRI and FcγRIIa are currently the only two FcγR known to be under inside-out control. These receptors are expressed on most myeloid cells, including monocytes, macrophages, and eosinophils.

FcγRIIa is also expressed on platelets, basophils, neutrophils, and Langerhans cells and FcγRI on dendritic cells (DC). FcγRI expression can be upregulated on neutrophils after cytokine stimulation.

FcγRI

FcγRI is the only receptor with a high affinity for monomeric IgG (K_D of 10^{-8}-10^{-9} M) and is the only FcR with three extracellular immunoglobulin (Ig)-like domains. Because of its high affinity, FcγRI is believed to be saturated with circulating monomeric IgG and thus incapable of participating in antibody responses. However, in FcγRI$^{-/-}$ mice antibody-dependent cellular processes like bacterial clearance, phagocytosis, antigen presentation, and cytokine production are impaired[14,15]. Furthermore, bispecific antibodies (bsAb) directly targeting FcγRI induced tumor cell killing and increased antigen presentation by DC[16].

The Fc receptor γ-chain is necessary for ligand binding-induced effector functions of FcγRI like phagocytosis and intracellular signaling. The α-chain of FcγRI itself contributes to phagocytosis, endocytosis and recycling of the receptor, antigen presentation, and IL-6 responses[17,18]. Although the cytoplasmic (CY) domain of FcγRI does not contain any known signaling motifs, there are four serines present in the CY domain that are constitutively phosphorylated[19]. Cross-linking of FcγRI induces a rapid and short-lived dephosphorylation of these serine residues. The phosphatase inhibitor okadaic acid (OA) inhibits this serine dephosphorylation and blocks FcγRI-mediated phagocytosis. Furthermore, OA can indirectly reduce the tyrosine phosphorylation of the γ-chain ITAM after FcγRI cross-linking. These data suggest a model where serine phosphorylation of the FcγRI CY domain prevents early tyrosine phosphorylation events on the associated γ-chain, and only after dephosphorylation of the CY domain full γ-chain signaling is possible[19]. Besides the four serines, there are several threonines and one tyrosine in the CY domain and their (de)phosphorylation could be important in other FcγRI α-chain effector functions.

Since FcγRI has a high affinity for IgG and binding of IgG leads to cellular effector functions, how can the activation of FcγRI be regulated? Firstly, monomeric IgG binding to FcγRI does not lead to intracellular ITAM signaling and FcγRI activation, although it can trigger rapid endocytosis and recycling of FcγRI[20,21]. Secondly, the binding of monomeric IgG to FcγRI probably results in a threshold for immune complex (IC) binding, meaning that only large IC or many smaller complexes can displace monomeric IgG. Finally, it has been proposed that FcγRI is also under inside-out control, indicating that FcγRI can be activated to bind IC more efficiently[22].

Inside-out signaling of FcγRI was first shown in an IL-3-dependent murine pre-B (Ba/F3) cell line that was transduced with FcγRI. Stimulation of these cells with IL-3 resulted in a slight increase in monomeric IgG binding and a strong increase in IC binding. The same results were obtained when FcγRI was preoccupied with monomeric IgG, indicating that monomeric IgG does not interfere with the enhanced binding of IC[22]. Experiments trying to show the release of monomeric IgG to allow IC binding were unsuccessful, thus the mechanism by which IC binding occurs when monomeric IgG is already bound to

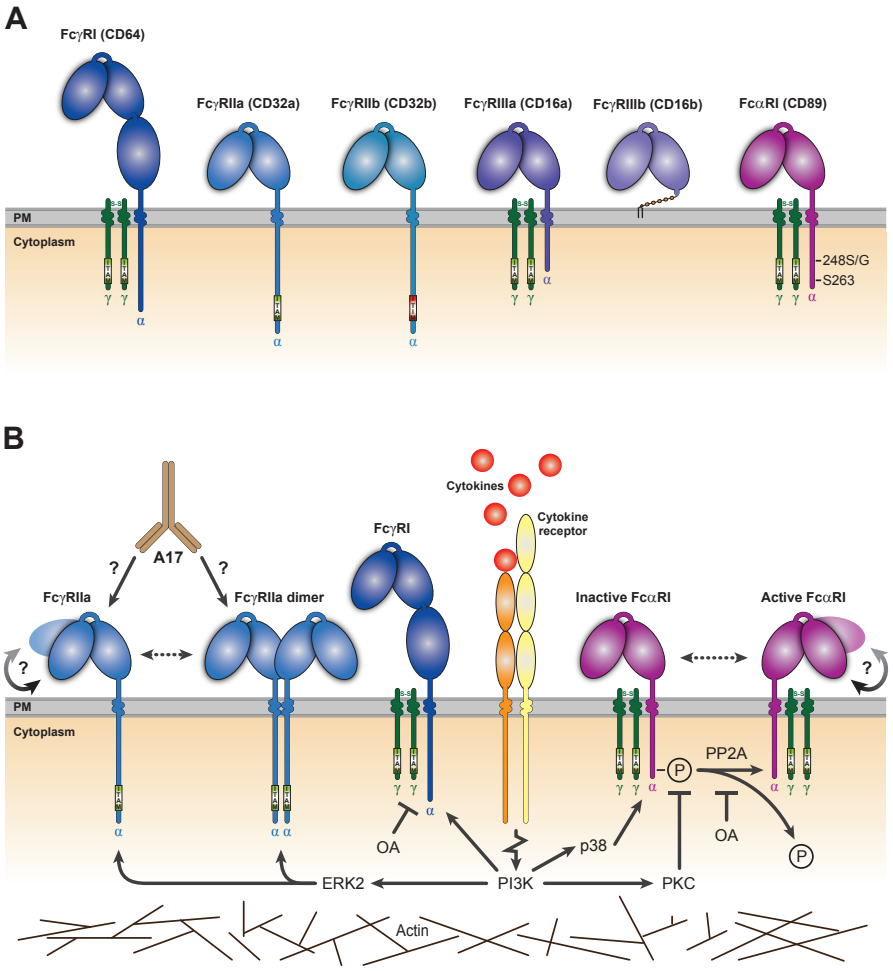

Figure 1. Schematic representation of FcγRI/II/III and FcαRI in inside-out signaling. (A) A selection of human FcR members are depicted: FcγRI, FcγRIIa/b, FcγRIIIa/b, and FcαRI. These FcR have two extracellular Ig-like domains, except for FcγRI that has three Ig-like domains. The N-terminal ectodomain most distal from the plasma membrane (PM) is termed EC1 and the membrane proximal domain is termed EC2 (with the notion that FcγRI has an additional EC3 domain, which is most proximal to the PM). The FcR dimeric γ-chain associates with FcγRI, FcγRIIIa, and FcαRI and contains the ITAM required for signaling. FcγRIIa has an intrinsic ITAM and the inhibitory FγRIIb contains an ITIM. FcγRIIIb is attached to the PM by a glycophosphatidylinositol (GPI)-anchor. In the intracellular domain of FcαRI, the serine at position 263 (S263) is indicated, of which the phosphorylation status controls the active or inactive FcαRI state. The SNP 248S/G is also depicted and is located closer to the PM. (B) As an example, initiation of inside-out signaling is illustrated after binding of a cytokine to its receptor. Triggering of the cytokine receptor results in PI3K activation, which in turn can activate FcαRI via p38 mitogen-activated protein kinase (MAPK) and protein kinase C (PKC). Active PKC together with upregulated PP2A activity leads to dephosphorylation of S263 and FcαRI activation. Possibly, a conformational change is involved (indicated by the rounded arrow) that enhances FcαRI affinity. S263 dephosphorylation can be prevented with okadaic acid (OA), which inhibits PP2A function. PI3K activation also induces FcγRI and FcγRIIa activation, which for FcγRIIa requires ERK2. FcγRI activation is inhibited by

OA via an unkown target. FcγRIIa can exist as a homodimer, a configuration that might be promoted during inside-out signaling, leading to a higher avidity. On the other hand, a conformational change of the FcγRIIa monomer resulting in higher affinity could be mediated by inside-out signaling. The A17 antibody can distinguish the active FcγRIIa from the inactive, but it is unknown which active configuration is recognized. Actin cytoskeleton rearrangments can facilitate the inside-out signaling of these FcR.

FcγRI remains unclear (own unpublished observations). The phosphatase inhibitor OA blocked the increased IC binding after IL-3 stimulation, showing that dephosphorylation of serines or threonines is important for the inside-out activation of FcγRI. Stimulation of primary human monocytes with IFNγ and TNFα also resulted in increased IC binding. Importantly, cytokine stimulation did not alter FcγRI expression on the cell surface, indicating that other mechanisms are regulating FcγRI avidity for IC[22].

Several proteins that interact with the CY domain of FcγRI might be involved in the inside-out signaling of FcγRI, since intracellular protein binding is a crucial step in integrin activation. Periplakin binds to the FcγRI CY domain and this interaction reduced the ligand binding affinity of FcγRI[23,24]. Therefore, periplakin has been postulated to keep FcγRI in an inactive state under normal conditions. However, preventing binding of periplaking using FcγRI with a mutated CY domain did not affect inside-out signaling (own unpublished observations). Two other proteins bind FcγRI intracellularly, filamin A and 4.1G, although their role in FcγRI inside-out signaling has not yet been studied[25,26]. Interestingly, all three proteins interact with the cytoskeleton, indicating that FcγRI avidity is tightly linked to and possibly regulated by the cytoskeleton.

The fact that monomeric IgG binding is slightly increased after inside-out signaling of FcγRI indicates a possible conformational change in the receptor. A recent study demonstrated that the third ectodomain (EC3) of FcγRI undergoes a conformational change after ligand binding[27], supporting this hypothesis. Currently, we are trying to generate antibodies specific for a putative 'active' or 'inactive' conformation of FcγRI using a similar approach as for FcαRI (see below). Antibodies recognizing a specific conformation of FcγRI might provide evidence for a conformational change after FcγRI inside-out activation. We are also studying the nanoscale dynamics and clustering of FcγRI, and indeed these processes are altered after inside-out signaling (Brandsma *et al.*, manuscript submitted). These and future experiments will unravel the molecular changes that underlie FcγRI inside-out activation.

FcγRIIa

FcγRIIa is a low affinity receptor with two extracellular Ig-like domains and an ITAM motif in its intracellular domain. A single nucleotide polymorphism (SNP) at position 131 from histidine to arginine (131H/R) of FcγRIIa has underlined the importance of this FcR in antibody therapy. FcγRIIa-H131 has a higher affinity for human IgG2 (hIgG2) and homozygosity for this allele is associated with increased response rates and progression-free survival during rituximab treatment[28]. In contrast, the FcγRIIa-R131 allele is associated with an increased risk of autoimmune diseases and bacterial infections[29].

More than two decades ago it was already suggested that FcγRIIa could be activated by several enzymes. Treatment of monocytes with proteolytic enzymes, including serine proteases, increased the FcγRIIa binding avidity for both monomeric IgG and IC[30,31]. The desialylating enzyme neuraminidase also enhanced the interaction between FcγRIIa and mouse IgG1 (mIgG1), leading to increased FcγRIIa-mediated effector functions (*e.g.* cytokine secretion by monocytes)[32]. Serine protease activity actually seems essential for FcγRIIa-mediated ligand binding and the proteolytic events most likely occur intracellular because these enzymes have to be endocytosed by monocytes to activate FcγRIIa[31]. These data led to the conclusion that FcγRIIa activity can be modulated by several proteases, which are released by neutrophils at the site of infection/inflammation to only locally activate FcγRIIa on monocytes.

Besides proteases, FcγRIIa can be activated by cytokines. IC binding to FcγRIIa on eosinophils was enhanced by GM-CSF, IL-5, and IL-3, without changing FcγRIIa expression levels[33]. These data provide a strong indication for inside-out activation of this receptor. The cytokine-induced IC binding was shown to be a temporal effect, since it was maximal after 35 minutes, but binding slowly declined afterwards. Furthermore, the binding of Mac-1 increased after cytokine stimulation, although the expression of Mac-1 also slightly increased, possibly indicating a correlation between IC binding and Mac-1 activation[33]. After cytokine stimulation, phosphatidylinositol 3-OH kinase (PI3K) becomes activated and can subsequently phosphorylate downstream signaling molecules, resulting in the inside-out activation of FcR. For FcγRIIa, stimulation of eosinophils with IL-5 to activate FcγRIIa required the mitogen-activated protein kinase (MAPK) ERK2[34]. The other signaling molecules involved in the inside-out activation of FcγRIIa remain to be elucidated.

Neutrophils express 10 times more FcγRIIIb than FcγRIIa on their cell surface[35]. In resting conditions, FcγRIIIb seems to be the main IC binding receptor on neutrophils, although this receptor cannot induce intracellular signaling. However, when FcγRIIa becomes activated via inside-out signaling, which in neutrophils could be achieved by fMLP stimulation, the binding capacity of FcγRIIa increased[36]. This leads to increased IC binding to FcγRIIa compared to FcγRIIIb, and therefore increased FcγRIIa-mediated signaling and effector functions. With neutrophils from a donor lacking FcγRIIIb expression, but normal expression of the other FcγR, it was shown that FcγRIIa indeed is in a low-avidity state in resting neutrophils which could be converted to a higher avidity state after neutrophil activation[37]. Next to cytokines/chemokines (or proteases activity) as activation stimuli, FcγRIIIb cross-linking was shown to induce FcγRIIa activation[11]. Multivalent ligation of FcγRIIIb on neutrophils also resulted in an increased F-actin content, promoting actin assembly and providing an activation signal for other neutrophil effector functions, including enhanced phagocytosis by FcγRIIa[10]. These data are in line with the observation that treatment of eosinophils with the actin polymerization inhibitor cytochalasin D strongly reduced IC binding after cytokine stimulation and imply a role for the actin cytoskeleton in the inside-out activation of FcγRIIa[33].

The exact mechanism for inside-out signaling of FcγRIIa is still unknown. As

mentioned before, the overall FcγRIIa expression is not altered between unprimed and activated granulocytes, as measured with the FcγRIIa-specific antibody IV.3. Importantly, an antibody (A17) has been described that specifically recognizes FcγRIIa only on activated granulocytes[38-40]. This strongly suggests that there is a conformational change in FcγRIIa after activation, leading to exposure of an epitope that can now be recognized by A17.

FcγRIIa activation status of asthmatic patients was measured using the A17 antibody. Asthmatic patients have a chronic airway inflammation, which is characterized by the presence of cytokines that can stimulate immune cells like eosinophils and neutrophils. Eosinophils from patients with asthma were already primed, expressing active FcγRIIa, compared to healthy controls. Neutrophils showed no difference in A17 binding at baseline, but after fMLP stimulation neutrophils from asthmatic patients bound A17 more efficiently compared to the healthy controls[40]. In a pilot study with 5 patients with infections or autoimmune diseases a similar observation was made: neutrophils from these patients showed a higher FcγRIIa-dependent IC binding compared to controls[36]. Together, these data indicate that in autoimmune diseases or during infection FcγRIIa is already in an active and high-affinity state. This can lead to increased IC binding and enhanced FcγRIIa-mediated effector functions. The A17 antibody might be a useful diagnostic tool to measure the activation status of FcγRIIa and correlate this to disease severity.

Based on the structural data of FcγRIIa, a potential mechanism underlying the conformational change can be postulated. The crystallization process of FcγRIIa-R131 identified dimers of FcγRIIa that might represent a beneficial condition for ligand binding. The dimer structure exposes an increased ligand binding surface and forces the Fc-binding sites to be parallel to the plasma membrane. This would make the IgG binding site more accessible compared to an FcγRIIa monomer. Dimerization of FcγRIIa might also juxtapose the ITAMs and signaling machinery for further downstream signaling[41]. Besides crystallography, mutagenesis analysis showed FcγRIIa to form non-covalent homodimers on the cell surface. Interestingly, one mutation (S129P) in FcγRIIa-R131 was able to block dimerization, while ligand binding remained intact, indicating that also FcγRIIa monomers are able to bind IgG[42]. However, in this study FcγRIIa-transduced cells were used, which are thought to only express the activated state of the receptor, and dimerization was not measured in the context of inside-out signaling (*i.e.* before and after cytokine stimulation). Dimerization does seem to be needed for optimal signal transduction, because the mutated FcγRIIa showed diminished receptor phosphorylation and calcium mobilization[42]. More recently, the crystal structure of the FcγRIIa-H131 dimer has been resolved, which forms a markedly distinct dimer compared to FcγRIIa-R13143. Modeling of these two FcγRII dimer structures in complex with IgG revealed that the FcγRIIa-R131 dimer is sterically not capable of binding IgG, while the FcγRIIa-H131 dimer can bind one or two IgG without any steric hindrance. These data led to the hypothesis that FcγRIIa can exist as a pre-assembled inactive dimer (represented by the 'low responder' FcγRIIa-R131), which can be converted into an active signaling competent dimer (represented by the 'high responder' FcγRIIa-H131)[43]. We postulate that this conversion is induced by

inside-out signaling, leading to FcγRIIa dimers that can efficiently bind IgG and have their ITAMs juxtaposed for optimal signaling.

In conclusion, FcγRIIa is under inside-out control and can be activated by both proteases and cytokines, which results in increased IC binding and effector functions. A conformational change, possibly induced by (altered) homodimerization, is part of the mechanism of activation, since a neo-epitope recognized by A17 is only exposed after activation (Fig. 1B). The cytoskeleton seems to be involved as well, but the exact mechanism underlying activation of FcγRIIa remains to be elucidated. Identifying this mechanism by studying (de)phosphorylation events, dimerization and/or further clustering and protein binding to the intracellular domain of FcγRIIa will lead to a better insight in the activation of FcγRIIa. Ultimately, this might provide us with ways to enhance or reduce FcγRIIa activation in a clinical setting, since the state of activation is correlated with infections and autoimmune diseases.

FC ALPHA RECEPTOR INSIDE-OUT SIGNALING

Although IgA is best known in its dimeric secretory form to neutralize pathogens at mucosal sites, a large part is present in the serum in a monomeric form. Monomeric IgA in complex with antigen can elicit immune effector functions through engagement of FcαRI on myeloid cells. FcαRI is expressed on monocytes, macrophages, neutrophils, eosinophils, Kupffer cells, alveolar macrophages, and some DC subsets[44]. Like several other FcR, the FcαRI α-chain contains two extracellular Ig-like domains and associates with the Fc receptor γ-chain that contains the ITAM necessary for signaling (Fig. 1A). Like all FcR, FcαRI is a member of the immunoglobulin (Ig) gene superfamily, but is only distantly related (~20% homology) to other FcR and its gene locus resides on a distinct chromosome[45]. Interestingly, there is no homologue of FcαRI in mice, which appear to have lost this gene during evolution[46].

FcαRI inside-out signaling

The first hint of a cytokine-induced activating mechanism for FcαRI came after stimulating neutrophils with GM-CSF or G-CSF, which induced IgA-mediated phagocytosis without changing receptor expression[47]. A similar effect was observed by stimulating eosinophils with IL-4 or IL-5, both enhancing the avidity of FcαRI for IgA-coated beads (IgA-IC), albeit with different kinetics[48]. Further research using Ba/F3 cells transfected with FcαRI demonstrated that IL-3 stimulation can also increase FcαRI binding to IgA-IC[34]. Several intracellular signaling events after cytokine stimulation are important for FcαRI inside-out signaling, including activation of P13K and the recruitment of PI(3,4,5)P3-dependent kinases. These kinases can subsequently phosphorylate and activate downstream kinases like protein kinase C (PKC), although the exact PKC is still unknown. In addition, the (PI3K)-p38 MAPK signaling pathway is involved in FcαRI inside-out activation[34,49]. Interestingly, cytokine-induced inside-out activation of FcαRI is not dependent on the association with the FcR γ-chain[34].

The intracellular domain of FcαRI is devoid of any known signaling motifs. It does contain several threonines and serines on which (de)phosphorylation could modulate receptor avidity. Inside-out signaling of integrins often involves (de)phosphorylation and this might also apply for FcαRI. Indeed, the phosphorylation status of serine 263 (S263) is crucial for the activation of FcαRI[50]. Deletion of the FcαRI intracellular domain resulted in a constitutively active receptor, which could efficiently bind IgA-IC without prior cytokine stimulation. This observation was followed up by using FcαRI mutants where S263 was replaced by either an alanine (S263A) or aspartic acid (S263D), representing the dephosphorylated or phosphorylated state, respectively. Binding experiments showed that the FcαRI-S263A has a high IgA binding capacity whereas FcαRI-S263D has a low IgA binding capacity (Fig. 2A)[50]. In accordance with this, protein phosphatase 2A (PP2A) becomes activated upon cytokine stimulation and dephosphorylates S263 to switch FcαRI into a 'high binding' state[51]. Treatment with OA, an inhibitor of PP2A, also blocked FcαRI inside-out signaling. Under non-stimulating conditions, S263 is constitutively phosphorylated by a yet unknown kinase, which counteracts PP2A and keeps FcαRI in a 'low binding' state. The activity of this kinase is likely inhibited after cytokine-induced inside-out activation of FcαRI. Within our laboratory, we are currently investigating which PKC and other downstream molecule(s), including the kinase that phosphorylates S263, are involved in the inside-out signaling of FcαRI.

Cytokine stimulation influences many cellular processes, including cytoskeleton dynamics, and changes in the actin cytoskeleton organization can modulate receptor activity. The cytoskeleton may therefore contribute to FcαRI activation by stabilizing the active receptor, as demonstrated for integrins[52]. The cytokine-induced binding to IgA-IC of wild type (WT) FcαRI as well as the constitutively active FcαRI-S263A was abrogated after cytochalasin D treatment[50]. This suggests that the cytoskeleton is indeed required for maintaining FcαRI in the active state. Unpublished work from our group using FRAP (fluorescence recovery after photobleaching) techniques indicates that the lateral mobility of FcαRI also changes upon cytokine stimulation.

As mentioned before, asthmatic patients have a chronic infection in the airways with infiltration of neutrophils and eosinophils. In this mucosal environment, IgA is abundantly present to stimulate FcαRI on these cells. Therefore, it was investigated whether the FcαRI activation state would be altered on immune cells from asthmatic patients compared to healthy controls[53]. Eosinophils isolated from the blood of allergic asthmatics bound IgA-IC efficiently without additional cytokine stimulation. This phenomenon correlated with an increased basal activity of PI3K in these eosinophils, indeed indicating an activated FcαRI through the inside-out signaling pathway. In addition, stimulation of eosinophils from allergic asthmatics with the proinflammatory cytokine TNFα strongly enhanced binding of IgA-IC. This is striking, since TNFα stimulation barely increased the binding capacity of FcαRI on eosinophils from healthy donors for IgA-IC. Additional experiments led to a model where TNFα stimulation amplifies the already activated PI3K signaling pathway in eosinophils form allergic asthmatics[53]. These data illustrate that regulation of FcαRI avidity through inside-out signaling can be altered in a disease setting.

One non-synonymous SNP in the FcαRI cytoplasmic tail involves the change of ser-

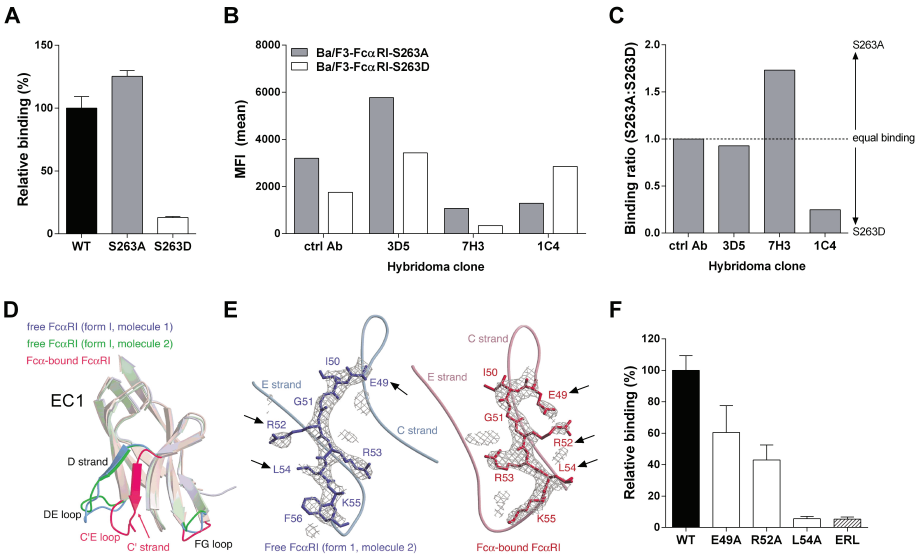

Figure 2. Conformational changes in FcαRI influence binding to IgA. (A) Binding of FcαRI-expressing Ba/F3 cells to immobilized IgA. Ba/F3 cells were transduced with either FcαRI-wild type (WT), FcαRI-S263A, or FcαRI-S263D. The percentage of cells that remained bound after 6 washing steps is shown (relative to FcαRI-WT). Data are represented as mean ± SD. (B-C) Antibodies were raised against FcαRI by immunizing female C57BL/6 mice (6–8 weeks of age, Janvier) with ITi cells (ImmunoTherapy Immunization cells) expressing FcαRI. The mice were boosted twice with the same cells to increase antibody titers. The spleen from the mouse with the highest antibody titer was harvested and splenocytes were fused with SP2.0 myeloma cells. The fusion was performed using the Hybridoma Clonig Kit (Stemcell technologies) according to the manufacturer's protocol. Two weeks after the fusion the antibodies in the resulting hybridoma supernatants were screened by flowcytometry for binding to FcαRI-S263A or FcαRI-S263D Ba/F3 cells. Anti-FcαRI (clone A59) was used as a control antibody (ctrl Ab). (B) Mean fluorescence intensity of antibody binding to the FcαRI mutants. The ctrl Ab indicates a higher expression of FcαRI on Ba/F3 cells for the FcαRI-S263A mutant compared to the FcαRI-S263D mutant. Hybridoma clone 3D5 shows a similar binding pattern as the ctrl Ab, whereas clone 7H3 preferentially binds to FcαRI-S263A and clone 1C4 to FcαRI-S263D. (C) Binding ratio of the hybridoma antibodies to FcαRI-S263A and FcαRI-S263D is represented (relative to the binding ratio of the ctrl Ab). A ratio of 1 represents equal binding to FcαRI-S263A and FcαRI-S263D, a ratio higher than 1 represents preferential binding to FcαRI-S263A, and a ratio lower than 1 represents preferential binding to FcαRI-S263D (as indicated by the arrows). (D) Superposition of the FcαRI EC1 from independently determined crystal structures of free FcαRI (blue and green) and FcαRI in complex with IgA Fc-tail (red). Regions that differ between the conformations are highlighted with the indicated colors. (E) Electron density maps showing the D strands in EC1 of free and IgA Fc-tail bound FcαRI, with ball-and-stick models of the altered regions superimposed. Three main residues that are involved in this conformational change are indicated by the arrows. Figure adapted from Herr *et al.*[57]. (F) Binding of FcαRI-expressing Ba/F3 cells to immobilized IgA. Ba/F3 cells were transduced with either FcαRI-WT or FcαRI mutants (E49A, R52A, L54A, or the triple mutant ERL). The percentage of cells that remained bound after 6 washing steps is shown (relative to FcαRI-WT). Data are represented as mean ± SD.

ine 248 to glycine (248S/G). In individuals homozygous for the G248 allele, more IL-6 was released after IgA-mediated cross-linking of FcαRI on neutrophils. FcαRI-G248 also induced cytokine release in the absence of the FcR γ-chain and this polymorphic variant is often present in SLE patients[54]. This SNP might affect the avidity of FcαRI for IgA and

by-pass the need for FcαRI activation by cytokine-induced inside-out signaling.

The molecular basis of the FcαRI-IgA interaction mediated by the extracellular domains is clearly distinct from other FcR and their ligands. For instance, the binding site for IgA on FcαRI is located in the N-terminal ectodomain (EC1), in contrast to other FcR where ligand binding occurs at the second ectodomain (EC2). The manner of interaction also differs, since the EC1 of FcαRI binds to the interface of the IgA CH2/CH3 domains, and not, like for FcγR, to the IgG N-terminal part of CH2 near the hinge region[55]. In addition, this allows a stoichiometry where IgA can bind two FcαRI simultaneously[56]. The switch from monovalent binding, one IgA molecule binding one FcαRI, to bivalent binding, one IgA molecule binding two FcαRI, could be responsible for the increased FcαRI avidity after inside-out signaling. Furthermore, bivalent binding could promote signaling of the FcαRI, although the exact organization of FcαRI in the plasma membrane is unknown. Constitutive phosphorylation of S263 might be an important mechanism in regulating bivalent binding of FcαRI, because the negatively charged phosphor groups could prevent optimal FcαRI organization within the plasma membrane. Once this repelling force is eliminated by inside-out signaling-induced dephosphorylation, bivalent binding and FcR γ-chain signaling may be promoted. More research on a nanoscale level is needed to further investigate this hypothesis.

Taken together, ample evidence supports the cytokine-induced inside-out activation of FcαRI. This is accomplished by a signaling pathway involving PI3K, p38, and PKC, resulting in the dephosphorylation of S263 by PP2A, which is a crucial step in the activation of FcαRI (Fig. 1B). The actin cytoskeleton is involved in maintaining FcαRI in the active state and a switch from monovalent to bivalent binding might represent part of the mechanism of inside-out activation of FcαRI. How the intracellular dephosphorylation of FcαRI is transmitted to its extracellular domains and what the contribution of receptor affinity and valency is, remains subject to future research.

FcαRI structure and conformational changes

Crystal structures of FcαRI and its complex with IgA revealed a conformational shift in EC1, located at the binding site for IgA[57]. This conformational change might already occur prior to ligand binding, resulting in a high affinity FcαRI. As mentioned before, conformational changes are a key feature of integrin inside-out activation and several antibodies have been described that discriminate between the different conformational states of integrins[9,58]. Since dephosphorylation of S263 in the intracellular domain of FcαRI is an essential step in the inside-out signaling of FcαRI, we hypothesized that the conformation of the ligand binding FcαRI-S263A mutant would differ from the inactive FcαRI-S263D mutant. To test this hypothesis, we generated antibodies against cell surface FcαRI and screened them for specific binding to either FcαRI-S263A or FcαRI-S263D. The antibodies differed in their degree of specificity for FcαRI-S263A and S263D and included antibodies highly specific for either FcαRI mutant (Fig. 2B and C). Unfortunately, establishing stable hybridoma clones to further characterize these antibodies was unsuccessful. These data

do imply that the extracellular domain of FcαRI-S263A adopts a different conformation than FcαRI-S263D. Thus, FcαRI is likely to exist in an inactive, low affinity conformation and an active, high affinity conformation induced by inside-out signaling before binding to IgA. Crystal structures of the active or inactive state of FcαRI prior to ligand binding are not yet available, although these would provide clear insights in the conformational changes mediated by inside-out signaling.

FcαRI crystal structures are available for the ligand-bound and ligand-free receptor. These structures show that FcαRI adopts a different conformation when bound to IgA compared to when the receptor is ligand-free. The change in FcαRI conformation is mainly evident in the FcαRI D-strand, which shifts from a front β-sheet to a back β-sheet (Fig. 2D)[57]. Three main amino acid residues seem to be involved in this conformational change: glutamic acid at position 49 (E49), arginine at position 52 (R52), and leucine at position 54 (L54) of the EC1 of FcαRI (Fig. 2E). These three amino acids reside in close proximity to the surface of FcαRI in the ligand-bound, but not in the ligand-free crystal structure. To test the influence of these amino acids on IgA binding, cells expressing the FcαRI mutants E49A, R52A, L54A, and a triple mutant ERL (E49A, R52A, and L54A) were generated. Whereas FcαRI-E49A and R52A were able to bind immobilized IgA, although less efficient than WT FcαRI and FcαRI-S263A, the binding to immobilized IgA was completely lost for the L54A and ERL mutants, comparable to FcαRI-S263D (Fig. 2A and F). This indicates the requirement of FcαRI residue L54 to bind IgA. Mutating R52 reduced the capacity of FcαRI to bind to immobilized IgA two-fold compared to WT FcαRI (Fig. 2F). Previously, an eightfold reduction in binding affinity of FcαRI-R52A was reported using surface plasmon resonance, although binding of monomeric IgA to FcαRI-R52A on the cell surface was comparable to WT FcαRI[59]. The discrepancy in this study may be attributed to the effects on avidity overcoming the lower affinity of FcαRI-R52A on the cell surface. This emphasizes the importance to also study receptor mutants within their natural cellular environment.

In conclusion, our data suggest that FcαRI adopts a high affinity conformation after inside-out signaling. We postulate that E49, R52, and L54 on the D-stand of active FcαRI might constitute an epitope that is exposed after cytokine-induced inside-out signaling. Furthermore, we show for the first time that L54 is a critical amino acid involved in binding of FcαRI to IgA.

INTERACTION BETWEEN FCR AND INTEGRINS

Integrins are essential for cell adherence and the formation of immunological synapses, while FcR in these synapses are required for the specific binding of opsonized target cells and the induction of signaling pathways. Immunological synapses formed between monocytes or neutrophils and target cells (*e.g.* infected cells, tumor cells, or endothelial cells) consist of different FcR and Mac-1. It is therefore not surprising that several FcR interact with integrins, especially Mac-1, and that cross-talk exists between these two types of receptors (reviewed in [60]). Interestingly, these interactions can enhance the function of either integrins or FcR, suggesting that these receptors can be activated via membrane

cross-talk in addition to cytokine/chemokine stimulation.

Based on the current literature, as described below, two general concepts can be postulated for the interaction between integrins and FcR. Firstly, for the process of transmigration of leukocytes, the interaction of FcR with IC provides the initial binding and leads to increased integrin expression and activated integrins on the cell surface via inside-out signaling. These active integrins can now bind their ligand more efficiently to ensure strong adherence and transmigration. Secondly, in the context of immunological synapses and binding of large IC, integrins seem to be essential for the correct formation of the synapse and the subsequent effector functions of FcR, including phagocytosis and ADCC.

Monocytes

On monocytes, Mac-1 is essential for IgG-IC binding, which mainly occurs via FcγRI[61]. When monocytes are exposed to IC-coated erythrocytes under flow conditions, blocking of Mac-1 can reduce the binding of these IC[62]. This indicates that Mac-1 can enhance FcγRI effector functions. The cross-talk between FcγRI and Mac-1 on monocytes also occurs in the other direction, as ligand binding to FcγRI can increase integrin avidity. This has recently been studied in the context of HLA class I antibodies and solid-organ transplant rejection[63]. HLA class I antibodies induce cross-linking of HLA on the surface of endothelial cells, leading to P-selectin upregulation that can induce partial adherence of monocytes. The Fc domains of these antibodies can subsequently bind to FcγR, mainly FcγRI, on monocytes, which induces further activation of integrins (Mac-1). This final step is essential for robust adherence followed by transmigration of the monocytes[63].

Neutrophils

Interaction of Mac-1 with three FcR on the plasma membrane of human neutrophils have been described. First of all, even though FcγRIIIb (the only GPI-linked FcR) has not been described to be under inside-out control, this receptor has been studied thoroughly for interacting with Mac-1 (reviewed in [64]). Using FcγRIIIb and Mac-1 transfectants, it was shown that only when both receptors are present, FcγRIIIb-mediated IC binding and phagocytosis was observed[12]. This supports the hypothesis that FcγRIIIb might exert effector functions via association with integrins and that Mac-1 could actually activate FcγRIIIb[13]. Secondly, FcγRIIa was demonstrated to be in close proximity of Mac-1 on neutrophils using FRET (fluorescence resonance energy transfer), which was confirmed with FcγRIIa and Mac-1 transfectants[64]. A functional interaction was suggested using a tail-less FcγRIIa, which is incapable of inducing phagocytosis of IC. Cotransfection of Mac-1 with the tail-less FcγRIIa restored the phagocytic capacity[65]. However, other reports indicate that FcγRIIa and Mac-1 function independently on neutrophils, since both receptors localize to different membrane protrusions on polarized neutrophils[64]. Thirdly, colocalization between FcαRI and Mac-1 on the neutrophil plasma membrane was observed. When neutrophils from Mac-1$^{-/-}$ mice were used in binding experiments, binding of secretory IgA, but not serum IgA, was abolished, even though FcαRI expression was normal[66]. The

secretory component of IgA is directly involved in the interaction of secretory IgA with Mac-1.

The cross-talk between Mac-1 and FcR is further supported by using anti-Mac-1 antibodies, which inhibit intracellular calcium signaling and ROS production on human neutrophils during IC stimulation[67]. Importantly, the ADCC capacity of Mac-1$^{-/-}$ neutrophils was abrogated, both with an FcαRI-HER2 bsAb and a mIgG1 anti-HER2 antibody, indicating that Mac-1 is crucial for FcαRI and FcγRIIa anti-tumor effector functions. Although Mac-1 is not required for ROS production in these Mac-1$^{-/-}$ neutrophils, the formation of an intact immunological synapse between neutrophils and tumor cells is dependent on Mac-1, probably explaining the impaired ADCC capacity of Mac-1$^{-/-}$ neutrophils[68]. Together, these data suggest that several interactions occur between Mac-1 and FcR on neutrophils and that Mac-1 is important for several effector functions of these FcR. We speculate that Mac-1 might induce activation of FcR via inside-out signaling, most likely by directly influencing the FcR.

As these studies indicate, uncoupling integrin activation from FcR activation and vice versa can be quite difficult, since integrins and FcR might directly interact with each other, their activation is very rapid, and some of their signaling pathways are shared. Furthermore, although many other integrins are also important for the functions of immune cells, most of the cross-talk is studied for Mac-1. Therefore, the exact role and extent of interactions between FcR and integrins remain to be elucidated. However, it is likely that ligand binding of integrins can lead to inside-out activation of FcR. Future investigations will shed more light on FcR and integrin interaction.

FCR INSIDE-OUT SIGNALING IN ANTIBODY THERAPY

Antibody therapy has gained considerable interest over the past two decades because of the high target specificity and boosted the attention for FcR, as the activation of these FcR on immune cells can trigger eradication of target cells. The first FDA-approved mAb for cancer patients was rituximab, a mAb directed against CD20 expressed on many B cells. Rituximab is very effective for treating non-Hodgkin's lymphoma patients, but it is also used in the treatment of several autoimmune diseases. Nowadays, many mAb are used in the clinic or are under development for the prophylaxis/treatment of malignancies (hematological and solid), autoimmune diseases (*e.g.* rheumatoid arthritis and multiple sclerosis), and infectious diseases (*e.g.* ebola, influenza and RSV infections).

The clinical potential of mAb relies on the different effector mechanisms exhibited by their Fab arms and Fc tail. The Fab arms induce a direct effect by binding to the target antigen, resulting in antigen internalization or blockade of signal transduction pathways. In addition, interaction of the Fc tail with FcR expressed on immune cells can trigger cytotoxic effector functions. As described above, there is clear evidence that several FcR (FcγRI, FcγRIIa, FcαRI) can be activated by cytokine-induced inside-out signaling. To our knowledge, however, no clear link between FcR inside-out signaling and the therapeutic potential of mAb has been made. Here, we will discuss the impact of cytokine stimulation (GM-CSF and G-CSF) on FcR activation with regard to mAb therapy.

Since GM-CSF and G-CSF treatment do not influence FcαRI expression levels on neutrophils and monocyte-derived macrophages, ADCC experiments using a fixed number of these effector and tumor cells might be one of the clearest models to investigate the role of FcαRI inside-out signaling in mAb therapy. We observed that *in vitro* GM-CSF-stimulated neutrophils lysed tumor cells opsonized with IgA2-EGFR mAb more effectively than unstimulated neutrophils (own unpublished data). Other studies using bsAb of the F(ab')×F(ab') format that recognize FcαRI with one arm and a tumor-associated antigen (*i.e.* HER2 or CD20) with the other arm showed that also *in vivo* GM-CSF stimulated neutrophils lysed tumor cells better than unstimulated neutrophils[69]. However, this effect could not be shown in ADCC assays using either whole blood or neutrophils from G-CSF treated patients[70]. In line with the GM-CSF stimulated neutrophil ADCC assay, *in vitro* GM-CSF stimulated monocyte-derived macrophages showed enhanced phagocytosis and tumor cell lysis with tumor-specific bsAb. Importantly, a change in the total FcR γ-chain formation upon cytokine stimulation was excluded as a possible mechanism for enhancing ADCC[71].

The effect of G-CSF stimulation on neutrophil FcγR-dependent ADCC has also been extensively studied, using both clinically approved and preclinical human and murine IgG mAb. Neutrophils from healthy donors have a low expression of the high affinity FcγRI, which can be upregulated by G-CSF treatment *in vivo* but not *in vitro*[72]. Nonetheless, both *in vitro* and *in vivo* G-CSF stimulated neutrophils performed superior in ADCC assays compared to unstimulated cells. Furthermore, combination treatment of G-CSF and mIgG1 TA99 augmented anti-tumor responses in a myeloma mouse model compared to TA99 alone[69]. A study comparing HLA class II-specific antibodies of different isotypes (hIgG1, mIgG1, mIgG2a, mIgG2b, and mIgG3) showed that all isotypes induced more ADCC with *in vivo* G-CSF stimulated neutrophils compared to unstimulated neutrophils. In depth analysis using FcγR blocking antibodies revealed the involvement of FcγR to be isotype-dependent; FcγRI blockade had the strongest influence on mIgG3 and mIgG2a, whereas FcγRIIa blockade had the strongest influence on mIgG1 followed by mIgG2b[73].

In contrast to G-CSF treatment in patients, GM-CSF treatment in patients and *in vitro* GM-CSF stimulation did not increase FcγRI expression on immune cells[71,74]. However, neutrophils, mononuclear cells, and granulocytes stimulated *in vitro* with GM-CSF were all shown to have enhanced ADCC capacity[75,76]. The effect of GM-CSF stimulation on mononuclear cells was less pronounced than on neutrophils. Interestingly, monocyte-derived macrophages cultured in the presence of GM-CSF (and M-CSF) demonstrated enhanced phagocytosis and ADCC of tumor cells with a bsAb targeting HER2 and FcγRI[71]. In two other studies, however, such bsAb only induced higher tumor cell lysis with neutrophils and whole blood from G-CSF but not GM-CSF treated patients[74,77]. Surprisingly, higher tumor cell lysis was not observed with a CD20×FcγRI bsAb[74]. Together, these data suggest that cytokine stimulation can increase mAb therapy both *in vitro* and *in vivo*. However, the magnitude of the effect is both antibody and cell type-dependent.

Although these studies demonstrate that cytokine stimulation could enhance mAb therapy, there are limitations to some studies that make it difficult to assess the involvement of FcR inside-out signaling. First of all, experiments performed with bsAb remain

complicated to interpret, as one of the Fab arms recognizes a specific epitope on the FcR. To our knowledge, no efforts are being made to determine if these bsAb already favorably recognize the active or inactive form of the targeted FcR. Secondly, the action of cytokines is time-dependent and of a short duration[47], whereas clinical trials focus on the long-term effects of mAb therapy. Thus, a better measurement of the impact of the applied cytokines is required to design the best treatment regimen. Thirdly, only a few clinical trials are available investigating cancer treatment with a combination of mAb and cytokines. Using G-CSF and rituximab as combination therapy in a clinical trial achieved a good response in patients[78]. However, without a control group receiving monotherapy (*i.e.* G-CSF or rituximab alone) and analysis of possible FcR changes it is difficult to determine the impact of cytokine treatment and inside-out signaling on mAb therapy in this study. Finally, antibodies discriminating between the FcR inactive and active form (*i.e.* low affinity and high affinity) to directly investigate FcR inside-out signaling are still not available for all FcR.

In summary, the knowledge gained from *in vitro* and *in vivo* studies that investigated the influence of G-CSF and GM-CSF stimulation on mAb therapy suggests that cytokine stimulation can enhance mAb therapy by increasing the FcR affinity for mAb. However, many studies did not measure FcR expression and/or activation. The magnitude of the effect of cytokine stimulation seems to be dependent on the cytokine, antibody specificity and isotype, timing, and immune cell type (and thus FcR expression profile).

CONCLUDING REMARKS AND OUTLOOK

Inside-out activation of receptors is a well-established concept for integrins. Here, we reviewed the inside-out activation of several FcR and showed that this is a key concept in the regulation of FcR. There are multiple shared mechanisms of inside-out signaling between integrins and FcR, including conformational changes, (de)phosphorylation events, and cytoskeleton rearrangements (Fig. 1B). SNPs in FcR may also directly affect FcR inside-out signaling, although this has not been investigated yet. However, it is becoming increasingly clear that each FcR has a unique mechanism of inside-out signaling. Whether all FcR are under inside-out control for the regulation of their binding affinity is still unknown. Given the sequence homology between the FcR, *e.g.* FcγRIIa, FcγRIIb, and FcγRIIc, it is tempting to speculate that other FcR are also regulated via inside-out signaling. Future research might clarify if indeed inside-out signaling is a general concept for all FcR.

Cytokine stimulation seems to enhance the FcR-mediated effects of anti-tumor mAb therapy. This is in line with the *in vitro* data on the inside-out signaling of FcR, since the avidity of FcR for IC is increased by cytokines, leading to improved FcR effector functions. However, besides GM-CSF and G-CSF, there is limited data on the effects of other cytokines (*e.g.* IL-3, IL-4, IL-5) on mAb therapy. It would be very interesting to test a broader panel of cytokines both *in vitro* and *in vivo* in tumor models. Future clinical trials combining mAb with cytokines should focus on FcR expression and activation status, to gain more insight in the role of inside-out signaling in mAb therapy. Considerable effort is being made to develop antibodies specific for the active or inactive conformation of

FcR. These antibodies are valuable tools to study the activation status of different FcR and monitor the changes in FcR inside-out signaling in patients. For FcαRI, such antibodies might be especially useful for studying rheumatoid arthritis and multiple sclerosis, since IgA contributes to the severity of diseases and the activation status of FcαRI might be of importance.

In conclusion, a thorough understanding of the mechanisms of inside-out signaling of FcR will provide us with means to manipulate the activation status of FcR, *e.g.* in autoimmune diseases or during mAb therapy.

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CHAPTER 3

Mechanisms of inside-out signaling of the high affinity IgG receptor FcγRI

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Submitted

Fc receptors (FcR) are an important bridge between the innate and adaptive immune system. Fc gamma receptor I (FcγRI, CD64), the high affinity receptor for IgG, plays roles in inflammation, autoimmune responses, and immunotherapy. Stimulation with cytokines has been shown to increase the binding of FcγRI to immune complexes (IC) such as opsonized pathogens or tumor cells, yet the molecular mechanisms are unknown. Using super-resolution imaging, we found that cytokine stimulation enhanced the clustering of FcγRI both before and after IC addition. This increased clustering is dependent on an intact actin cytoskeleton. We found that PP1 is the serine/threonine phosphatase involved in FcγRI inside- out signaling, while the phosphorylation of FcγRI itself is unaffected. Furthermore, cytokine-stimulated neutrophils demonstrated a stronger antibody-dependent cellular cytotoxicity of CD20-expressing tumor cells. These results indicate a role for cytokine-induced inside-out signaling in altering the nanoscale organization of FcγRI which enhances FcγRI effector functions.

INTRODUCTION

Expressed on immune cells, Fc receptors (FcR) are required for the cellular effector functions of antibodies, including protection against bacteria and phagocytosis. In humans, the FcγR family comprises both the activating receptors FcγRI, FcγRIIa, FcγRIIc, and FcγRIIIa as well as one inhibitory receptor, FcγRIIb. Fc gamma receptor I (FcγRI, CD64), the high affinity receptor for IgG (K_D of 10^{-8}-10^{-9} M), associates with the FcR gamma chain and is constitutively expressed on monocytes, macrophages, eosinophils, and dendritic cells, as well as on neutrophils after activation. The docking mode between IgG and the different FcγR, including FcγRI, is very similar and conserved[1]. Because of its high affinity, FcγRI is believed to be saturated with monomeric IgG, even after isolation or extravasation of immune cells[2]. Therefore, the in vivo role of FcγRI in immune responses remains unclear. However, several studies have implicated an important role for FcγRI during inflammation, autoimmune responses, and monoclonal antibody immunotherapy in tumor models[3-5]. In addition, FcγRI can efficiently induce MHC class II antigen presentation[6].

It was demonstrated that FcγRI, saturated with prebound IgG, was capable of effective immune complex (IC) binding after cytokine stimulation[7]. This phenomenon was termed 'inside-out signaling' because ligand binding of the receptor is rapidly enhanced after intracellular signaling without altering receptor expression. This is a well-known process for integrins[8], as well as two other FcR: FcαRI and FcγRIIa[9,10]. Both of these FcR receptors as well as FcγRI were studied previously in a Ba/F3 transfection model, a murine cell line dependent on IL-3 for survival and in which IL-3 can stimulate inside-out signaling[7,9]. In primary human leukocytes, analogous cytokines such as IL-5, IFNγ, and TNFα stimulate ligand binding and receptor function[10]. However, the mechanisms by which FcR increase their ligand binding are largely unknown. Cytokine stimulation induced a small but significant increase in monomeric IgG binding of FcγRI-expressing cells. Strikingly, stimulation with cytokines strongly enhanced the binding of IC without altering FcγRI expression[7]. This effect was inhibited by okadaic acid (OA), a phosphatase inhibitor of PP2a at low concentrations and PP1 at higher concentrations[11]. Furthermore, cytokine stimulation can also enhance anti-tumor responses of therapeutic antibodies mediated by FcR-bearing immune cells[10,12].

How cytokine signaling alters FcγRI to increase its binding capacity is still unknown. Potential explanations include changes in FcγRI conformation, dynamics, and clustering. There is increasing evidence indicating that the membrane environment can modulate each of these aspects of receptor behavior[13-15]. Furthermore, the plasma membrane is organized into microdomains, such as actin corrals, lipid rafts, and protein islands, that play an essential role in promoting signal transduction for a number of membrane proteins[13,14]. Previous work has provided evidence that FcγRI resides in lipid rafts and that disruption of lipid rafts could increase ligand binding[16]. Here, we sought to elucidate the mechanism of FcγRI inside-out signaling by cytokine stimulation. To this end, we studied the mobility and nanoscale organization of FcγRI in the plasma membrane using single particle tracking (SPT) and super-resolution imaging, investigated the role of phosphorylation and the actin cytoskeleton, and measured the effect of cytokine stimulation on antibody-depen-

dent cellular cytotoxicity (ADCC) of human neutrophils.

RESULTS

Inside-out signaling of FcγRI enhances immune complex binding

For our studies, we made use of the previously characterized FcγRI-expressing Ba/F3 cell line[7]. Consistent with previous studies[7], we found the same trend that IL-3 stimulation resulted in a minor increase in monomeric IgG binding (Fig. 1A). To measure IC binding, we utilized a small synthetic antigen, DNP_{24}-BSA combined with fluorescently labeled anti-DNP rabbit IgG (polyclonal). This system allowed us to study the dynamics and dis-

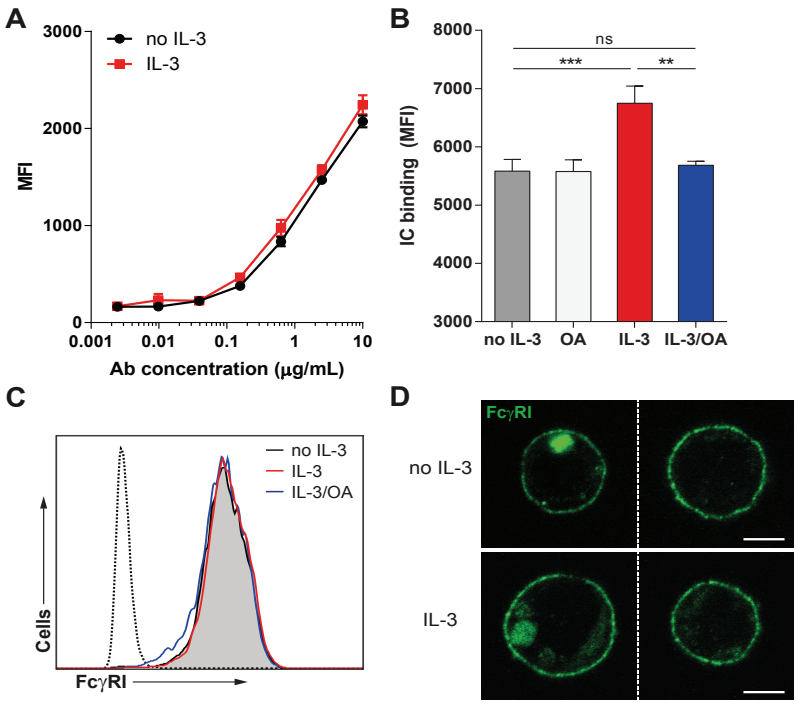

Figure 1. Inside-out signaling of FcγRI enhances IC binding. (A) Binding of monomeric IgG (IgGαDNP) at increasing concentrations after IL-3 stimulation of Ba/F3-FcγRI cells. Binding was measured using flow cytometry and the mean fluorescence intensity (MFI) is depicted. One representative of n=3 independent experiments is shown. (B) Binding of IC is increased after IL-3 stimulation of Ba/F3-FcγRI cells. IC were composed of AF647-IgGαDNP:DNP_{24}-BSA (~3:1 ratio) and binding of these IC to Ba/F3-FcγRI cells was measured using flow cytometry. The MFI of the cells is depicted. $p<0.01$: **, $p<0.001$: ***, ns: not significant, one-way analysis of variance (ANOVA) and Tukey's post hoc test. (C) Ba/F3-FcγRI expression was measured with mouse anti-FcγRI AF647 using flow cytometry after 1 hour IL-3 stimulation (IL-3) or not (no IL-3). Where indicated, cells were pretreated with 1 µM okadaic acid (OA) before IL-3 stimulation (IL-3/OA). Dotted line represents the isotype control. A representative expression profile is depicted of n=4 individual experiments. (D) FcγRI distribution measured using confocal microscopy, by labeling Ba/F3-FcγRI cells with mouse anti-FcγRI AF488. Two representative images for each condition are shown. Scale bar represents 5 µm.

tribution of FcγRI receptors in both the presence and absence of IC with high-resolution imaging. IL-3 stimulation significantly enhanced the binding of Ba/F3-FcγRI cells to preformed IC and this effect could be blocked by the addition of OA (Fig. 1B). FcγRI surface expression was constant under all treatments (Fig. 1C and fig. S1), confirming that the cytokine-induced binding enhancement was not a result of increased receptor expression. Next, we used fluorescence microscopy to examine whether the receptor distribution in the plasma membrane was altered. Using confocal imaging, we did not observe large-scale changes in receptor organization (Fig. 1D).

Cytokine stimulation enhances FcγRI clustering in the plasma membrane

To better understand how cytokine stimulation may be associated with spatio-temporal changes in receptor behavior, we began by characterizing the mobility of FcγRI using SPT (Fig. 2A). We found that IL-3 caused a small but significant increase in FcγRI diffusion, both in resting and antigen bound receptors (Fig. 2B). This IL-3-dependent increase in diffusion was not prevented by OA pretreatment. Using the Kolmogorov-Smirnov test to compare the conditions, we found that all conditions are significantly different ($p<0.001$) except for IL-3 vs IL-3/OA (both with and without antigen). We also used fluorescence recovery after photobleaching (FRAP) to measure the ensemble mobility of FcγRI-eYFP in Ba/F3 cells. In these experiments, the addition of IL-3 did not affect the diffusional behavior of the receptor (Fig. 2C). We conclude that changes in FcγRI mobility are not correlated with enhanced IC binding (Fig. 2).

It has been previously demonstrated that small aggregates of immune receptors are capable of robust signaling without inducing changes in mobility[17]. Therefore, to gain insight into the nanoscale organization of FcγRI in the plasma membrane, we used dSTORM, a localization-based super-resolution imaging technique that provides ~20nm resolution[18]. Ba/F3-FcγRI cells were labeled with AF647-IgGαDNP, leading to FcγRI bound with labeled monomeric IgG on the plasma membrane. Super-resolution images were reconstructed from independent localizations of AF647-IgGαDNP on the basal side of the Ba/F3-FcγRI cells. Figure 3A shows representative super-resolution images of FcγRI distribution from control (no IL-3), IL-3-stimulated, and antigen-bound cells. DNP_{24}-BSA was used as antigen to induce IC on the plasma membrane (Fig. S2A). To quantify the images, we used a DBSCAN clustering algorithm to classify localizations into clusters based on their relative local spatial density[19]. This approach allowed us to identify individual clusters and make comparisons between the distributions of clusters across different conditions (Fig. 3B). We found that the equivalent cluster radius, calculated as the radius of a circle with the same area as the boundary of all fits within a cluster, was a useful representation of cluster size. In Figure 3C, we plot the FcγRI cluster sizes for each condition, where each symbol represents the mean cluster size in an individual 2 μm^2 region of interest. Changing the maximal distance between neighboring cluster points to an epsilon of 35 nm instead of 50 nm or using the Getis-G clustering method instead of DBSCAN gave similar results for all conditions.

Consistent with other reports of antigen-induced receptor crosslinking[20], addition of

antigen resulted in the formation of large FcγRI clusters in all conditions (Fig. 3C and fig. S2B). Cluster size was dependent on antigen dose (Fig. S2C), verifying that these observed changes were antigen specific. In line with the increased antigen binding seen in flow cytometry, antigen-induced clusters were larger in IL-3 stimulated cells at all doses (Fig.

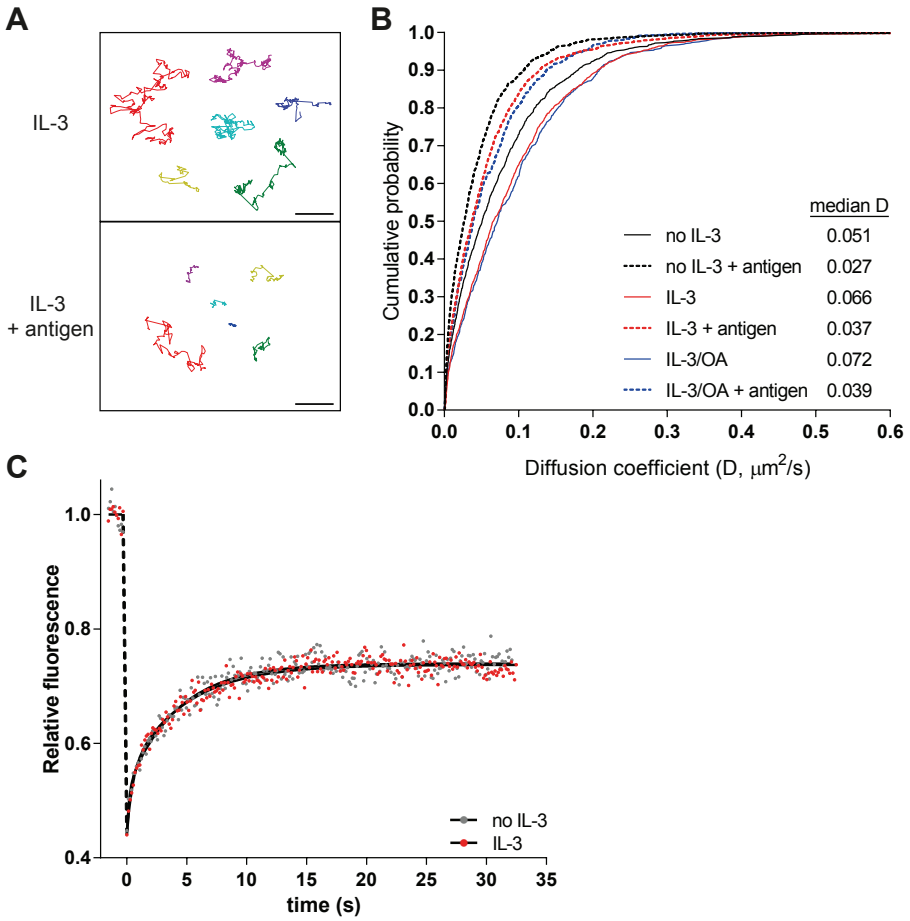

Figure 2. Lateral mobility of FcγRI. (A+B) QD-IgG was used to track the motion of individual FcγRI. For each trajectory, we calculated the diffusion coefficient (D) under control, IL-3 stimulated and antigen-bound conditions. We found that addition of multivalent antigen (DNP_{24}-BSA) that induced IC on the plasma membrane reduced receptor mobility under all conditions, consistent with crosslinker-induced immunoreceptor aggregation[20]. (A) Single particle tracking (SPT) of individual FcγRI using QD655-IgGαDNP on Ba/F3-FcγRI cells. Example particle trajectories of IL-3 ± antigen are depicted. Scale bar represents 1 μm. (B) Diffusion coefficients for FcγRI mobility were calculated using MSD analysis of individual SPT trajectories. Plotted is the cumulative probability of D values for n=85-200 cells per condition. For each condition, the median D (μm2/s) is also depicted. (C) Fluorescence recovery after photobleaching (FRAP) was used to measure the ensemble mobility of FcγRI-eYFP in Ba/F3 cells stimulated with (IL-3) or without (no IL-3) IL-3 for >30 min. Fluorescence recovery is depicted as the mean relative fluorescence (normalized and corrected). The curve (black line) is calculated by non-linear two phase fitting of the data.

S2C). Notably, we found that even before antigen addition, IL-3 stimulation promoted an increase in the size of receptor clusters and these increased further with antigen (Fig. 3C). This IL-3-induced increase in cluster size was reduced by pretreatment with OA, the same treatment that reversed the effect of IL-3 on IC binding (Fig. 1A and fig. 3C). Similar results were obtained when preformed IC (composed of AF647-IgGαDNP + DNP_{24}-BSA) were added to Ba/F3-FcγRI cells prelabeled with AF647-IgGαDNP (Fig. S2D) or when AF647-conjugated human IgG1 was used instead of rabbit IgG (Fig. S2E). These results demonstrate that cytokine stimulation leads to an enhanced clustering of FcγRI.

When performing dSTORM imaging, a minimum cluster size is generated from a single fluorophore due to repeated localizations and the precision of the measurement. Therefore, to confirm that the small shift in cluster radius observed with IL-3 stimulation was due to multiple proteins in a cluster, we performed two-color dSTORM. In these experiments, FcγRI was labeled stochastically with either AF647-IgGαDNP or Cy3B-IgGαDNP (Fig. 3D) and receptor proximity was quantified using localization-based cross-correlation analysis[21,22]. As seen in Figure 3E, the cross-correlation function for FcγRI in the absence of IL-3 is close to 1, but shows a small increase at short distances (<200 nm), supporting that a fraction of receptors exist in small clusters in the absence of crosslinking. Upon stimulation with IL-3, the correlation function at short distances dramatically increased (Fig. 3E, red line). To confirm that this difference is not due to an artifact of multiple localizations of the same fluorophore, we used H-SET analysis that collapses clusters of observations of blinking fluorophores into single estimates of their true locations[23]. Analysis of the collapsed data shows the same relative increases of cross-correlation at short distances (Fig. 3F). The higher cross-correlation seen for FcγRI in the presence of IL-3 is consistent with the increased clustering induced by IL-3 as seen in the single color dSTORM results (Fig. 3C). Altogether, the super-resolution imaging demonstrates that FcγRI is found in small clusters on the plasma membrane and the clustering is increased with IL-3 stimulation. This change in spatial organization with IL-3 suggests that cytokine stimulation sensitizes the cells to engage antigen or IC by enhanced preclustering of FcγRI.

An intact actin cytoskeleton is required for increased FcγRI clustering

Since the intracellular tail of the FcγRI α-subunit (CY) has been shown to interact with three actin-binding proteins (filamin A, periplakin and protein 4.1G)[24-26], we postulated that actin rearrangement could be facilitating the observed cytokine-induced changes in receptor clustering. Using IgG-coated beads as IC we measured rosette formation in the presence of Latrunculin A (LatA), an inhibitor of actin polymerization (Fig. 4). Consistent with previous data[7], we detected a nearly two-fold increase in the percentage of rosettes with IL-3 stimulation (Fig. 4A and B). In contrast, pretreatment of the cells with LatA before IL-3 stimulation significantly reduced the rosette formation to the similar extent as OA. LatA did not abrogate the rosette formation completely, indicating that, at least to some extent, IC can still bind without an intact cytoskeleton. LatA treatment did not alter FcγRI surface expression (Fig. S3). Next, we examined the effect of cytoskeletal disruption

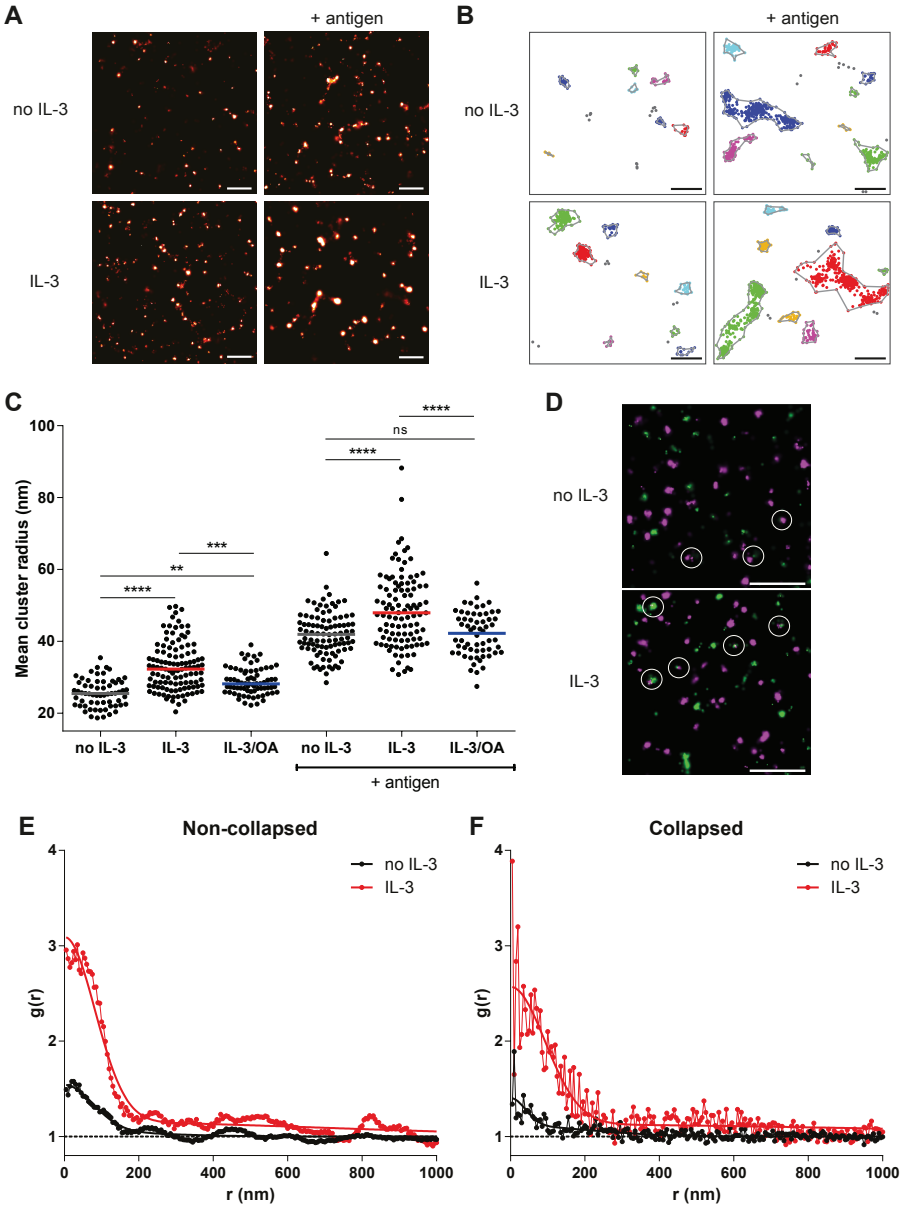

Figure 3. Cytokine stimulation promotes FcγRI clustering. All Ba/F3-FcγRI cells were labeled with AF647-IgGαDNP for super-resolution imaging and stimulated with IL-3 for 1 hour (IL-3) or not (no IL-3). Where indicated, cells were subsequently incubated with 1 µg/mL DNP_{24}-BSA (+ antigen) to induce immune complexes on the cell surface. (A) dSTORM images of FcγRI distribution on the basal plasma membrane. One representative reconstruction for each condition is shown. Scale bar represents 500 nm. (B) Images in A were analyzed using the density-based DBSCAN algorithm. Depicted are examples of the FcγRI clusters. Scale bar represents 200 nm. For each condition, 20-30

cells from n=3 individual experiments were combined and analyzed. Mean cluster radius is shown. Where indicated, cells were pretreated with 1 μM okadaic acid (OA) before IL-3 stimulation (IL-3/OA). Line represents the median. $p<0.01$: **, $p<0.0001$: ****, ns: not significant, one-way analysis of variance (ANOVA) and Tukey's post hoc test. (D-F) Two-color dSTORM of FcγRI. Ba/F3-FcγRI cells were labeled with a mix of AF647-IgGαDNP and Cy3B-IgGαDNP. (D) Two representative images are shown. Circles indicate examples of overlap between AF647 (magenta) and Cy3B (green). Scale bar represents 1 μm. (E) Two-color dSTORM images were analyzed using cross-correlation analysis. The dashed black line indicates a cross-correlation $g(r) = 1$, which is considered a random distribution. 3-4 cells per condition were analyzed and the actual data points (symbols) and the fit through the cross-correlation function (solid line) are shown. (F) Cross-correlation analysis of H-SET collapsed data.

on FcγRI organization. LatA did not change FcγRI cluster size in control cells or prevent the formation of larger aggregates with antigen (Fig. 4C). However, LatA did prevent the ability of IL-3 to enhance FcγRI cluster size both before and after antigen. From these results, we conclude that the cytokine enhanced clustering of FcγRI specifically requires an intact actin cytoskeleton, while IC-induced clustering is independent of the actin cytoskeleton.

PP1 is the phosphatase that regulates FcγRI inside-out signaling but does so without changes in total FcγRI phosphorylation

Previous work showed a dependence of inside-out signaling on phosphatase activity by treatment with OA[7], which we have shown to also be effective in blocking FcγRI nanoscale reorganization (Fig. 3). However, OA inhibits the phosphatase activity of PP2a at low concentrations and PP1 at higher concentrations[11]. To determine if PP1 is the involved phosphatase, we treated cells with the inhibitor tautomycetin (TC), which has a ~40x higher specificity for PP1 over PP2A. We found that TC was a much more potent inhibitor of IC binding by IL-3 stimulated Ba/F3-FcγRI cells (Fig. 5A), showing inhibition at 1 nM while OA required concentrations of 100 nM or more to show an effect (Fig. 5B).

Since PP1 is a serine/threonine phosphatase, and the CY domain of FcγRI contains four serine and two threonine residues, we next investigated whether PP1 acts directly on the receptor. Neither IL-3 stimulation nor phosphatase inhibition influenced the amount of p-FcγRI (Fig. 5C and fig. S4). The p-FcγRI band was no longer present when the intracellular serines and threonines were mutated to alanines (FcγRI 4S/2T>A mutant) (Fig. 5C and fig. S4). These data indicate that FcγRI phosphorylation is not altered with inside-out signaling and that PP1, while critical for inside-out signaling, does not directly act on the CY domain of FcγRI. In line with this, cytokine stimulation of the FcγRI 4S/2T>A mutant resulted in equal rosette formation compared to FcγRI wild-type. However, when a truncated version of FcγRI was used that lacked the CY domain no increase in rosette formation was observed after IL-3 stimulation (Fig. 5D). This indicates that while the intracellular domain of FcγRI is required for inside-out signaling the 4S/2T residues within this domain do not play a role.

Inside-out signaling affects clustering of FcγRI on human monocytes and IgG-mediated ADCC by neutrophils

The above experiments were performed using the Ba/F3 cell line expressing FcγRI. To confirm that cytokine regulated FcγRI clustering also occurs in other immune cells, we performed experiments to quantify receptor organization in primary human monocytes from healthy donors. Human monocytes endogenously express FcγRI and it has been previously shown that a combination of two cytokines, IFNγ and TNFα, induced inside-out signaling of FcγRI in these cells[7]. We found that stimulation of monocytes with cytokines did not alter FcγRI expression or diffusion (Fig. S5A and B). Next, we used single color dSTORM to measure FcγRI clusters on these cells. Notably, the cluster sizes observed in human monocytes were comparable to those measured in Ba/F3-FcγRI cells. Stimulation

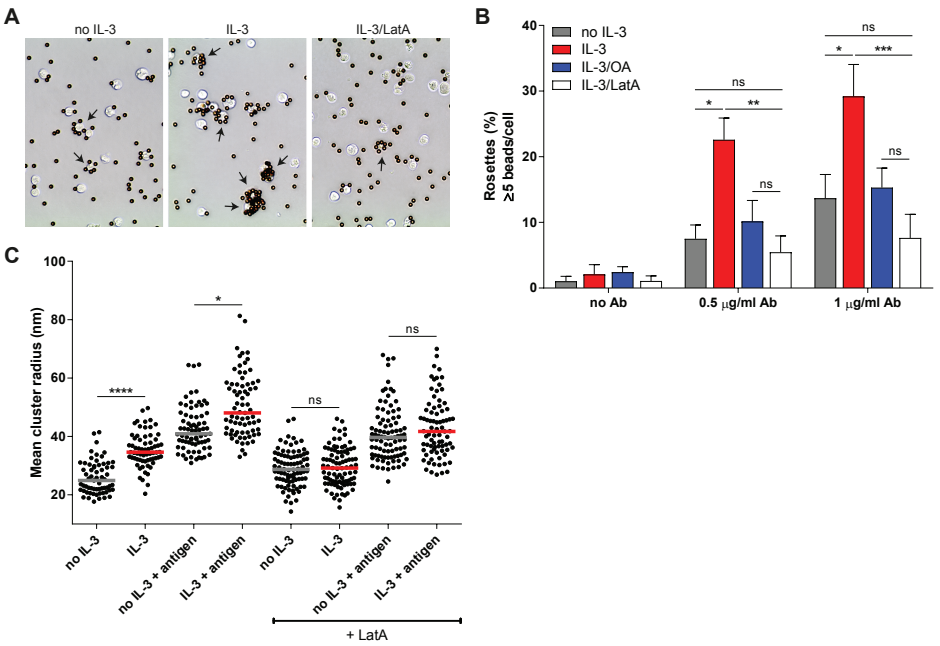

Figure 4. An intact actin cytoskeleton is required for increased clustering of FcγRI. (A) Beads were opsonized with 1 μg/mL antibody and added to Ba/F3-FcγRI cells. To inhibit actin polymerization, 0.1 μg/mL latrunculin A (+ LatA) was added before IL-3 stimulation. Rosettes were defined as cells bound with ≥5 beads. Example images are shown; arrows indicate rosettes. Beads are 4.5 μm in size, Ba/F3-FcγRI cells are ~10 μm in size. (B) Quantification of rosette formation. Beads were opsonized with or without the indicated antibody (Ab) concentrations and added to Ba/F3-FcγRI cells. Where indicated, cells were pretreated with 1 μM okadaic acid (OA) or LatA. Data from n=3 independent experiments is shown. p<0.0001: ****, ns: not significant, one-way analysis of variance (ANOVA) and Tukey's post hoc test. (C) dSTORM images of FcγRI distribution were acquired and analyzed as in Figure 3. For each condition, 9-12 cells from n=2 individual experiments were combined and analyzed. Line represents the median. p<0.0001: ****, ns: not significant, one-way analysis of variance (ANOVA) and Sidak's multiple comparisons test.

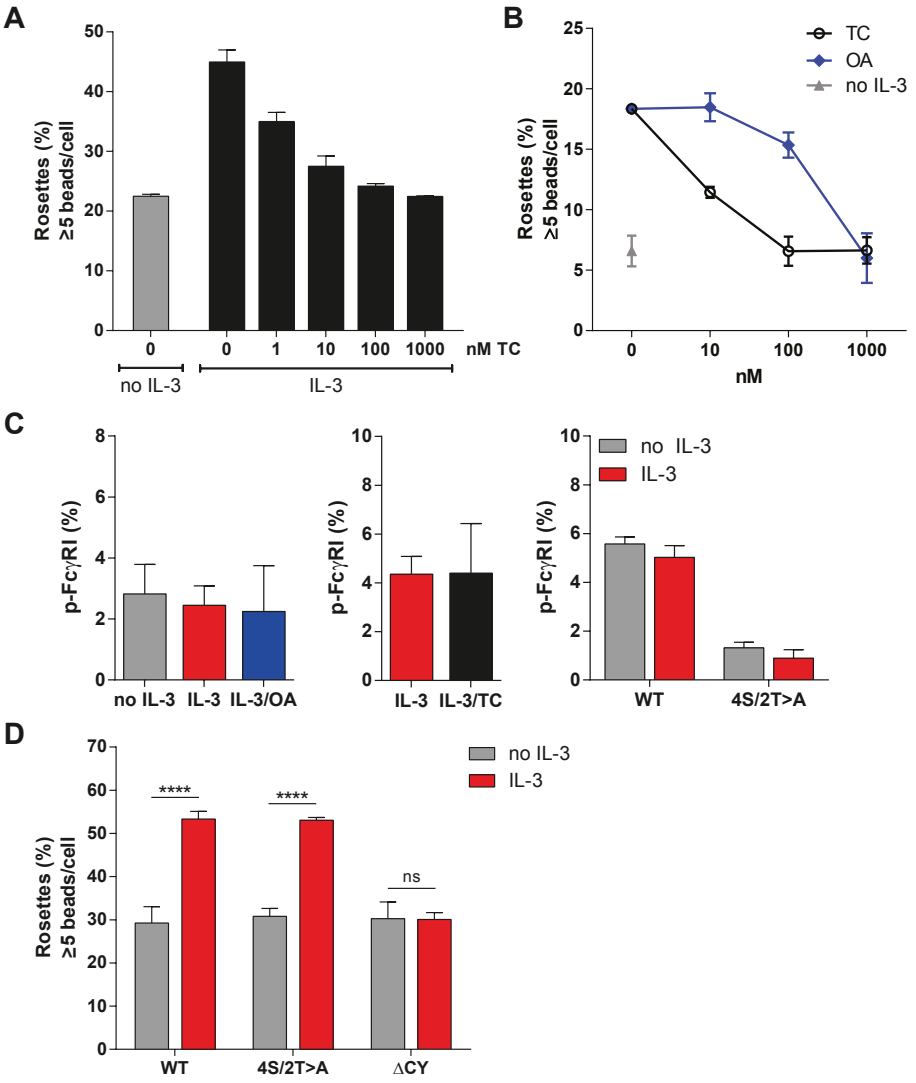

Figure 5. PP1 regulates FcγRI inside-out signaling without changes in FcγRI phosphorylation. (A+B) Ba/F3-FcγRI cells were pretreated with tautomycetin (TC) or okadaic acid (OA) at the indicated concentrations before IL-3 stimulation. Beads were opsonized with 1 µg/mL antibody and added to Ba/F3-FcγRI cells. Data from n=3 independent experiments is shown. (C) Quantification of the percentage phosphorylated FcγRI (p-FcγRI) of total FcγRI from Phos-tag SDS-PAGE gel and Western Blot analysis of FcγRI. Ba/F3-FcγRI cells after stimulation with IL-3 and pretreatment with inhibitors (1 µM OA or 100 nM TC) were used. The mean ± SEM is depicted. Data from n=3 independent experiments is shown. (D) Ba/F3 cells expressing FcγRI WT (wild-type), FcγRI 4S/2T>A, or FcγRI ΔCY (without intracellular domain) were stimulated with or without IL-3, and opsonized beads were added to the cells. One representative experiment of n=3 independent experiments is depicted. p<0.0001: ****, ns: not significant, two-way analysis of variance (ANOVA) and Sidak's multiple comparisons test.

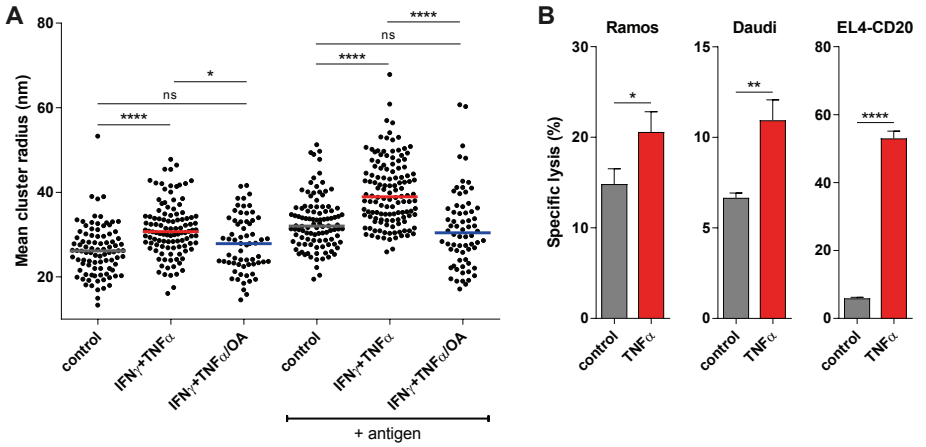

Figure 6. Inside-out signaling affects clustering of FcγRI on monocytes and IgG-mediated ADCC by neutrophils. (A) dSTORM of FcγRI distribution on human monocytes. Human monocytes were stimulated with IFNγ+TNFα cytokines for 1 hr or not (control) and then labeled with AF647-IgGαDNP. Cells were then cross-linked with DNP_{24}-BSA at 1 μg/mL (+ antigen). Where indicated, cells were pretreated with 1 μM okadaic acid (OA) before cytokine stimulation. For each condition, 5-8 cells per donor were analyzed and the data of two donors were combined. p<0.01: **, p<0.0001: ****, ns: not significant, one-way analysis of variance (ANOVA) and Tukey's post hoc test. (B) ADCC of CD20- expressing tumor cell lines with 1 μg/mL IgG-anti-CD20 (rituximab) and human neutrophils (40:1 E:T ratio). TNFα-stimulated neutrophils were compared to unstimulated neutrophils (control). One representative experiment of n=2 independent experiments per cell line is depicted. p<0.05: *, p<0.01: **, p<0.0001: ****, Student's t test.

with IFNγ and TNFα increased FcγRI clustering, both before and after adding antigen (Fig. 6A). Also in monocytes, this cytokine-induced increase in cluster size was prevented by pretreatment with OA. These data confirm that cytokine stimulation increases FcγRI clustering in human monocytes.

Finally, we investigated if cytokine stimulation can also enhance FcγR effector functions. To study this, we measured ADCC of neutrophils towards different CD20-expressing tumor cell lines in the presence of the therapeutic IgG-anti-CD20 antibody rituximab. Two activating FcγRs are expressed by neutrophils: FcγRI and FcγRIIa, both of which are under inside-out control. Neutrophils induced tumor cell lysis of Ramos, Daudi, and EL4-CD20 cells with an anti-CD20 antibody (Fig. 6B). Cytokine-stimulated neutrophils showed a significant increase in ADCC capacity, especially when EL4-CD20 cells were used as targets (Fig. 6B). Expression of both FcγRI and FcγRIIA on neutrophils did not change during these experiments (Fig. S5C). These results in primary human cells confirm that cytokine-induced inside-out signaling of FcγRs, especially FcγRI, is a physiologically relevant mechanism in immune cell function.

DISCUSSION

Our results provide the first evidence for a nanoscale reorganization of FcγRI in response to cytokine stimulation. Changes in FcγRI clustering were consistently observed in both our model cell line and primary human monocytes. The increase in receptor clustering was prevented when cells were treated with inhibitors of PP1 and actin polymerization, indicating an important role for phosphatase and cytoskeletal activity in FcγRI inside-out signaling. We propose a model in which following cytokine stimulation of immune cells PP1 activity and actin polymerization together lead to enhanced FcγRI clustering in the plasma membrane (Fig. 7). This leads to increased FcγRI-IC binding capacity and, ultimately, enhanced FcγRI effector functions.

The specific dependence of cytokine-induced FcγRI activation on actin polymerization suggests that this process may induce cytoskeleton rearrangements that facilitates FcγRI clustering and sensitizes the cells to bind opsonized pathogens. Actin might also facilitate the binding or recruitment of other proteins, which in turn can enhance FcγRI clustering. Previously, the CY domain of FcγRI has been shown to interact with the actin-binding protein filamin A. One study indicated that the interaction between filamin A and FcγRI is reduced upon large IC binding to FcγRI, possibly uncoupling FcγRI from the actin cytoskeleton to allow efficient phagocytosis[27]. Intriguingly, a recent study on the human interactome identified a significant interaction between filamin A and PP1[28]. FcγRI was not expressed in these cells (HeLa cells), therefore a direct interaction between FcγRI and other proteins like PP1 was not investigated.

For many other immune receptors, receptor clustering by microdomains is important for their regulation and function. One well-known example is the role of clustering in integrin inside-out signaling, where even a subtle increase in clustering can lead to substantial increases in cellular adhesion[29]. Furthermore, clustering has been shown to facilitate antigen binding to FcεRI, regulate BCR activity, and enhance FcγRIIa binding to ligand[15,20,30]. Besides clustering, conformational changes in the extracellular domain of receptors can be involved in receptor activation. For example, the activation of integrins requires a distinct conformational change from a bent to an extended conformation, essential for higher ligand binding affinity[8]. In case of the BCR, a more open conformation of the receptor is induced for its activation[31]. In this study, we focused on altered lateral mobility and clustering of FcγRI in the plasma membrane as possible mechanism of FcγRI inside-out activation. However, recently the crystal structure of FcγRI in complex with Fc was resolved[32]. Additional crystallography data showed that EC3 undergoes a conformational shift after IgG is bound[33], and might represent an FcγRI conformation that binds with higher affinity to IgG-IC. Future research might elucidate whether the increased clustering of FcγRI after cytokine stimulation coincides with a conformational change of the receptor.

We demonstrate that the CY domain of FcγRI is necessary for inside-out signaling, but that the serine/threonine motifs within this domain do not play a role. In previous studies of FcγRI-transfected P388D1 cells, serine phosphorylation of the CY-domain was shown to have constitutive phosphorylation using an anti-phosphoserine antibody on

Western Blot[34]. Because this approach was not quantitative, we instead chose to determine the degree of phosphorylation of FcγRI using a Phos-tag SDS-PAGE gel, which allows for direct quantification of protein phosphorylation. We found a phosphorylated fraction of FcγRI in unstimulated cells that is absent in the FcγRI 4S/2T>A mutant, indicating that this signal represents serine/threonine phosphorylation. Edberg *et al.* also showed that the CY-domain serines are transiently dephosphorylated upon FcγRI crosslinking

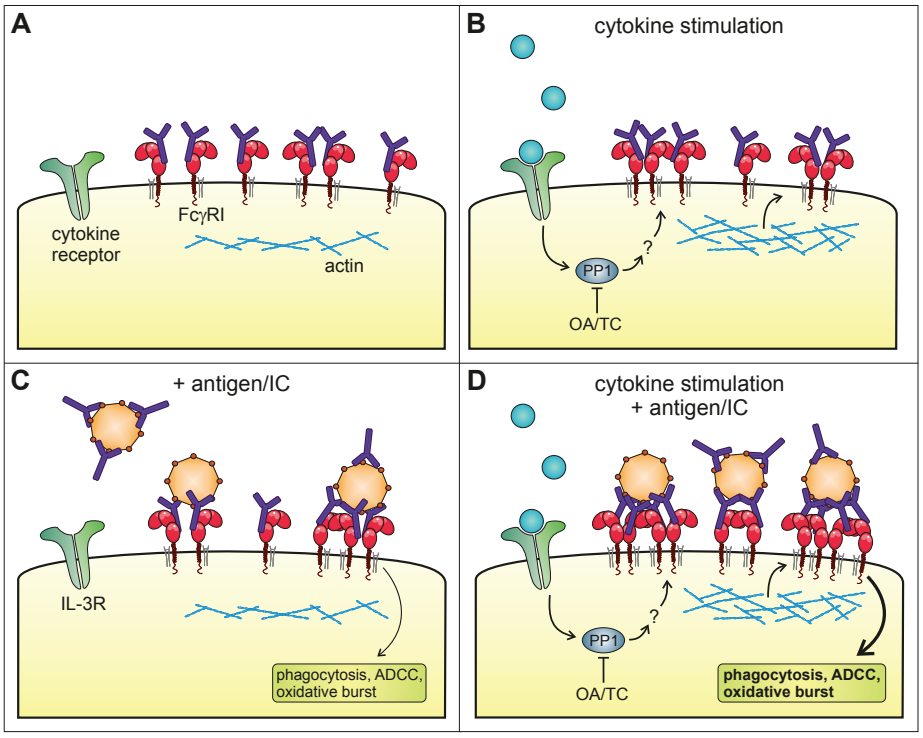

Figure 7. Model for FcγRI inside-out signaling. (A) In unstimulated cells, FcγRI is bound by monomeric IgG because of its high affinity. FcγRI might exist as monomers or possibly small multimers, as indicated by the two-color dSTORM data. (B) Cytokine stimulation of FcγRI-expressing cells first induces 'outside-in' signaling when a cytokine binds its cytokine receptor. Subsequently, inside-out signaling is initiated resulting in increased clustering of FcγRI in the plasma membrane. The phosphatase PP1 is essential for this process and can be blocked by okadaic acid (OA) and tautomycetin (TC). Increased actin polymerization is likely to facilitate the clustering of FcγRI, although these clusters retain the same mobility as without cytokine stimulation. Together, this leaves the cell in a primed state that has a stronger immune complex (IC) binding capacity. (C) The presence of antigens or IC can induce clustering of FcγRI. These cluster sizes are approximately similar to the inside-out signaling induced clusters of FcγRI. However, the mobility of these clusters is decreased, since antigen or IC binding initiates crosslinking of FcγRI that leads to ITAM signaling and FcγRI effector functions. (D) Antigens or IC can bind more efficiently to FcγRI on cells stimulated with cytokines. Both the inside-out signaling and the antigen increase the FcγRI clusters, resulting in large FcγRI clusters in the plasma membrane and stronger effector functions like ADCC. FcγRI in these clusters is also less mobile compared to stimulated cells without antigen.

(outside-in signaling), and this dephosphorylation was prevented by OA treatment[34]. This is in contrast to the inside-out signaling, where OA inhibits enhanced IC binding of FcγRI but does not directly influence FcγRI phosphorylation. Furthermore, the 4S>A mutant generated by Edberg *et al.* resulted in reduced phagocytosis compared to FcγRI wild-type[34], while our 4S/2T>A mutant has no influence on IC binding after IL-3 stimulation (Fig. 5D). IL-3 stimulation leads to inside-out activation of FcγRI, as measured by increased IC binding in the rosette assay, which is performed at 4°C thereby not allowing for any outside-in signaling. However, phagocytosis is dependent on FcγRI outside-in signaling leading to ITAM phosphorylation of the Fc receptor γ-chain. We conclude that outside-in and inside-out signaling are distinct processes and that dephosphorylation of these four serines, although important for outside-in signaling leading to phagocytosis, are not required for cytokine-induced inside-out signaling.

Receptor phosphorylation is not the only post-translational modification that can influence protein function. Since direct phosphorylation of FcγRI is excluded by our data, other modifications such as ubiquitination and methylation might also affect FcγRI function or localization. For other FcR, including FcεRI, FcγRIIa and FcγRIIIa, ligand-induced ubiquitination has been reported to be essential for receptor internalization and degradation, providing a negative feedback on Fc receptor activity[35]. For FcγRI, ubiquitination may play a role as well since this receptor is continuously internalized and recycled to the plasma membrane within minutes under steady state conditions. Therefore, in future studies it would be very interesting to monitor the ubiquitination of FcγRI in resting, inside-out activated, and IC-bound cells.

One promising clinical application of cytokine stimulation is to enhance the activity of neutrophils (or other FcγRI-expressing immune cells) during administration of anti-tumor therapeutic antibodies (Fig. 6B; [10]). Previous studies have already demonstrated that administration of cytokines in combination with therapeutic antibodies against several tumor antigens increased the efficacy of these antibodies both *in vitro* and *in vivo*[12,36,37]. However, many of the tested cytokines in these *in vivo* studies are associated with processes that take hours or even days. G-CSF for example, has been shown to be associated with increased FcγRI expression on neutrophils and increased recruitment of effector cells[37]. The inside-out signaling of FcγRI we describe here occurs rapidly – on the order of minutes – to promote FcγRI-IC binding. Therefore, we expect that cytokine stimulation in combination with antibody therapy would increase the binding capacity of FcγRI-expressing cells to tumor cells directly after cytokine administration (minutes), while increasing FcγRI expression and recruiting more effector cells from the bone marrow occurs later (hours or days). Together this can lead to more effective anti-tumor responses, especially when the timing of cytokine administration is taken into account.

In summary, we have shown that enhanced nanoscale receptor clustering underlies the increased macroscale IC binding. Thorough understanding of the regulatory mechanisms of FcγRI will aid us in manipulating immune responses using cytokines, for example during infections, vaccinations, antibody immunotherapy, or autoimmune diseases.

MATERIALS AND METHODS

Cell lines

Ba/F3 (murine pro-B cell line), Ramos, Daudi, and EL4-CD20 cells were cultured in RPMI medium (RPMI 1640; GIBCO) supplemented with 10% fetal calf serum (FCS), penicillin/streptomycin, and murine IL-3 (kindly provided by Paul Coffer, UMC Utrecht)[7,9]. The retroviral vector pMX human FcγRI was described previously[24]. In addition, the human FcγRI in this vector was replaced with a fusion protein of human FcγRI and eYFP. Using site-directed mutagenesis the 4 serines (Ser^{328}, Ser^{331}, Ser^{339}, and Ser^{340}) and 2 threonines (Thr^{312} and Thr^{374}) in the intracellular domain of FcγRI were mutated to alanine (4S/2T>A mutant); to generate FcγRI ΔCY, first Thr^{312} was mutated to alanine and second Glu^{316} was mutated to a stopcodon. Amphotropic viral particles produced in HEK293T cells were used to transduce Ba/F3 cells. Ba/F3-FcγRI (with mutations) and Ba/F3-FcγRI-eYFP cells were sorted on a FACSAria (BD Biosciences) for FcγRI expression.

Reagents

Antibodies: anti-FcγRI, either unlabeled rabbit IgG1 (clone EPR4624; Abcam) or Alexa Fluor 488 (AF488) or AF647 labeled (mIgG1 clone 10.1; Biolegend); eFluor450 labeled anti-CD14 (61D3; eBioscience); FITC labeled goat-anti-rabbit IgG (Jackson ImmunoResearch); goat-anti-rabbit IgG HRP conjugated (Pierce); rabbit IgG-anti-dinitrophenyl (IgGαDNP, polyclonal; Vector Labs). IgGαDNP was biotinylated and subsequently conjugated to QD655 (Invitrogen) as described for IgE[20]. IgGαDNP and human IgG1-anti-trinitrophenyl (IgG1αTNP)[38] were fluorescently labeled with AF647 or Cy3B (Life Technologies) following the same protocol as for biotinylation. Where indicated, 1 μM okadaic acid (OA; Enzo Life Sciences) or 1-1000 nM tautomycetin (TC; Tocris) was added for 30 min or 0.1 μg/mL latrunculin A (LatA; Life Technologies) was added 10 min before IL-3 stimulation. Phos-tag Acrylamide was from Wako-Chem. The anti-human CD20 antibody rituximab (Roche) was purchased from the pharmacy of UMC Utrecht. Eight-well Lab-Tek chambers (Nunc, Rochester, NY) were coated with poly-l-lysine (1 mg/mL in 10% 1x PBS, 90% water) for 30 min at room temperature (RT). Thrombin and fibrinogen were both from Enzyme Research Laboratories.

FcγRI (CD64) expression and IC binding

Ba/F3-FcγRI cells were starved from cytokines overnight in RPMI with 1% FCS (RPMI-1%FCS). The next day, cells were stimulated with IL-3 (in RPMI-1%FCS) for 1 hour at 37°C. For confocal microscopy, cells were added to eight-well Lab-Tek chambers, fixed with 4% paraformaldehyde (PFA; Sigma) and stained with AF488 anti-FcγRI. Images were collected on a Zeiss LSM510 two-photon confocal microscope (Zeiss Axiovert 200M inverted microscope with X,Y-motorized stage) with a 63× oil-immersion objective using an argon laser.

For flow cytometry, 1×10^5 Ba/F3-FcγRI cells/well were added to a 96-well plate,

washed with cold PBS and stained with AF647 anti-FcγRI for 1 hour at 4°C. Afterwards, cells were washed with PBS and fixed with 1% PFA. Expression of FcγRI was measured on a FACSCanto II (BD Biosciences). FcγRI expression after IL-3 stimulation was routinely measured. For monocyte flow cytometry, 2×10^5 PBMCs/well were added to a 96-well plate and stimulated with TNFα and IFNγ (500 and 400 U/mL, respectively) for 1 hour in RPMI-1%FCS at 37°C. Afterwards, cells were washed once with cold PBS and stained with eFluor450 anti-CD14 and AF647 anti-FcγRI for 1 hour at 4°C. Cells were washed with PBS and fixed with 1% PFA. Expression of FcγRI on $CD14^{high}$ monocytes cells was measured on a FACSCanto II (BD Biosciences).

Binding of Ba/F3-FcγRI to monomeric IgGαDNP was measured by plating $1x10^5$ Ba/F3-FcγRI cells/well in a 96-well plate, washing the cells with cold PBS and incubating them with different concentrations of this antibody for 1 hour at 4°C. Afterwards, cells were washed with PBS and incubated with a FITC-labeled anti-rabbit IgG antibody for 45 min at 4°C. Cells were washed with PBS and fixed with 1% PFA. IgG binding was measured on a FACSCanto II (BD Biosciences). IC binding was assessed with preformed IC (AF647-IgGαDNP:DNP_{24}-BSA mixed at 3:1 ratio) and measured on a HyperCyt Autosampler (Intellicyt).

Primary monocytes

De-identified blood was obtained from healthy donors (UNM Hospital Blood and Tissue Bank and MDD UMC Utrecht) and PBMCs were isolated by Ficoll-Paque gradient separation (GE Healthcare). PBMCs were allowed to rest for 1 hour in RPMI-1%FCS at 37°C in (uncoated) eight-well Lab-Tek chambers. Next, PBMCs were stimulated with human TNFα and IFNγ (500 and 400 U/mL, respectively) for 1 hour in RPMI-1%FCS at 37°C. During the last 10 min of incubation, 5 μg/mL IV.3 antigen binding fragments ($F(ab')_2$) and 1 μg/mL 3G8 $F(ab')_2$ were added to block the other FcγR[39]. Wells were washed with RPMI-1%FCS to remove all unbound cells, leaving the adherent cells (monocytes) in the wells. Next, monocytes were stained for SPT or super-resolution imaging.

Sample preparation for single particle tracking (SPT)

Cytokine-starved Ba/F3-FcγRI cells were stimulated with IL-3, labeled with a low concentration of QD655-IgGαDNP (2 nM) for 5 min at RT, and subsequently saturated with unlabeled IgGαDNP (100 nM) for 5 min at RT. This resulted in the labeling of single receptors (2-20 per cell). Cells were washed with PBS, resuspended in Hanks buffer and plated in eight-well Lab-Tek chambers. For each condition, data was combined from 10-20 experiments taken over 3-5 different days. Within an experiment, tracks from 5-10 different cells were acquired resulting in over 200 FcγRI trajectories/experiment.

SPT image registration and processing

SPT was performed as described previously[40,41]. Images were acquired at 20 frames/s using

an Olympus IX71 inverted microscope with a 60×1.2-numerical-aperture water objective lens combined with an extra 0.6 magnification. An objective heater (Bioptechs) maintained samples at 34-35°C. A mercury lamp with a 436/10-nm BP excitation filter provided wide-field excitation. Emission was collected by an electron multiplying charge-coupled device camera (Andor iXon 887) using a DuoView image splitter (Optical Insights) to image the QD655 (655/40 BP) probe. All data reported were collected after focusing on the apical surface of the cells. Image processing was performed using MATLAB (The MathWorks) functions in conjunction with the image processing software DIPImage (Delft University of Technology). Single molecule localization and trajectory elongation were performed as previously described[22]. The diffusion coefficient (D) was calculated based on the mean square displacement (MSD) over all QD655 tracks within an experiment[20].

Fluorescence recovery after photobleaching (FRAP)

For FRAP experiments, Ba/F3-FcγRI-eYFP cells were washed twice in PBS and seeded in a fibrin-matrix: 2.5 mg/mL fibrinogen and 1×10^{-4} U/uL thrombin in RPMI 1640 without phenol red supplemented with 1% FCS/2 mM L-glutamin on a µ-Dish (35 mm, high; Ibidi). The cells were incubated overnight in this matrix; to prevent dehydration of the matrix RPMI without phenol red with 1% FCS/L-glutamin was added. The next day, FRAP measurements were performed on these cells (no IL-3) or after IL-3 stimulation for 30 min (IL-3). FRAP experiments were performed on a Zeiss LSM710 confocal microscope with a 63× oil objective lens and an environmental chamber for temperature (37°C) and CO_2 (5%) control. An argon laser provided the 488 nm excitation. 10 pre-bleach images were acquired, after which a small area (~1 µm^2) spanning the membrane was bleached for 0.2s to obtain a bleach of ~50%. The fluorescence in this region was monitored by acquiring images at 7.3 frames/s for 30-35 s per cell. For each condition, >70 cells were measured.

FRAP data analysis

The fluorescence intensity of the bleached area was corrected for loss of fluorescence during the measurement (by subtracting the background fluorescence intensity and correcting for the overall fluorescence intensity) and normalized (by setting the mean fluorescence before bleaching to 1; this corrects for differences in cell fluorescence between measurements). The relative mean fluorescence intensity of the bleached area of all cells per condition was plotted and a non-linear two-phase association (GraphPad Prism 6 software) was used to fit the experimental data. To determine the mobile fraction (M_f) of receptors, the following equation was used:

$$M_f = \frac{I_{plateau} - I_0}{1 - I_0} \times 100\%$$

where $I_{plateau}$ is the maximal fluorescence intensity of the two-phase association fit (plateau) and I_0 is the average fluorescence intensity directly after bleaching (minimum y-axis value of the two-phase association fit).

Super-resolution imaging

Cytokine-starved Ba/F3-FcγRI were labeled with AF647-IgGαDNP (2 μg/mL) at RT after IL-3 incubation. For two-color super-resolution imaging, cells were labeled with a mix of AF647-IgGαDNP (0.667 μg/mL) and Cy3B-IgGαDNP (1.333 μg/mL) at RT after IL-3 incubation. After labeling, cells were washed with PBS and incubated in Hanks buffer with or without antigen (DNP_{24}-BSA at 1 μg/mL, unless other concentrations are indicated) for 10 min at 37°C, to induce IC. Cells were washed with Hanks buffer, plated in eight-well Lab-Tek chambers and allowed to adhere for 10 min at 37°C. Cells were then fixed with 4% PFA and 0.2% gluteraldehyde for 1-2 h. Prior to super-resolution imaging, 200 μL of fresh SRB (Super-resolution buffer: 50 mM Tris, 10 mM NaCl, 10% glucose, 168.8 U/mL glucose oxidase, 1404 U/mL catalase, 10 mM cysteamine hydrochloride, pH 8.0) was added to the well. Labeling of human monocytes followed the same protocol as for Ba/F3-FcγRI, with some adjustments: labeling and incubation with antigen were both done at 37°C and monocytes were fixed immediately after incubation with IC.

dSTORM imaging was performed using an inverted microscope (IX71; Olympus America) equipped with an oil-immersion objective 1.45-NA total internal reflection fluorescence objective (U-APO 150×; Olympus America)[18]. A 637-nm diode laser (HL63133DG; Thorlabs) was used for AF647 excitation and a 561 nm frequency-doubled diode laser (Spectra-Physics Cyan Scientific; Newport, Irvine, CA) was used for Cy3B excitation. A quad-band dichroic and emission filter set (LF405/488/561/635-A; Semrock) was used for sample illumination and emission. Emission light was separated onto different quadrants of an AndorIxon 897 electron-multiplying charge-coupled device (EM CCD) camera (Andor Technologies, South Windsor, CT), using a custom built 2-channel splitter with a 585 nm dichroic (Semrock) and additional emission filters (692/40 nm and 600/37). The sample chamber of the inverted microscope (IX71; Olympus America, Center Valley, PA) was mounted in a three-dimensional piezostage (Nano-LPS; Mad City Labs, Madison, WI) with a resolution along the *xyz*-axes of 0.2 nm. Sample drift was corrected for throughout the imaging procedure using a custom-built stage stabilization routine. Images were acquired at 57 frames/s in TIRF and between 10,000–20,000 frames were collected for each image reconstruction.

For fig. S1D, Ba/F3-FcγRI cells were incubated with 1 μg/mL DNP24-BSA or preformed IC (AF647-IgGαDNP:DNP_{24}-BSA mixed at 3:1 ratio). For fig. S1E, Ba/F3-FcγRI cells were labeled with AF647-human IgG1αTNP. This anti-TNP antibody is cross-reactive with DNP and binds DNP24-BSA with a similar affinity as rabbit IgGαDNP (as measured with a DNP_{24}-BSA binding ELISA).

Super-resolution image reconstruction and data analysis

dSTORM images were analyzed and reconstructed with custom-built MATLAB functions as described previously[42,43]. For each image frame, subregions were selected based on local maximum intensity. Each subregion was then fitted to a pixelated Gaussian intensity distribution using a maximum likelihood estimator. Fitted results were rejected based on log-likelihood ratio and the fit precision, which was estimated using the Cramér–Rao low-

er bound values for each parameter, as well as intensity and background cut-offs.

Analysis of dSTORM FcγRI cluster data was performed using the density-based DBSCAN algorithm[19] implemented in MATLAB[44] as part of a package of local clustering tools (http://stmc.health.unm.edu). Parameters chosen were a maximal distance between neighboring cluster points of epsilon = 50 nm and a minimal cluster size of 6 observations. Cluster boundaries were produced with the MATLAB 'boundary' function, using a default methodology that produced contours halfway between a convex hull and a maximally compact surface enclosing the points. The cluster areas within these boundaries were then converted into the radii of circles of equivalent area for a more intuitive interpretation. Regions of interest (ROIs) of size 2 μm × 2 μm were selected from the set of images from which statistics for the equivalent radii were collected per ROI.

Two-color image analysis

Dual-color images were acquired by imaging AF647 and Cy3B sequentially. AF647 was imaged first to prevent photobleaching by Cy3B. To correct for shifts due to chromatic aberrations channels were aligned using multicolor beads (Tetraspek; Invitrogen). Using the piezostage, we placed a single Tetraspek bead at 36 locations (6×6) uniformly distributed across the image window. The emission position in both channels was fitted and recorded. This transform was then used to convert fits from the Cy3B channel into the AF647 channel. A channel registration data set was always taken within 2 hours of any data acquisition. This was necessary to ensure that the registration transform was relevant and that no alignment drift occurred. Two-color super-resolution datasets were analyzed by localization-based cross-correlation analysis similar to previously described methods[21,45-48]. Briefly, multiple sub-regions within the centers of each cell (of size 3 μm × 3 μm, excluding the lateral membrane region) were selected, and opposite color localizations were collected radially in 5 nm bins after being angularly averaged over a set of ROIs. The computation of the cross-correlation function was based on MATLAB code originally developed by Sarah Veatch[21]. In some of the analyses, the data was first run through H-SET (Hierarchical Single Emitter Hypothesis Test), a top-down hierarchical clustering algorithm implemented in MATLAB that collapses clusters of observations of blinking fluorophores into single estimates of their true locations (localizations)[23]. Briefly, for a cluster of observations to be collapsed into a single localization, a hypothesis test is performed with the null hypothesis that all observations come from the same fluorophore. The null hypothesis is not rejected if the p-value, calculated using a log-likelihood ratio statistic, is larger than a specified level of significance (0.01 was used here).

Rosette assay

The rosette assay using Dynabeads was adapted from Van der Poel *et al.*[7]. DNP_{24}-BSA-Dynabeads were opsonized with IgGαDNP using the indicated concentrations. Ba/F3-FcγRI cells were stimulated with IL-3, and where indicated incubated with inhibitors prior to IL-3 stimulation. Ba/F3-FcγRI cells were combined with beads and incubated for 1 hour

at 4°C on a shaker. Rosette formation was evaluated using microscopy, cells bound to ≥5 beads were defined as rosettes.

Phos-tag SDS-PAGE

To measure FcγRI phosphorylation, cytokine-starved Ba/F3-FcγRI cells stimulated with 1 ng/mL IL-3 for 45 min at 37°C (where indicated, IL-3 stimulation was preceded with treatment by inhibitors). Afterwards, cells were lysed in cold triton lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% triton with Complete EDTA-free protein inhibitor cocktail and PhosSTOP cocktail, Roche). Lysates were incubated with protein G beads coupled to mouse-anti-FcγRI antibody m22 for 4 hours at 4°C. Subsequently, beads were washed in triton lysis buffer and boiled in reducing sample buffer. Samples were loaded onto a 12% polyacrylamide separating gel (no Phos-tag) and a 7.5% polyacrylamide separating gel containing 100 μM Phos-tag (Wako-Chem) and 200 μM MnCl2 (Phos-tag)[49,50]. After electrophoresis, the Phos-tag gel was washed in a 1 mM EDTA solution, followed by a wash with EDTA-free solution, before transferring the proteins to a PVDF membrane. Membranes were blocked with BSA and incubated with rabbit anti-FcγRI monoclonal antibody (clone EPR4624) for 2 hours at RT. After washing, membranes were incubated with goat anti-rabbit HRP conjugated secondary antibodies for 1 hour at RT. FcγRI was visualized using ECL prime (GE Healthcare, imaged with a ChemiDoc MP (Bio-Rad), and quantified using Image Lab software. For quantification, non-saturated images were used.

ADCC

ADCC with ^{51}Cr-labeled target cells was described previously[51]. Briefly, 1×10^6 target cells were labeled with 100 μCi (3.7MBq) ^{51}Cr for 2 hours. After extensive washing, cells were adjusted to 10^5/mL. Blood was obtained from healthy donors at the UMC Utrecht. The PMN fraction was isolated from blood by Ficoll/Histopaque separation (GE Healthcare; Sigma-Aldrich). PMNs were pretreated for 15 min at 37°C with TNFα (250 U/mL). Effector cells (40:1 effector-to-target ratio), the mAb rituximab at 1 μg/mL, medium and tumor cells were added to round-bottom microtiter plates (Corning Incorporated). After 4 hours incubation at 37°C, ^{51}Cr release was measured in counts per minute (cpm). Percentage of specific lysis was calculated using the following formula: ((experimental cpm – basal cpm)/(maximal cpm – basal cpm)) × 100, with maximal lysis determined in the presence of 3% triton and basal lysis in the absence of Abs and effector cells.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software. An unpaired Student's t test was used to compare mean values between two groups. Statistical analysis for multiple comparisons was performed using one- or two-way ANOVA, with Tukey's or Sidak's post hoc analysis as indicated. Graphs of FcγRI cluster sizes show the median, unless indicated otherwise. Other graphs represent mean ± SD, unless indicated otherwise.

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AUTHOR CONTRIBUTIONS

J.H.W.L. and D.S.L. conceived the project and supervised all research. A.M.B., S.L.S., D.S.L. and J.H.W.L. designed the experiments and wrote the manuscript. A.M.B., S.L.S., G.L.A.B. and T.T.B. performed the experiments. S.L.S., M.J.W, C.C.V. and A.M.B. designed and performed the computational analysis. D.S.L., K.A.L. and G.V. provided expertise, specialized reagents and equipment.

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SUPPLEMENTARY FIGURES

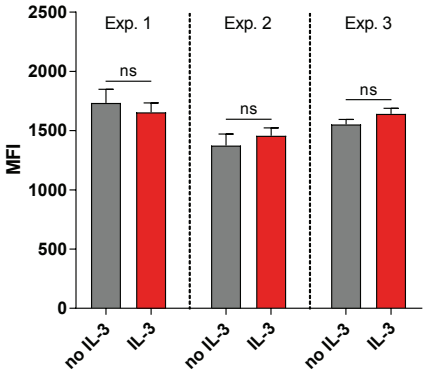

Figure S1. FcγRI surface expression does not change after IL-3 stimulation. Ba/F3-FcγRI expression was measured with mouse anti-FcγRI AF647 using flow cytometry after 1 hour IL-3 stimulation (IL-3) or not (no IL-3). Mean fluorescence intensity (MFI) is depicted of n=3 independent experiments (Exp. 1-3). ns: not significant, Student's t test.

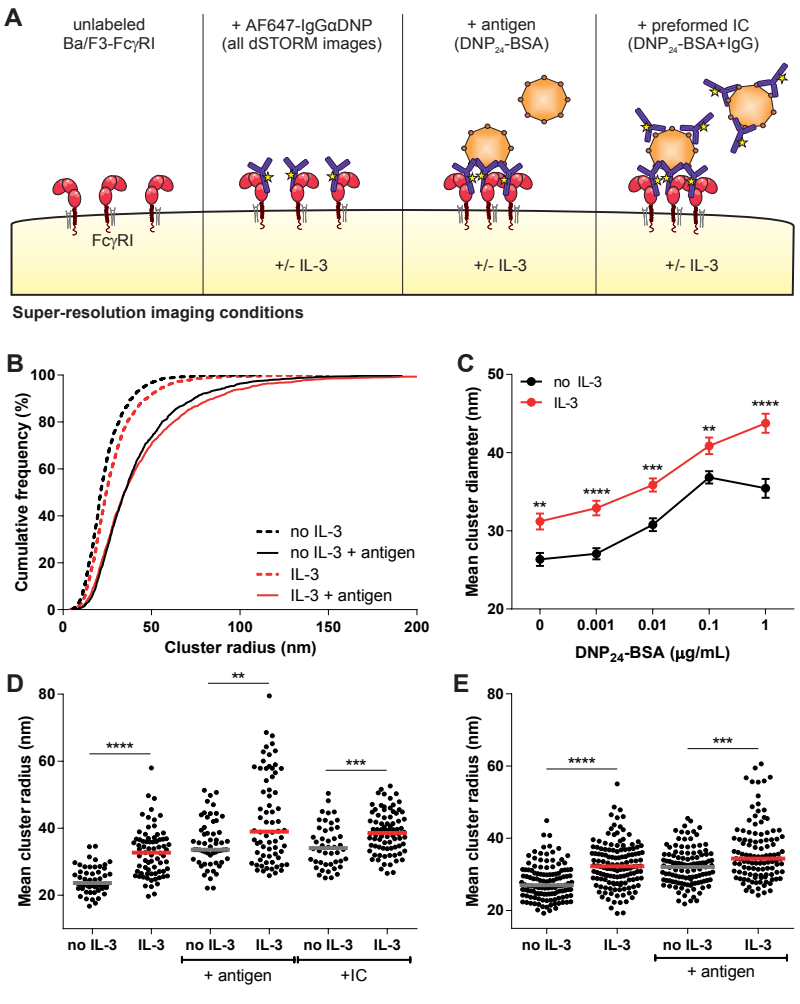

Figure S2. FcγRI clustering increases after cytokine stimulation. (A) Schematic overview of the dSTORM imaging conditions. (B) The cumulative distribution of cluster sizes for all identified FcγRI clusters used in Figure 3C. For each condition, 20-30 cells from n=3 individual experiments were combined and analyzed, resulting in 1500-2500 cluster sizes per condition. (C) The increasing FcγRI cluster sizes are dependent on antigen (DNP_{24}-BSA) concentration and IL-3. Mean FcγRI cluster sizes of each region of interest (ROI) are depicted for different DNP_{24}-BSA concentrations. For each condition, 15-20 cells from n=2 individual experiments were combined and analyzed. Values are mean ± SEM. p<0.05: *, p<0.01: **, p<0.0001: ****, Student's t test. (D) Preformed IC also increased mean FcγRI cluster radius after IL-3 stimulation. All Ba/F3-FcγRI cells were labeled with AF647-IgGαDNP. Stimulated (IL-3) or unstimulated (no IL-3) Ba/F3-FcγRI were incubated with 1 μg/mL DNP_{24}-BSA (+ antigen, to induce IC on the plasma membrane) or preformed IC (+ IC) that were composed of AF647-IgGαDNP:DNP_{24}-BSA (~3:1 ratio). For each condition, 3-5 ROIs of 8-12 cells were analyzed. p<0.01: **, p<0.001: ***, p<0.0001: ****, Mann-Whitney U test. (E) To verify that FcγRI clustering was the same with a human IgG (instead of rabbit IgG), Ba/F3-FcγRI were labeled with AF647-human IgG1 anti-TNP (this antibody also binds to DNP_{24}-BSA). For each condition, 8-11 cells from n=2 individual experiments were combined and analyzed. Line represents the median. p<0.001: ***, p<0.0001: ****, Mann-Whitney U test.

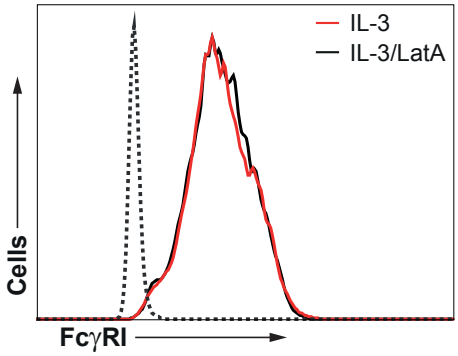

Figure S3. FcγRI surface expression does not change after latrunculin A pretreatment. Ba/F3-FcγRI expression was measured with mouse anti-FcγRI AF647 using flow cytometry after 1 hour IL-3 stimulation with pretreatment of 0.1 μg/mL latrunculin A (IL-3/LatA) or not (IL-3). Dotted line represents the isotype control.

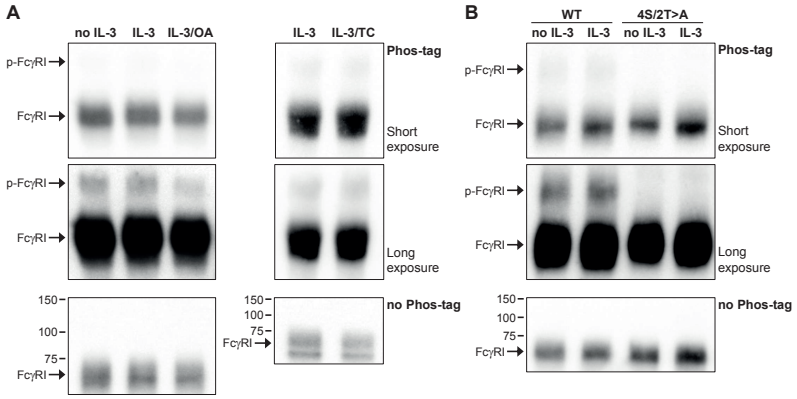

Figure S4. FcγRI phosphorylation after inside-out signaling. (A+B) Phos-tag SDS-PAGE gel and Western Blot analysis of FcγRI immunoprecipitated from Ba/F3-FcγRI cells after stimulation with IL-3 and/or pretreatment with inhibitors (1 μM OA or 100 nM TC). (B) Comparison of Ba/F3 cells expressing WT-FcγRI or mutant 4S/2T>A-FcγRI. Shifts in height indicate differential phosphorylation of FcγRI (p-FcγRI: phosphorylated FcγRI). Since a long exposure time was required to visualize the upper FcγRI band (middle panels), we also depict a short exposure image (top panels) where the lower band is not completely saturated. The bottom panels are SDS-PAGE gels without Phos-tag, showing only a single band of FcγRI at the expected height of ~72 kDa (numbers indicate the marker).

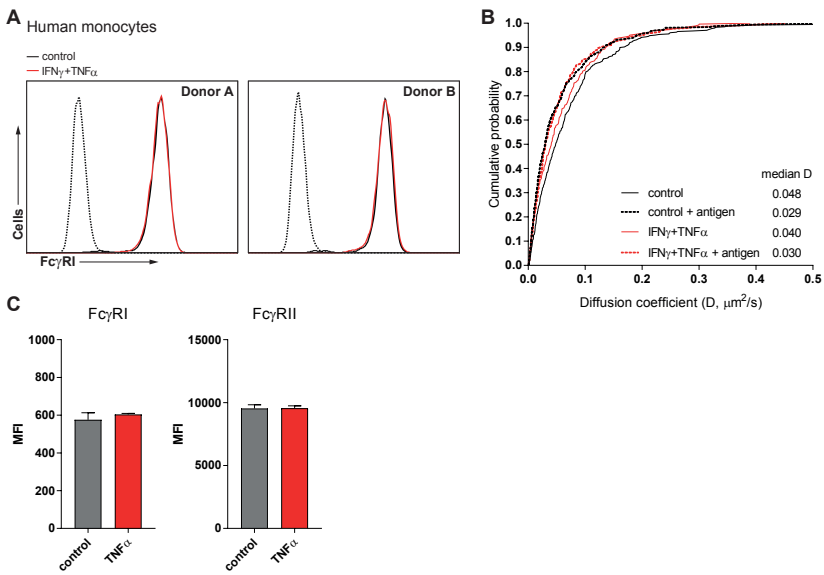

Figure S5. FcγRI expression does not change after cytokine stimulation of monocytes or neutrophils. (A) PBMCs from two healthy donors (A and B) were stimulated with cytokines (IFNγ+TNFα) for 1 hour, after which FcγRI expression was measured using flow cytometry. Monocytes were defined as $CD14^{high}$ and FcγRI expression of these cells is depicted. Dotted line represents the isotype control. (B) Single particle tracking (SPT) of FcγRI on the apical surface of human monocytes was done using QD655-IgGαDNP. Diffusion coefficient (D) values were calculated for each trajectory from n=33-46 cells per condition from two different donors. (C) FcγRI and FcγRII expression on neutrophils after 4 hours of cytokine stimulation with TNFα. Mean fluorescence intensity (MFI) is depiced. One representative of n=3 independent experiments is shown.

CHAPTER 4

Strategies to generate conformation-specific antibodies against the high affinity IgG receptor FcγRI

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Fc gamma receptors (FcγR) bind to antibodies of the IgG subclass and FcγRI is the only receptor with high affinity for monomeric IgG. The binding of FcγRI towards immune complexes can be increased by inside-out signaling. This refers to a rapid increase in ligand binding in response to extracellular stimuli, like chemokines and cytokines. Importantly, this does not change the receptor expression. Based on previous data of other receptors, like integrins, we hypothesize that FcγRI undergoes a conformational change upon cytokine stimulation. In this study, we set out to find antibodies recognizing a putative active or inactive conformation of FcγRI, with or without cytokine stimulation, respectively. First, we tested several known anti-FcγRI antibodies by using Ba/F3-FcγRI cells that activate FcγRI after IL-3 stimulation and found no difference in binding with or without IL-3. Furthermore, we observed that none of these antibodies could specifically block FcγRI ligand binding. Subsequently, we generated novel FcγRI-specific antibodies with a cellular immunization method. Unfortunately, after making Fab and $F(ab')_2$ fragments of our selected antibodies CD64-01 and CD64-02, no specific binding to FcγRI could be detected. We conclude that these novel antibodies are not FcγRI-specific. These data indicate that we have selected for antibodies binding with their Fc domain to FcγRI and not their Fab domain, regardless of our efforts to minimize Fc-FcγRI interactions in the antibody screening assays. To overcome these shortcomings, an alternative strategy was developed using antibody phage display. An 'Fc-free' antibody fragment library was generated from FcγRI-immunized mice and phages specific for FcγRI-expressing cells will be selected using a selection strategy excluding Fc-containing detection reagents, thereby avoiding unwanted Fc-FcγRI interactions during the screening process.

INTRODUCTION

Fc receptors bind the Fc domain of antibodies and are widely expressed on immune cells. This receptor family is essential for the cellular effector functions of antibodies. Most Fc gamma receptors (FcγR) specifically recognize antibodies of the IgG subclass with low affinity, but bind immune complexes (IC) such as opsonized pathogens with high affinity. FcγRI is the only receptor capable of binding monomeric IgG with high affinity. FcγRI is expressed on most myeloid cells, including monocytes/macrophages, dendritic cells, and neutrophils after activation. Besides binding to monomeric IgG, FcγRI can very efficiently bind to IC, even in the presence of monomeric IgG, and thereby initiate downstream receptor signaling and effector functions[1]. An important role for FcγRI has been described during inflammation, autoimmune responses, and monoclonal antibody therapy in tumor models[2-5].

The binding of FcγRI towards IC can be increased using stimuli like cytokines via a process termed inside-out signaling[1]. This process refers to a rapid increase in ligand binding in response to extracellular stimuli without changing the receptor expression. For integrins, inside-out signaling is well established and characterized by a large conformational change of the integrin after activation. Several antibodies have been generated that recognize this 'activated' state of the integrin, for example antibody CBRM1/5 that recognizes active Mac-1 (CD11b/CD18, αMβ2)[6,7]. These antibodies are very useful in studying the molecular mechanism of inside-out signaling as well as to assess the activation state of integrins on cells of patients[8,9]. Since FcγRI is also activated by cytokines to bind IC more efficiently without changing surface expression, we hypothesize that FcγRI undergoes a conformational change upon cytokine stimulation. Having specific antibodies against a possible 'active' or 'inactive' conformation of FcγRI would strongly support this hypothesis. Furthermore, such antibodies would be very useful tools in studying the activation state of FcγRI in patients with autoimmune diseases, inflammation, or in correlation with monoclonal antibody therapy.

Since we are interested in finding antibodies specific for an active or inactive conformation of FcγRI, antibodies that block ligand binding of FcγRI might be of particular interest because they bind in or close to the ligand binding domain of FcγRI. Several antibodies are used in research as blocking antibody for FcγRI, including clone 197, 22, and 10.1[10-13]. Indeed, in some studies a reduction of FcγRI-mediated processes like phagocytosis was observed after incubation with these antibodies at high concentrations, indicating a role for FcγRI in these processes[11,14]. We first tested these known anti-FcγRI antibodies by using the IL-3 sensitive cell line Ba/F3 transduced with FcγRI. In these cells, the cytokine IL-3 can induce FcγRI inside-out signaling[1]. All three antibodies could not distinguish between cells stimulated with IL-3 or not. Moreover, in our hands they could not block IgG binding to FcγRI. Therefore, we set out to generate novel FcγRI-specific antibodies. We used a cell-based immunization strategy and screened for possible antibodies by flow cytometry and ELISA.

MATERIALS AND METHODS

Cell lines and cell culture

Ba/F3 (murine pro B-cell line), HEK293T, ITi (ImmunoTherapy Immunization cells), and ITi-cofactor cells were cultured in RPMI 1640 medium (RPMI 1640 Glutamax; GIBCO) supplemented with 10% FCS, penicillin/streptomycin, and 0.1 ng/mL murine IL-3 (Immunotools) for Ba/F3 cells. The retroviral vector pMX human FcγRI was described previously[15]. Amphotropic viral particles produced in HEK293T cells were used to transduce Ba/F3, ITi and ITi-cofactor cells. Puromycin selection generated stable Ba/F3-FcγR, ITi-FcγRI, and ITi-cofactor-FcγRI cell populations.

Antibodies

Labeled antibodies: Alexa Fluor 647 (AF647)-labeled anti-human FcγRI (mIgG1 clone 10.1; Biolegend), APC-labeled goat $F(ab')_2$ anti-mouse IgG (H+L) (Southern Biotech), AF647-labeled anti-human CD20 (clone mIgG2c clone A8-D2; own production; referred to as 'AF647-mIgG2c'), horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (Santa Cruz). Unlabeled antibodies: anti-human FcγRI clone 197 (mIgG2a; own production), clone m22 (mIgG1; own production), clone 10.1 (AbD Serotec), anti-human CD20 clone A8-D2 (mIgG2c; own production; referred to as 'mIgG2c'), clone 10F381 (rituximab, chimeric IgG; Roche), anti-human EGFR (mIgG1 clone AY13; Biolegend; referred to as 'mIgG1'), anti-Ly17.2 (mIgG2a; kindly provided by Dr. Sjef Verbeek).

Immunizations

Animal experiments were approved by the local Animal Ethical Committee. C57BL/6 mice (Janvier Labs) were immunized by intravenous (i.v.) injection of 1.5×10^5 cells (1:1 ratio ITi-FcγRI and ITi-cofactor-FcγRI cells). Boosts were given after 5 and 11 weeks by i.v. injection of 1.5×10^5 ITi-FcγRI cells. On day 0, 21, and 56 blood was collected to analyze antibody titers in the serum. Four days after the last boost, spleen cells were fused with SP2/0 myeloma cells by using a cell ratio of 5:1 (48×10^6 splenocytes and 9.6×10^6 SP2/0 cells) using the Hybridoma Clonig Kit (Stemcell Technologies) according to manufacturer's protocol. 8×10^4 fused cells were plated per well in 96-well plates. Hybridomas were selected by growing in the presence of hypoxanthine, aminopterin, and thymidine (HAT) in semi-solid methylcellulose-based DMEM (ClonaCell™-HY Medium D, Stemcell Technologies). After 8 days, HT expansion medium (ClonaCell™-HY Medium E, Stemcell Technologies) was added to the hybridomas. On day 14 after fusion, the hybridoma supernatants were screened for FcγRI-specific antibodies. Selected hybridomas were subcloned twice by limiting dilution.

Antibody production

The selected hybridoma subclones (CD64-01, CD64-02, and CD64-03) were adapted to

low-serum medium: DMEM (Gibco) + 2.5% FCS + 1 ng/mL IL-6 (Immunotools). For antibody production, the hybridoma subclones were expanded in the low-serum medium and afterwards cultured in serum-free medium (DMEM + 1 ng/mL IL-6) for 1 week. Supernatants were harvested and filtered using a 0.22 µm membrane (Express PLUS membrane filters; Merck Millipore). Antibodies were purified using a HiTrap rProtein A FF column (GE Healthcare) fitted to the ÄKTAprime™ plus (GE Healthcare) liquid chromatography system according to manufacturer's protocol. Bound antibodies were eluted using 0.1 M sodium acetate pH 2.5 (Sigma Aldrich) and directly neutralized with 1 M Tris-HCl pH 8.8 (Roche Diagnostics). Fractions containing the antibodies were concentrated using a 100 kDa filter (Vivaspin 20 100,000 MWCO; Sartorius) and dialyzed against PBS using the Slide-A-Lyzer Dialysis Cassette 10K 3 mL (Thermo Fisher). After filtration through 0.2 µm filters, antibodies were stored under sterile conditions at 1 mg/mL. Protein concentrations were measured using Nanodrop spectrophotometer (Thermo Scientific).

Antibody screen using flow cytometry

Ba/F3-FcγRI cells were starved from cytokines overnight in RPMI 1640 with 1% FCS. The next day, half of the cells were stimulated with IL-3 for 1 h at 37°C. ITi cells were labeled with LavaCell (Active Motif), ITi-FcγRI cells with CFSE (Life Technologies), and Ba/F3-FcγRI +IL-3 cells with CellTrace Violet (Life Technologies), all according to manufacturer's instructions. Ba/F3-FcγRI no IL-3 cells were left unlabeled. Cells were mixed in a 1:1:1:1 ratio and in total 2×10^5 cells/well were added to a 96-well plate and washed with cold PBS. 5 µg/mL hIgG Fc fragment (Bethyl Laboratories) was added to wells for 30 min at 4°C as Fc block. Next, mouse serum diluted in PBS or hybridoma supernatant was added to the wells for 1 h at 4°C. After washing with cold PBS, mouse IgG bound to the cells was detected using APC-labeled anti-mouse IgG. Cells were fixed with 1% paraformaldehyde (PFA; Klinipath) in PBS and subsequently analyzed on a FACSCanto II (BD Biosciences).

Antibody screen using ELISA

Maxisorp NUNC plates (Sanbio) were coated overnight at 4°C with 0.1 µg/mL recombinant human FcγRI (extracellular domain, His tag; Thermo Fisher) in PBS followed by a blocking step with 1% BSA (Roche Diagnostics) in 0.05% Tween-20 (Immunologic) in PBS. 5 µg/mL hIgG Fc fragment in 1% BSA/0.05% Tween-20 in PBS was added to the plates for 30 min at room temperature (RT) as Fc block. Subsequently, the samples were added, diluted in 1% BSA/0.05% Tween-20 in PBS, for 1 h at RT. HRP-labeled anti-mouse IgG (1:1000) was used for 1 h at RT for detection. The plates were developed using ABTS substrate (Roche Diagnostics) and signal intensity was measured on a Multiscan RC (Thermolab systems) at 415 nm.

Flow cytometry assays

1×10^5 cells/well were added to a 96-well plate and washed with PBS. All subsequent steps were performed at 4°C. To determine FcγRI expression, cells were stained with AF647 anti-FcγRI for 1 h. For ligand blocking assays, first the indicated anti-FcγRI antibodies were added for 1 h. After washing, 5 μg/mL fluorescently labeled ligand (AF647-mIgG2c) was added for 1 h. Ligand blocking (%) was calculated by the following formula: *Ligand blocking* (%) = 100 - (*MFI Ab sample* / *MFI PBS sample*) where '*MFI Ab sample*' indicates the mean fluorescence intensity (MFI) of the samples preincubated with anti-FcγRI antibodies and '*MFI PBS sample*' indicates the average MFI of the samples preincubated with PBS. For antibody binding assays, cells were incubated with the different antibodies at the indicated concentrations. After washing, mouse IgG bound to the cells was detected using APC-labeled anti-mouse IgG for 1 h. For the ligand displacement assay, 5 μg/mL of AF647-mIgG2c was added for 1 h. After washing, anti-FcγRI antibodies were added for 1 h. Ligand displacement (%) was calculated by the same formula as for the ligand blocking assay. To measure cross-blocking of the anti-FcγRI antibodies with 10.1, the indicated anti-FcγRI antibodies were added to the cells for 1 h. After washing, AF647-labeled 10.1 was added for 1 h. The MFI of samples with AF647-labeled 10.1 only were set to 100%. For the Fc block assay, cells were incubated with 10 μg/mL hIgG Fc fragment for 30 min. Next, the anti-FcγRI antibodies were added for 1 h. After washing, bound mouse IgG was detected using APC-labeled anti-mouse IgG for 1 h. MFI of samples without Fc block were set to 100%. For all experiments, cells were washed with PBS after staining with antibodies and fixed with 1% PFA. All measurements were performed on a FACSCanto II (BD Biosciences).

Fab and F(ab')$_2$ fragment generation and analysis

For each antibody, 1 mg was digested with papain or pepsin to obtain Fab or F(ab')$_2$ fragments, respectively (Pierce Fab and F(ab')$_2$ preparation Kits; Thermo Scientific), following the manufacturer's protocol. 2.5 μg of Fab and F(ab')$_2$ preparations were subjected to reducing SDS-PAGE (4-20% polyacrylamide gradient gel, Bio-Rad). Proteins were visualized using InstantBlue Ultrafast Protein Stain (Sigma Aldrich).

Antibody phage display library generation

Mice were immunized, boosted four times, and high FcγRI-specific antibody titers were confirmed using flow cytometry, as described above. Four days after the last boost, the spleen was harvested and stored at -80°C after snap freezing. Total RNA was isolated using the RNeasy Mini Kit (Qiagen), following the manufacturer's protocol. Afterwards, cDNA was generated according to published protocols[16]. The variable regions of the light and heavy chains were amplified from the cDNA with a PCR using a gradient for the annealing temperatures (T_A: 45°C, 50°C, 55°C). Next, the amplified variable regions of the light and heavy chains were assembled by the splicing-overlap-extension (SOE)-PCR, as described previously[17]. The assembled scFv sequences were cloned in the phagemid vector pAK100

and transformed in *E. coli* according to published protocols[16].

RESULTS

Known FcγRI antibodies do not recognize activated FcγRI

Three of the most used anti-FcγRI antibodies are clone 197 (mIgG2a), m22 (mIgG1), and 10.1 (mIgG1). To determine if these antibodies might recognize an active or inactive state of FcγRI, we first generated cell lines expressing FcγRI. We transduced Ba/F3 cells, an IL-3 dependent murine pro B-cell line, and measured FcγRI expression after stimulation with IL-3 or not (Fig. 1A). FcγRI is stably expressed on these cells and there is no difference in FcγRI expression after 1 hour of IL-3 stimulation. Besides Ba/F3 cells, ITi (murine lymphoma) cells were transduced. Parental ITi cells do not express FcγRI whereas the stably transduced ITi-FcγRI cells express high levels of FcγRI (Fig. 1B). ITi and Ba/F3 cells do not express any endogenous murine Fc receptors (data not shown).

These known anti-FcγRI antibodies were tested for possibly recognizing an active conformation of FcγRI. Ba/F3-FcγRI cells were stimulated with IL-3 to induce inside-out signaling of FcγRI (+IL-3) or were left untreated (no IL-3). Unexpectedly, no difference in binding to Ba/F3-FcγRI cells with or without IL-3 was observed for 197, m22, or 10.1 (Fig. 1C). A small increase in mIgG2c binding to the Ba/F3-FcγRI +IL-3 cells was observed, in line with previous reports that monomeric IgG binding is slightly increased after FcγRI inside-out signaling[1]. Other described FcγRI antibodies like clone 32.2 and 62 were also tested but did not show differences in binding to Ba/F3-FcγRI cells with or without IL-3 (data not shown)[18]. Together, these data indicate that none of the known anti-FcγRI antibodies binds differentially to FcγRI after inside-out signaling.

Known FcγRI antibodies are not specifically blocking ligand binding

Since antibodies 197, m22, and 10.1 are used as blocking antibodies for FcγRI, we had expected that one or more of them could recognize an activated state of FcγRI. Therefore, we wanted to verify ligand blocking capacity of these antibodies. We incubated ITi-FcγRI cells with a broad concentration range of 197, m22, and 10.1 and subsequently measured the binding of ligand (Fig. 1D). A fluorescently labeled aspecific mIgG2c antibody was used as ligand. As a positive control, unlabeled mIgG2c was added to block the binding of fluorescently labeled mIgG2c. Both 197 and mIgG2c can very efficiently block ligand binding of FcγRI, especially at high concentrations, whereas m22 and 10.1 only show a moderate blockade of ligand binding at high concentrations (Fig. 1D).

Antibody 197 is of the mIgG2a isotype, which can bind via its Fc domain with the same affinity as mIgG2c to FcγRI. To test whether the Fab region of 197 has ligand blocking properties, we generated $F(ab')_2$ and Fab fragments of 197 and measured binding of these fragments to ITi-FcγRI and Ba/F3-FcγRI cells. The $F(ab')_2$ and Fab fragments of 197 show a strong reduction in ligand blocking capacity (Fig. 1E), demonstrating that the majority of ligand blocking was caused by binding of the Fc domain of 197. Also on Ba/F3

-FcγRI cells, antibody 10.1 can only modestly prevent ligand binding, strongly indicating that this is not a specific blocking antibody. Similar results were obtained when fluorescently labeled human IgG1 (hIgG1) was used as ligand (data not shown).

Together, these data indicate that 197, m22 and 10.1, three widely-used anti-FcγRI antibodies which are often used as blocking antibodies, do not specifically block the binding of IgG to FcγRI.

Optimizing screening assays for selecting FcγRI-specific antibodies

Before the mice were immunized with ITi-FcγRI cells to generate novel FcγRI-specific antibodies, we first invested in establishing robust screening assays to select antibodies that bind to FcγRI with their Fab and not their Fc domain. Especially since the high affinity of FcγRI endows strong binding of monomeric antibodies, also of mouse origin. While

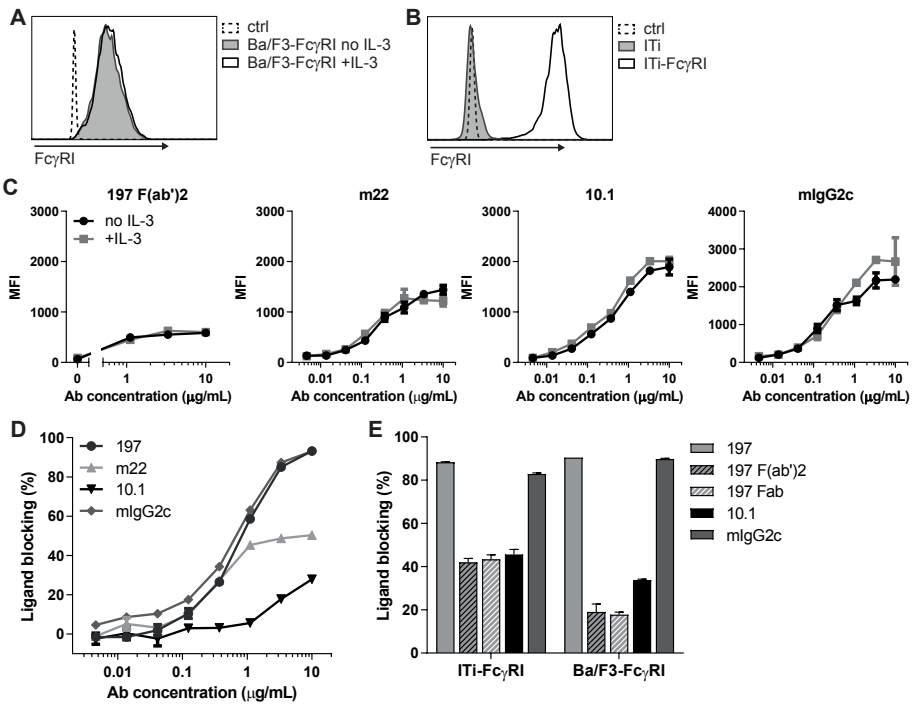

Figure 1. Known FcγRI antibodies do not recognize activated FcγRI or specifically blocking ligand binding. (A+B) Expression of FcγRI on different cell lines. Ba/F3-FcγRI no IL-3 (A) or ITi cells (B) without antibody are depicted as control (ctrl). (C) Binding of anti-FcγRI antibodies to Ba/F3-FcγRI cells treated with (+IL-3) or without (no IL-3) IL-3 to induce inside-out signaling. Mean fluorescent intensity (MFI) is depicted. (D) ITi-FcγRI cells were incubated with the different anti-FcγRI antibodies and after washing, fluorescently labeled ligand (mIgG2c) was added and binding of ligand was measured. Percentage (%) ligand blocking was calculated using ligand only as control. (E) ITi-FcγRI or Ba/F3-FcγRI cells were incubated with 10 μg/mL of the indicated antibodies. Afterwards, ligand binding was measured as in (D).

FcγRI hardly binds mouse IgG1 (mIgG1) antibodies, it binds antibodies of the mIgG2a/c and mIgG2b isotype with high affinity. mIgG2c is an allotype of mIgG2a expressed in C57BL/6 mice[19].

A cell-based flow cytometry assay was developed using a mix of cells in one well. ITi cells with or without FcγRI were used in the screen to identify antibodies specific for FcγRI while not binding other molecules on ITi cells. Ba/F3-FcγRI cells stimulated with IL-3 or not were used to identify antibodies recognizing a possible active or inactive conformation of FcγRI, as well as being another FcγRI-positive cell line. The different cells were labeled with different fluorescent dyes and could nicely be distinguished using flow cytometry (Fig. 2A). Adding 10.1 to these cells reveals an FcγRI-specific binding pattern: only cells expressing FcγRI were positive for mouse IgG (Fig. 2B). Again, no difference in binding of 10.1 to Ba/F3-FcγRI cells with or without IL-3 could be observed.

To circumvent the Fc-specific binding of mIgG2b/c antibodies to FcγRI, we tested if adding free human IgG Fc tails (Fc block) could prevent this. Indeed, preincubation of ITi-FcγRI cells with Fc block could almost completely block the binding of an aspecific mIgG2c antibody (Fig. 2C). As expected, an aspecific mIgG1 antibody did not bind to FcγRI while the FcγRI-specific antibody 10.1 could efficiently bind to FcγRI after Fc block. Interestingly, the binding of 10.1 was slightly increased when FcγRI was preoccupied with ligand (Fig. 2C). Together, this cell-based flow cytometry assay with the addition of Fc block allows for the selection of antibodies binding with their Fab domain to FcγRI expressed on the cell surface. Furthermore, the aspecific mIgG2c antibody should be taken along in all assays, to measure the background binding of mIgG2b/c antibodies.

Besides this flow cytometry assay, we developed an ELISA-based assay. The extracellular domain of FcγRI was coated onto microtiter plates and the binding of antibodies to FcγRI measured. Again, Fc block was added to prevent excess binding of antibodies with their Fc domain. Specific binding of 10.1 to FcγRI could be measured using this approach, while the binding of aspecific mIgG2c was ~2-fold lower (Fig. 2D). The difference between 10.1 and mIgG2c was smaller in this assay compared to the flow cytometry assay. Therefore, a threshold will be set at the binding of 10 μg/mL aspecific mIgG2c. Furthermore, both assays will be used in parallel to screen for FcγRI-specific antibodies to maximize the chance of obtaining FcγRI-specific antibodies with different characteristics.

Generating and screening for FcγRI-specific antibodies

Serum from mice immunized with a mix of ITi-FcγRI and ITi-cofactor-FcγRI cells was collected prior to, after the first, and after the second immunization. Especially after the second immunization, high anti-FcγRI titers in the serum were measured both by flow cytometry and ELISA (Fig. 3A-C). The polyclonal serum contained relatively low amounts of antibodies directed at the ITi cells. Interestingly, there are more antibodies binding FcγRI on Ba/F3-FcγRI +IL-3 cells compared to without IL-3 (Fig. 3A). Serum from day 0 (containing all mouse antibody isotypes) and the negative control with aspecific mIgG2c indicate that background binding of antibodies with their Fc domain is minimal (Fig. 3A-C). After a final boost, hybridomas were generated from the spleen B-cells and

two weeks later the hybridoma clones were tested for the production of FcγRI-specific antibodies. Figure 3D-E depict the 12 hybridoma clones that were selected for further subclonation based on their high ELISA signal (1B10, 4E8, and 7B8) or high flow cytometry signal (the rest). After two rounds of limiting dilution, three hybridoma subclones

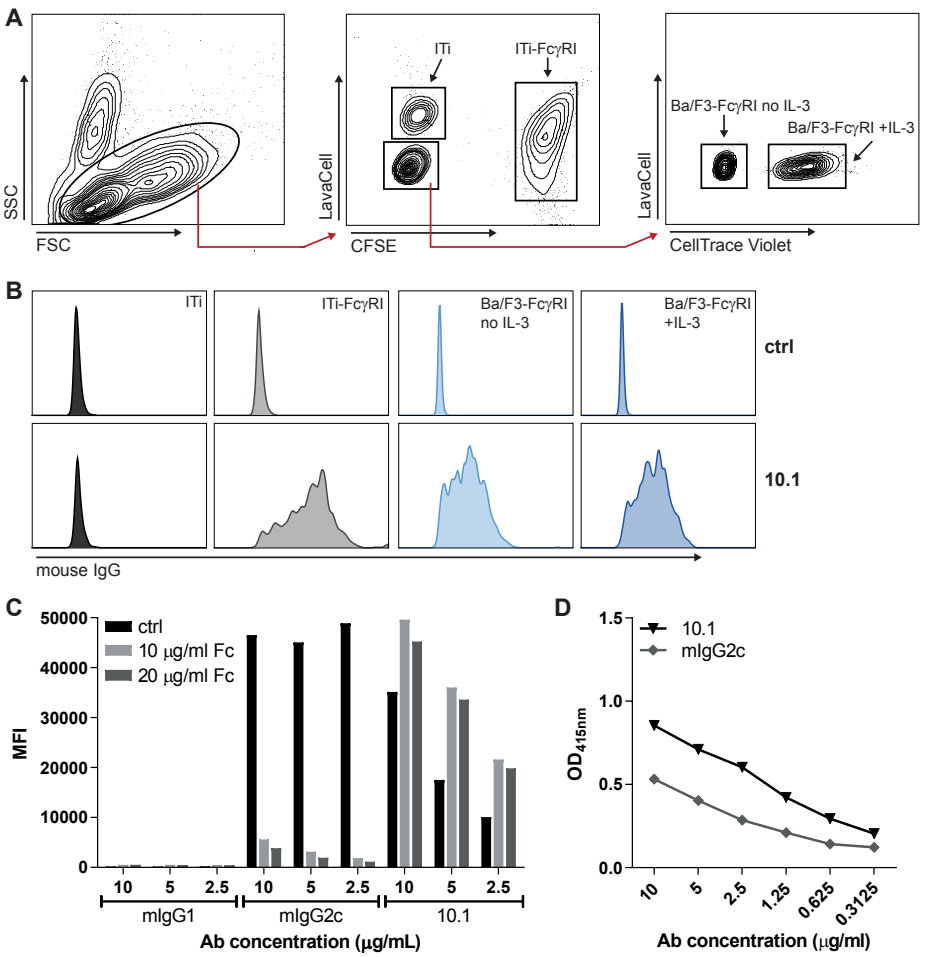

Figure 2. Optimizing flow cytometry and ELISA assays to screen for FcγRI-specific antibodies. (A) For the flow cytometry-based antibody screen, four differentially labeled cell types were mixed: ITi, ITi-FcγRI, Ba/F3-FcγRI +IL-3, and Ba/F3-FcγRI no IL-3 cells. Gating strategy is depicted. Living cells were gated based on forward scatter (FSC) and side scatter (SSC). Next, ITi cells were identified based on LavaCell positivity and ITi-FcγRI cells based on CSFE positivity. The double negative cells were gated and assessed for CellTrace Violet positivity (Ba/F3-FcγRI +IL-3) or not (Ba/F3-FcγRI no IL-3). (B) Bound mouse IgG was measured on the cell populations identified in (A). Cells incubated without antibody (ctrl) or with the anti-FcγRI antibody 10.1 are depicted. (C) ITi-FcγRI cells were incubated with 0 (ctrl), 10, or 20 μg/mL human IgG Fc fragment (Fc) to preoccupy FcγRI. Afterwards, an aspecific mIgG1, mIgG2c, or the anti-FcγRI antibody 10.1 was added at the indicated concentrations and binding of these antibodies was measured. Mean fluorescence intensity (MFI) is depicted. (D) ELISA for FcγRI- specific antibodies. Optical density (OD) values at 415 nm were measured.

producing monoclonal antibodies were selected for antibody production and further characterization. These three subclones originated from hybridoma clone 1E2 (CD64-01), clone 1E3 (CD64-02), and clone 3B9 (CD64-03).

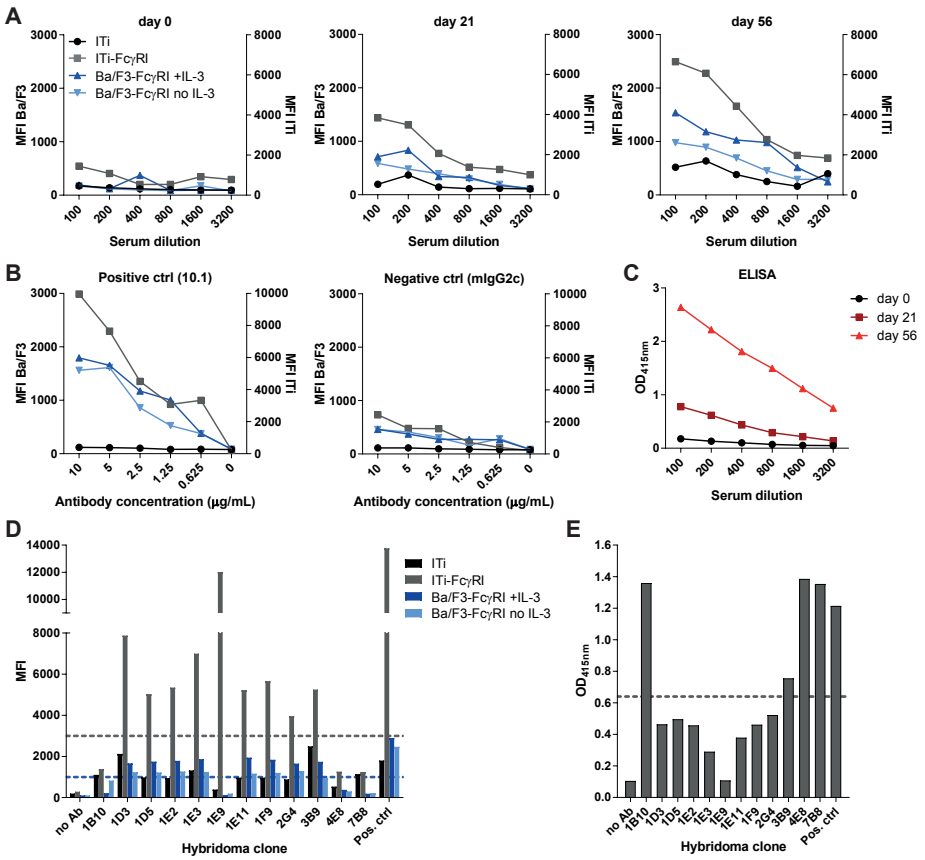

Figure 3. Screening mouse serum and hybridoma clones for FcγRI-specific antibodies. (A) Binding of antibodies in mouse serum to different cell lines expressing FcγRI (ITi-FcγRI, Ba/F3-FcγRI with or without IL-3) or not (ITi) using the flow cytometry-based screen. Serum was diluted with the indicated dilution factor. Serum from day 0 (before immunization), day 21 (after first immunization), and day 56 (after boost) was tested. Left y-axes displays mean fluorescence intensity (MFI) of Ba/F3-FcγRI cells, right y-axes displays MFI of ITi and ITi-FcγRI cells. (B) Controls for the flow cytometry serum screen. Positive control is the mouse anti-FcγRI antibody 10.1. Negative control is aspecific mIgG2c. (C) ELISA for FcγRI-specific antibodies. Recombinant human FcγRI was coated and serum from day 0, 21, and 56 after immunization were tested for binding of FcγRI-specific mouse antibodies. Optical density (OD) values at 415 nm were measured. (D+E) All hybridoma clones were screened for FcγRI-specific antibodies and the selected hybridoma clones are depicted here. (D) Binding of mouse antibodies to different cell lines, as in A. Controls without antibody (no Ab) or with the specific FcγRI-antibody 10.1 (pos. ctrl) are shown. Dashed lines represent aspecific binding: binding of 10 µg/mL mIgG2c antibody to ITi-FcγRI cells (grey line) or Ba/F3-FcγRI +IL-3 cells (dark blue line). (E) ELISA for FcγRI-specific antibodies, as in C. Dashed lines represent aspecific binding of 10 µg/mL mIgG2c antibody.

Functional characterization of antibodies CD64-01, CD64-02, and CD64-03

The hybridoma supernatant of CD64-01, CD64-02, and CD64-03 contained between 2.58 and 4.89 µg/mL antibody. Next, these three novel antibodies were compared to 10.1 and mIgG2c in a ligand blocking assay with ITi-FcγRI cells. In line with our previous experiments, 10.1 only induced a modest blockade of IgG binding to FcγRI while mIgG2c could efficiently do this (Fig. 4A). CD64-01 and CD64-03 blocked binding of IgG to the same extent as mIgG2c, while CD64-02 was slightly less efficient. None of these antibodies could displace ligand already bound to FcγRI (Fig. 4B). CD64-01, CD64-02, CD64-03, as well as mIgG2c did not interfere with 10.1 binding to ITi-FcγRI cells (Fig. 4C). Interestingly, as we observed before with the Fc block, the binding of fluorescently labeled 10.1 was increased after preincubation with these antibodies. Blocking the IgG binding site of FcγRI with Fc block reduced the binding of CD64-01, CD64-02, CD64-03 (Fig. 4D). However, this was not to the same extent as the reduction of mIgG2c binding after Fc block. 10.1 binding was markedly increased after Fc block, similar as in Figure 2C. Taken together, the initial characterization indicates that the three novel antibodies have similar properties and might be promising FcγRI blocking antibodies.

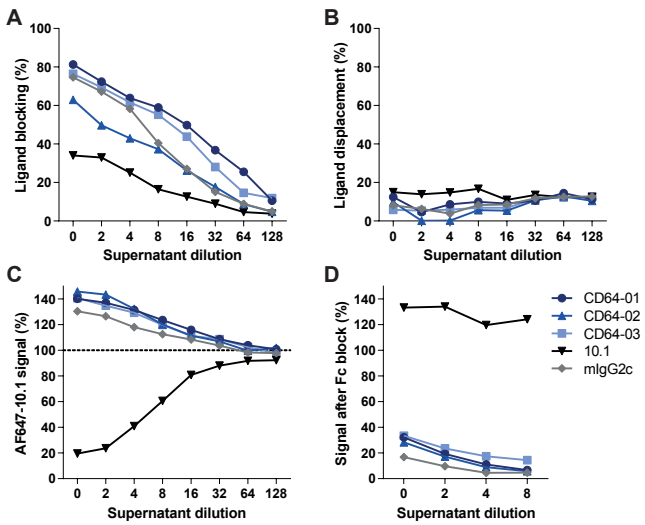

Figure 4. Functional characterization of selected antibodies. Three novel mouse antibodies (CD64-01, CD64-02, and CD64-03) were compared to 10.1 and mIgG2c in flow cytometry assays with ITi-FcγRI cells. A serial dilution of hybridoma supernatant or a 2-fold dilution series of 10.1 and mIgG2c with a starting concentration of 10 µg/mL was used. (A) Cells were incubated with antibodies and after washing, fluorescently labeled ligand (mIgG2c) was added and binding of ligand was measured. Percentage (%) ligand blocking was calculated using ligand only as control. (B) Cells were incubated with fluorescently labeled ligand and after washing the antibodies were added. Percentage ligand displacement was calculated using ligand only as control. (C) Cells were incubated with the antibodies and afterwards AF647-labeled 10.1 antibody was added. Signal of AF647-10.1 antibody only was set to 100% and percentage binding of AF647-10.1 after preincubation with antibodies was calculated. (D) Cells were incubated with 10 µg/mL human IgG Fc fragment (Fc block) or not, to preoccupy FcγRI. Antibodies were added and after washing the bound mouse IgG antibodies were detected. Fluorescence signal without Fc block was set to 100%. One representative of n=2 independent experiments is shown.

Fab and F(ab')$_2$ fragments of selected antibodies indicate that they are not FcγRI-specific

Because the functional characteristics of CD64-01, CD64-02, CD64-03 were quite similar to aspecific mIgG2c (Fig. 4), we determined the isotype of these antibodies. All three antibodies are of the mIgG2c isotype (data not shown). Next, we generated Fab and F(ab')$_2$ fragments of CD64-01 and CD64-02 to measure specific binding of the Fab domain of these antibodies to FcγRI. We did not include CD64-03, because further testing of this antibody showed aspecific binding to cells without FcγRI expression (including ITi and Ramos cells, data not shown). SDS-PAGE analysis depicts the Fab and F(ab')$_2$ fragments generated after enzymatic digestion (Fig. 5A-B). Antibody 197 was taken along as control antibody, since this antibody can bind with both its Fab and Fc domain to FcγRI. Specific binding of 197 Fab and F(ab')$_2$ was measured on both Ba/F3-FcγRI and ITi-FcγRI cells, although strongly reduced compared to intact IgG (Fig. 5C-D). Unfortunately, we could not detect binding of Fab or F(ab')$_2$ fragments of CD64-01 or CD64-02. Therefore, we conclude that these novel antibodies are not FcγRI-specific. These data indicate that we have selected for antibodies binding with their Fc domain to FcγRI and not their Fab domain, regardless of our efforts to minimize Fc-FcγRI interactions in the antibody screen.

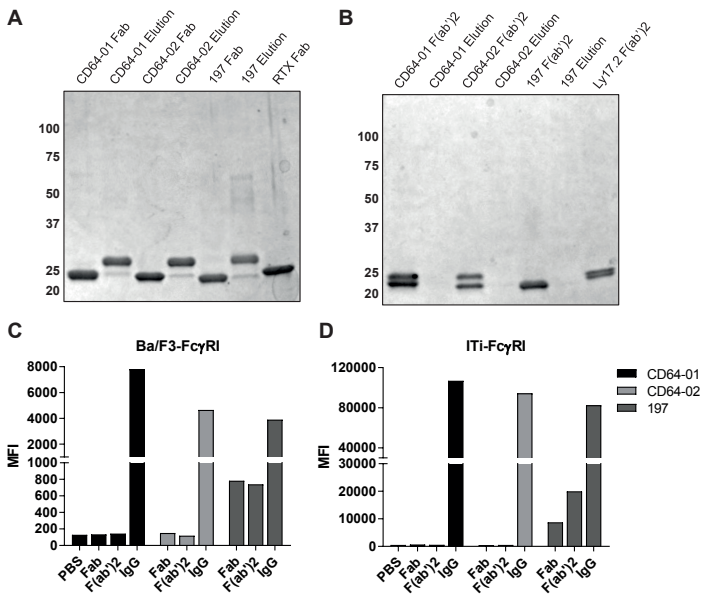

Figure 5. Fab and F(ab')$_2$ fragments of selected antibodies indicate that they are not FcγRI-specific. (A) Reducing SDS-PAGE analysis of generated Fab fragments of CD64-01 and CD64-02 compared to the anti-FcγRI antibody 197 and a positive control (rituximab Fab). Depicted are the specific Fab fractions and elution fractions (containing the Fc tails). (B) Reducing SDS-PAGE analysis of generated F(ab')$_2$ fragments. Positive control is Ly17.2 F(ab')$_2$. Depicted are the F(ab')$_2$ fractions (containing a heavy and light chain) and the elution fractions (containing no detectible protein since the remaining Fc tails are cleaved into small Fc fragments). Numbers indicate the molecular weight, in kDa, of the marker used. (C+D) Binding of 10 μg/mL of Fab, F(ab')$_2$, or intact IgG of CD64-01, CD64-02, and 197 to Ba/F3-FcγRI (C) or ITi-FcγRI (D) cells. Mean fluorescence intensity (MFI) is depicted.

Phage display library to generate FcγRI-specific antibodies

As an alternative approach to generate novel antibodies for FcγRI, an antibody phage display library can be generated to select conformation-specific FcγRI antibodies (Fig. 6). Phages only express the variable domain of antibodies as a single-chain variable fragment (scFv), thereby all Fc-FcγRI interactions are circumvented during the selection process. To generate the phage display library, mice were immunized with FcγRI-expressing cells as described above and high titers of anti-FcγRI antibodies were induced. The RNA of mouse spleen cells was isolated and cDNA generated by reverse transcription. The variable regions of the light (V_L) and heavy (V_H) chains were amplified from the cDNA using

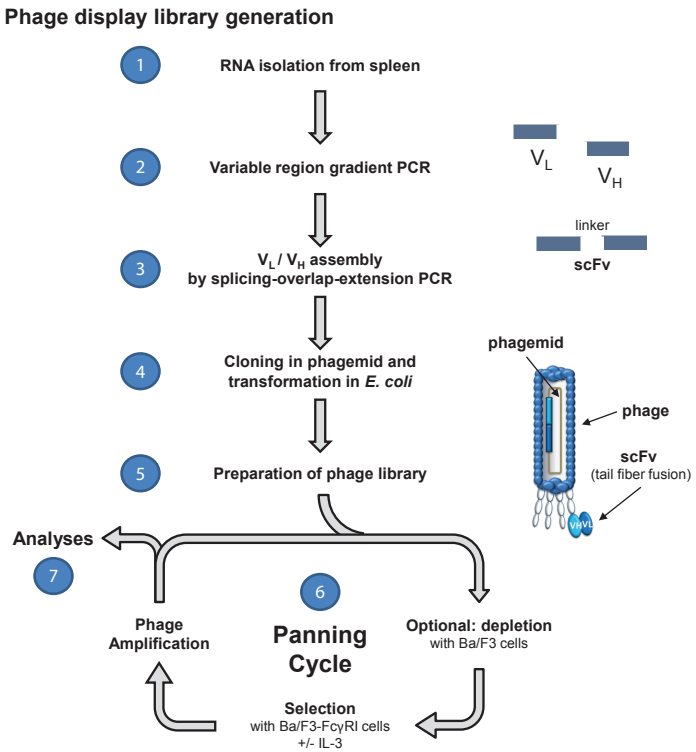

Figure 6. Outline of the phage display library generation strategy. (1) RNA is isolated from spleen of the immunized mouse. (2) cDNA is generated from the RNA and the variable regions of the light (V_L) and heavy (V_H) chains are amplified using gradient PCR. (3) Single-chain variable fragments (scFv) are generated by assembly of the V_L and V_H by splicing-overlap-extension PCR. (4) The scFv sequences are cloned into the phagemid vector and transformed into *E. coli.* (5) The phage display library is prepared. Phage particles contain the genetic information in the genome and at the same time express the functional scFv on the phage surface via fusion to the tail fiber. (6) With the phage display library, bio panning cycles are performed. First, an optional depletion step can be done to remove phages binding to non- transduced Ba/F3 cells. Next, phages specific for Ba/F3-FcγRI with or without IL-3 stimulation are selected (two selections in parallel, one with Ba/F3-FcγRI +IL-3 and one with Ba/F3-FcγRI -IL-3). The selected phages are eluted and amplified, and this cycle is repeated 2-3 times. (7) The FcγRI-specific phages can be subjected to further analyses. Phages can be used in similar ways as antibodies, but can be detected in an antibody-free method.

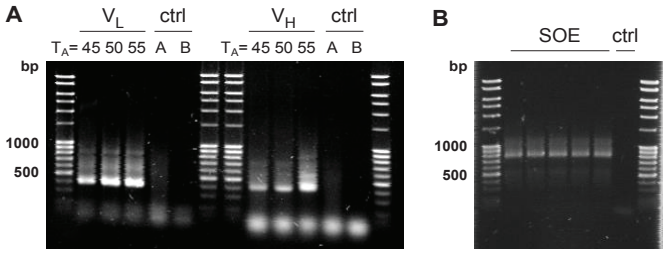

Figure 7. Amplification and assembly of heavy and light chain variable regions. (A) The variable regions of the light (VL) and heavy (VH) chains were amplified with PCR using a gradient for the annealing temperatures (TA: 45°C, 50°C, 55°C). 5 µl of all PCR samples and the negative controls (ctrl) were analyzed by gel electrophoresis. The expected size of the V_L and V_H PCR products are 350-400 bp. The first negative control (ctrl A) used RNA without reverse transcriptase in the reaction, indicating no contamination with genomic DNA in the prepared RNA. The second negative control (ctrl B) represents a water control, indicating no contamination with variable region templates of other sources in the master mix. (B) The V_L and V_H regions were assembled using splicing-overlap-extension (SOE)-PCR. 5 µl of 5 PCR samples and the negative control (ctrl) were analyzed by gel electrophoresis. The negative control represents a water control, indicating no contamination in the master mix.

a gradient PCR (Fig. 7A). No contamination with genomic DNA or other variable region templates was present in the RNA preparation. Next, the V_L and V_H regions were assembled by splicing-overlap-extension (SOE)-PCR (Fig. 7B). The assembled scFv sequences were cloned in the phagemid pAK100 and transformed in *E. coli*. An initial phage display library of 80,000-100,000 clones was generated. This library will be expanded and subsequently phages specific for Ba/F3-FcγRI with or without IL-3 will be selected, amplified and analyzed further (Fig. 6). With this strategy, novel sequences from FcγRI-specific scFv will be obtained that can be used as purified scFv or cloned into mIgG1 backbones for further functional testing.

DISCUSSION

In this study, we aimed to find antibodies that specifically recognize an active or inactive conformation of FcγRI. The previously described anti-FcγRI antibodies did not have these properties. Furthermore, none of them can block FcγRI ligand binding with their Fab domain. To generate novel antibodies against FcγRI, we immunized mice with cells expressing FcγRI and obtained high titers of anti-FcγRI antibodies. After generating hybridomas and selecting subclones for further testing we ended up with non-FcγRI specific mIgG2c antibodies binding with their Fc domain to FcγRI. This occurred despite our efforts to minimize the Fc-mediated binding of mIgG2b/c antibodies in the flow cytometry and ELISA screening assays. However, an initial antibody phage display library was generated from FcγRI-immunized mice and phages specific for FcγRI-expressing cells will be selected in the next step. This strategy allows us to generate 'Fc-free' antibody fragments and by combining this with a selection strategy that excludes Fc-containing

detection reagents, we avoid any Fc-FcγRI interactions during the screening process.

Prior to immunizations, we optimized our antibody screening assays as detailed in Figure 2 and 3 because we were already apprehensive of the efficient Fc-FcγRI interaction of mIgG2b/c antibodies. The addition of free human IgG Fc tails to preoccupy the IgG binding site of FcγRI, which was kept present during the subsequent antibody incubation step, seemed to prevent the majority of aspecific mIgG2c binding. Therefore, it is still unclear to us how our screening and selection criteria have led to the selection of the non-FcγRI specific antibodies CD64-01 and CD64-02. One possible explanation might be that the hybridoma subclones selected after limiting dilution were producing high levels of mIgG2c, likely able to compete off the IgG Fc tails from FcγRI. However, the hybridoma supernatant from CD64-01 and CD64-02 after growing them up did not contain more than 4.89 μg/mL antibody.

After discovering that CD64-01 and CD64-02 were not specific for FcγRI, we screened the mouse serum and hybridoma supernatants specifically for mIgG1 antibodies binding FcγRI since mIgG1 cannot bind FcγRI with its Fc domain. We used an fluorescently labeled anti-mouse IgG1 antibody in the flow cytometry assay (data not shown). Although low levels of anti-FcγRI mIgG1 could be measured in the serum of immunized mice, none of the hybridomas contained anti-FcγRI antibodies of the mIgG1 isotype. This indicates that with this cellular immunization method, we skewed towards inducing more mIgG2b/c antibodies compared to mIgG1.

None of the currently available anti-FcγRI can distinguish between Ba/F3-FcγRI cells stimulated with or without IL-3 (Fig. 1C). However, after immunization we could detect more antibodies in the serum binding to Ba/F3-FcγRI cells stimulated with IL-3 compared to unstimulated cells (Fig. 3A). These data might support the hypothesis that FcγRI undergoes a conformational change after inside-out signaling induced by IL-3. In a similar approach, antibodies against the active and inactive conformation of FcαRI were discovered[7]. Together, this suggests that conformation-specific antibodies of FcγRI might be generated with our immunization method. Future studies, including the phage display library approach, might provide further evidence for this and possibly lead to the generation of antibodies against an active or inactive FcγRI.

Many proteins undergo conformational changes to regulate their function. This can be induced in several ways, including post-translational modifications, binding of metal ions or small molecules, and oligomerization. The conformational change of integrins upon inside-out activation has been well described; inactive integrins have a bent/closed conformation, while active integrins have an extendend/open conformation[20]. This major conformational change leads to the exposure of neo-epitopes and several conformation-specific antibodies have been generated that recognize these neo-epitopes specifically[6,21]. Conformation-specific antibodies can also recognize aggregated proteins, which is a hallmark of protein misfolding disorders like Alzheimer's disease, Parkinson's disease, and prion diseases. The formation of amyloid-β (Aβ) oligomers is associated with the pathogenesis of Alzheimer's disease and multiple conformation-specific antibodies recognizing Aβ oligomers have been generated[22,23]. These antibodies are being tested as

therapeutic monoclonal antibodies, to clear Aβ oligomer plaques and to directly prevent the oligomerization[23,24]. Also for other proteins, for example caspase-1 and α-Synuclein, antibodies have been generated that recognize a specific conformation of the protein[25,26]. Taken together, conformation-specific antibodies are very useful diagnostic and therapeutic tools.

Phage display libraries have been used before to generate conformation-specific antibodies[27]. For example, a high affinity conformation-specific antibody against Ras was identified[28]. Blocking antibodies specific for the active conformation of integrin GPIIb/IIIa and Mac-1 have also been generated using this approach[29,30]. Therefore, we initiated the generation of an antibody phage display library to be able to select phages that express scFv specific for FcγRI on Ba/F3 cells stimulated with or without IL-3. Phages can be used in similar ways as antibodies, but can be detected in an antibody-free method and do not express the Fc domain of antibodies. Therefore, no Fc-FcγRI interactions are possible during the phage selection. Splenocytes from FcγRI-immunized mice were used for the RNA isolation, since these are mainly B cells of which a significant portion produces anti-FcγRI antibodies. This will increase the amount of phages that recognize FcγRI compared to using a phage display library from non-immunized mice. However, the assembly of V_L and V_H sequences is a random process and does not guarantee that the paired V_L and V_H from an FcγRI-specific antibody are paired together. Before the selection of FcγRI-specific phages, we will most likely deplete the phage library from phages that bind to non-transduced Ba/F3 cells. This reduces the probability of selecting phages that are specific for another antigen on the Ba/F3 cells instead of FcγRI. At the end of this study, an initial phage display library of 80,000-100,000 clones was generated. However, to be able to select (very) rare clones specific for an active or inactive conformation of FcγRI, we will expand the library to several million clones before starting the screening. Furthermore, this increases the likelihood of having the original pairing of V_L and V_H within the library.

Antibody 10.1 is the most used antibody to block FcγRI and sold by many companies as an FcγRI blocking antibody. Our data clearly demonstrate that 10.1 is not an FcγRI blocking antibody (Fig. 1 and 4). This is not surprising given that the binding site of 10.1 is located in extracellular domain 3 (EC3) of FcγRI[31], while the IgG binding site is located in EC2[32,33]. At saturating antibody conditions, a maximum of ~40% ligand blocking was achieved with 10.1 in our assays, likely indicating that 10.1 can only sterically hinder IgG from efficiently binding to FcγRI. We would therefore strongly advise that 10.1 is no longer sold and used as an FcγRI blocking antibody. Interestingly, we observed that the binding of 10.1 is increased when FcγRI is preoccupied with IgG (Fig. 2 and 4). Crystallography data demonstrated that EC3 of FcγRI slightly changes its conformation after IgG binding[33]. Together, this might indicate that 10.1 has a higher affinity for the IgG-bound conformation of FcγRI than the unoccupied conformation of FcγRI.

Blocking antibodies are widely used tools to study the function of proteins, especially in *in vivo* settings. For all other human Fc receptors, specific blocking antibodies are available that have led to substantial insights into their role in immunological processes and antibody therapy. Therapy with the anti-FcγRIIIa blocking antibody 3G8, for example, can

successfully treat immune thrombocytopenia (ITP), indicating a crucial role for FcγRIIIa in this disease[34,35]. All known antibodies binding FcγRI cannot block binding of IgG with their Fab domain (Fig. 1; [36]). Therefore, there is still a great need for an FcγRI-specific blocking antibody, especially since the high affinity of FcγRI can for example interfere with the immunophenotypic staining of FcγRI-bearing cells.

Several years ago, a novel FcγRI blocking antibody was developed and patented[5,37]. Using this antibody, the importance of FcγRI in several immunological processes was demonstrated, including autoimmune arthritis, ITP, IC mediated airway inflammation, and systemic anaphylaxis[5]. Furthermore, FcγRI can mediate protective effects of anti-tumor antibodies in a metastatic melanoma model. However, this blocking antibody anti-hFcγRI.1 is of the mIgG2a isotype (Pierre Bruhns, personal communication, November 17, 2014) and is used as intact IgG in these experiments without comparison to an isotype control. Therefore, we cannot exclude that the blocking effects of this antibody are due to Fc-FcγRI interactions. The specificity of anti-hFcγRI.1 has not been demonstrated, for example by generating Fab and $F(ab')_2$ fragments of anti-hFcγRI.1. Our study stresses the importance of including these controls when testing novel anti-FcγRI antibodies.

In conclusion, our efforts to generate novel anti-FcγRI antibodies, recognizing a possible active conformation of FcγRI or blocking FcγRI ligand binding, were unsuccessful up to now. However, the data presented here do provide insight into FcγRI characteristics regarding antibody binding and we emphasize the need for a specific FcγRI blocking antibody.

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CHAPTER 5

Single nucleotide polymorphisms of the high affinity IgG receptor FcγRI reduce immune complex binding and downstream effector functions

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Binding of IgG antibodies to Fc gamma receptors (FcγR) on immune cells induces FcγR crosslinking that leads to cellular effector functions, such as phagocytosis, antibody-dependent cellular cytotoxicity, and cytokine release. However, polymorphisms in low affinity FcγR have been associated with altered avidity toward IgG, thereby substantially impacting clinical outcomes of multimodular therapy when targeting cancer or autoimmune diseases with monoclonal antibodies as well as the frequency and severity of autoimmune diseases. In this context, we investigated the consequences of three nonsynonymous single nucleotide polymorphisms (SNPs) for the high affinity receptor for IgG, FcγRI. Only SNP V39I, located in the extracellular domain of FcγRI, reduces immune complex (IC) binding of FcγRI while monomeric IgG binding is unaffected. This leads to reduced FcγRI effector functions, including Fc receptor gamma chain (FcRγ) signaling and intracellular calcium mobilization. SNPs I301M and I338T, located in the transmembrane or intracellular domain, respectively, have no influence on monomeric IgG or IC binding, but FcRγ signaling is decreased for both SNPs, especially for I338T. We also found that the frequency of these SNPs in a cohort of healthy Dutch individuals is very low within the population. To our knowledge, this study addresses for the first time the biological consequences of SNPs in the high affinity Fc gamma receptor, and reveals reduction in several FcγRI functions, which have the potential to alter efficacy of therapeutic antibodies.

INTRODUCTION

The Fc receptors (FcR) bind the constant domain (Fc) of antibodies. Antibody-opsonized pathogens or cells can crosslink FcR on leukocytes, leading to effector functions like phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), and cytokine release. In this way, FcR are essential bridges between the adaptive and innate immune system. Genetic polymorphisms of IgG receptors (FcγR) have been described and extensively characterized for several members of this family, including FcγRIIA, FcγRIIIA, and FcγRIIIB[1-4]. These single nucleotide polymorphisms (SNPs) can alter ligand binding and thereby influence the outcome of immune responses.

For both FcγRIIA and FcγRIIIA one functional SNP has been described: 131H/R (histidine/arginine) for FcγRIIA and 158F/V (phenylalanine/valine) for FcγRIIIA. FcγRIIA-131HH has a higher binding capacity for IgG2 than FcγRIIA-131RR[5]. Functionally, individuals homozygous for H131 have increased responses against opsonized bacteria, including increased phagocytosis, degranulation, and cytokine release, compared to individuals homozygous for R131[6-8]. Furthermore, anti-tumor responses mediated by the therapeutic monoclonal antibody (mAb) rituximab where higher in patients with FcγRIIA-131HH[9,10]. For FcγRIIIA, 158VV has a higher avidity for IgG1, IgG3, and IgG4 compared to 158FF[2]. Consequently, IgG stimulated NK cells from individuals with FcγRIIIA-158VV had higher intracellular calcium mobilization, NK cell activation, and apoptosis induction in target cells, compared to FcγRIIIA-158FF[11]. The FcγRIIIA-158F/V SNP is also associated with clinical outcome of mAb treatment against cancer. Patients with FcγRIIIA-158VV have better responses and progression-free survival after treatment with rituximab, trastuzumab, or cetuximab[9,12-14]. On the other hand, the FcγRIIIA-158FF genotype is associated with increased incidence of auto-immune diseases, including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE)[11,15]. Thus, these FcγR SNPs appear to directly impact the effectiveness of immune responses in humans.

FcγRI is the high affinity IgG receptor and is expressed on monocytes, macrophages, eosinophils, and dendritic cells, and on neutrophils after activation. Expression of FcγRI is upregulated within hours on neutrophils during sepsis and can very efficiently be used as a biomarker for sepsis or systemic infection[16]. In the plasma membrane, FcγRI associates with the Fc receptor gamma chain (FcRγ) that contains the immunoreceptor tyrosine-based activation motifs (ITAMs) required for intracellular signaling after receptor crosslinking[17]. For many years, FcγRI has been far less studied than the low affinity IgG receptors. In part, this is caused by the dogma that FcγRI is always saturated with monomeric IgG due to its high affinity, and thereby not able to participate in mAb therapy or other immune responses. However, FcγRI can very efficiently bind to immune complexes (IC) even when preoccupied with monomeric IgG[18]. Cytokines can increase this IC binding greatly, leading to a specific activation of FcγRI at the site of infection/inflammation[18]. Furthermore, several studies have implicated an important role for FcγRI during inflammation, autoimmune responses, and monoclonal antibody immunotherapy in tumor models[19-22]. In addition, FcγRI can efficiently induce MHC class II antigen presentation and cross-presentation[23,24].

The gene encoding human FcγRI (*FCGRIA*) is located on human chromosome 1q21. Two very similar genes, *FCGR1B* and *FCGR1C*, are considered pseudogenes of *FCGRIA* and most likely originate from gene duplication. Only transcription of *FCGR1A* leads to the human FcγRI protein, since the two pseudogenes contain stopcodons in the extracellular domain (EC) 3 and are barely transcribed[25]. *FCGR1A* encodes for six exons: the signal peptide is encoded by the first two exons, EC1, 2, and 3 are encoded by exon 3, 4, and 5 respectively, and exon 6 encodes the transmembrane region and intracellular domain. Several polymorphisms have also been identified for FcγRI: three nonsynonymous SNPs (rs7531523, V39I; rs12078005, I301M; and rs142350980; I338T), and two SNPs resulting in stop codons (rs74315310, R92*; and rs1338887; Q224*)[26,27]. Until now, the functional consequences of these three nonsynonymous SNPs have not been studied[26], although they might have important consequences as was shown for the low affinity FcγR single nucleotide changes. Therefore, we explored the frequency of these SNPs in a cohort of healthy Dutch volunteers and the possible differences in receptor function.

MATERIALS AND METHODS

Nucleic acid isolation

Blood was obtained from healthy donors at the UMC Utrecht and mononuclear cells were isolated using Ficoll density centrifugation (GE Healthcare). Frozen mononuclear cells were used for DNA isolation via the MagnaPure Compact System (Roche Diagnostics). Cell samples were thawed at 37°C, dissolved in 9 mL RPMI 1640 (GIBCO) supplemented with 20% heat-inactivated fetal calf serum (FCS; Bodinco) and centrifuged for 10 min at 380g. Prior to DNA extraction, cells were dissolved in phosphate buffered saline (PBS, Sigma-Aldrich) at a concentration of 5×10^6 cells/mL.

Genotyping FcγRI SNPs by direct sequencing

PCR reactions were designed for genotyping FcγRI SNPs using gDNA as template. Two regions of the *FCGR1A* gene were sequenced: exon 3 and exon 6, encoding for the EC1 and transmembrane/cytosolic domain, respectively. For exon 3, the forward primer 5'-CCCAACACCCAGATGAGGAT-3' and reverse primer 5'-GGAGAGAACGCAGAGGAAGA-3' were used. For exon 6, the forward primer 5'-CCAAACATAACTCAGCTAGACC-3' and reverse primer 5'-GGACGGTCCAGATCGATG-3' were used. Both PCR reactions were performed with 100 ng DNA, 10 pmol of each primer, 10 nmol dNTPs, 10 pmol of $MgCl_2$, 1X Phusion HF buffer, and 2 U of Phusion HF polymerase in a 25 µL reaction volume (New England Biolabs). PCR started with 98°C for 3 min, 30 cycles of denaturing at 98°C for 15 s, annealing at 65°C for 30 s, and extension at 72°C for 1 min with a final extension at 72°C for 10 min. PCR products were analyzed on a 1% agarose gel for amplification of the correct product size (538 bp for exon 3; 389 bp for exon 6). 1 µL of the PCR reaction and 10 pmol primer were combined in 10 µL final volume and the sequencing reactions were performed by EZ-Seq (Macrogen).

Generation of FcγRI expression constructs

The retroviral vector pMX human FcγRI was described previously[28]. Using site-directed mutagenesis, the FcγRI SNPs were introduced into this plasmid. The following mutagenesis primers were used (nt changes are underlined): for V39I (nt G115A) 5'-GTTCCAAGAGGAAACCATAACCTTGCACTGTGAG-3'; for I301M (nt A903G) 5'-CTATCTGGCAGTGGGAATGATGTTTTTAGTGAACAC-3'; for I338T (nt T1013C) 5'-GGTCATGAGAAGAAGGTAACTTCCAGCCTTCAAGAAG-3'. Given here are the forward primers, for each reaction also the reverse complement primers were used.

Generation of stable cell lines expressing FcγRI

Ba/F3 (murine pro B-cell line) and HEK293T cells were cultured in RPMI 1640 medium (RPMI 1640 Glutamax; GIBCO) supplemented with 10% FCS, penicillin/streptomycin, and 0.1 ng/mL murine IL-3 (Immunotools) for Ba/F3 cells. P388D1 (murine macrophage-like cell line) cells were cultured in DMEM medium (DMEM Glutamax; GIBCO) supplemented with 10% FCS and penicillin/streptomycin. Amphotropic viral particles produced in HEK293T cells were used to transduce both Ba/F3 and P388D1 cells. Puromycin selection generated stable Ba/F3-FcγRI and P388D1-FcγRI cell populations. Expression of FcγRI was evaluated using an Alexa Fluor 647-labeled anti-FcγRI antibody (clone 10.1, Biolegend) measured on a FACSCanto II (BD Biosciences).

Murine FcγR expression

To evaluate the expression of murine FcγR on Ba/F3 cells, cells were incubated with RPE-labeled anti-mFcγRI (clone X54-4/7.1.1, Biolegend), Alexa Fluor 647-labeled anti-mFcγRII antibody (clone K9.361, Ly17.2 specific, own production), or allophycocyanin-labeled anti-mFcγRIV antibody (clone 9E9, Biolegend) for 45 min at 4°C. For staining of mFcγRIII, cells were first incubated with unlabeled anti-mFcγRII antibody (clone K9.361, Ly17.2 specific, own production) for 45 min at 4°C. After washing the cells, FITC-labeled anti-mFcγRII/III antibody (clone 2.4G2, BD Biosciences) was added for 45 min at 4°C. As a positive control, P388D1 cells were stained with these antibodies. After washing, the cells were fixed with 1% paraformaldehyde (PFA; Sigma) and fluorescence intensity was measured on a FACSCanto II.

Monomeric IgG binding assay

To measure monomeric IgG binding, 1×10^5 Ba/F3-FcγRI cells were incubated with different concentrations of hIgG1 (human IgG1 anti-RSV F-protein, no F(ab') binding; Abbott) or mIgG2c (mouse IgG2c anti-human CD20, no F(ab') binding; own production) for 1 h at 4°C followed by two washes with PBS. The cells were then incubated with saturating concentrations of PE-conjugated goat-anti-human IgG (Southern Biotech) or allophycocyanin-conjugated goat-anti-mouse IgG (H+L) (Southern Biotech) for 45 min at 4°C. After washing, the cells were fixed with 1% PFA and fluorescence intensity was measured

on a FACSCanto II.

Soluble IC binding assay

Soluble immune complexes (IC) were generated by combining 10 µg/mL hIgG1 with 5 µg/mL allophycocyanin-conjugated goat $F(ab')_2$ anti-human IgG $F(ab')_2$ (Jackson ImmunoResearch) in PBS, followed by a 2x dilution series. The antibodies were incubated at 37°C for 30 min, followed by 10 min incubation at 4°C. 1×10^5 Ba/F3-FcγRI cells were combined with the soluble IC, mixed well and incubated for 1 h at 4°C followed by two washes with PBS. The cells were fixed with 1% PFA and fluorescence intensity was measured on a FACSCanto II.

IgG-coated beads assay (rosettes)

The rosette assay using Dynabeads was adapted from a previously described protocol[29]. 4.5 µm epoxy-Dynabeads were coupled to DNP_{24}-BSA following manufacturer's instructions (ThermoFisher Scientific). DNP_{24}-BSA-Dynabeads were opsonized with rabbit IgG anti-DNP (polyclonal Ab; Vector Labs) using the indicated concentrations. 1×10^5 Ba/F3-FcγRI cells were combined with 3.5×10^5 beads and incubated for 1 h at 4°C on a shaker. Afterwards, cells were fixed with 3% PFA. Rosette formation was evaluated using bright-field microscopy at 20× magnification; cells bound to ≥5 beads were defined as rosettes. For each condition, triplicates were measured and on average 400-600 cells were counted per well.

Bone marrow-derived macrophages (BMDMs)

Mouse FcγRI/III$^{-/-}$ mice on C57BL/6 background were kindly provided by Dr. J.S. Verbeek and maintained in the Animal Facility of University Medical Center Utrecht. Experiments were approved by the Animal Ethical Committee of the University Medical Center Utrecht. Bone marrow (BM) cells were harvested from mFcγRI/III$^{-/-}$ mice and the erythrocytes were lysed. 3×10^6 BM cells were seeded in a 6-well plate. BM cells were cultured in RPMI 1640 medium supplemented with 10% FCS, penicillin/streptomycin, and 10 ng/mL GM-CSF (Immunex). Amphotropic viral particles produced in HEK293T cells were used to transduce the adherent BM cells on day 3 and 4 with human FcγRI (WT or SNPs). Medium was refreshed on these days and again on day 7. At day 8, adherent cells were harvested with 50 mM EDTA. The BMDMs were used in the IgG-coated beads assay, described above. The IC binding was corrected for the percentage FcγRI-positive BMDMs, as measured by flow cytometry.

Cell stimulation and Western Blot

Microtiter plates were coated with 10 µg/mL DNP_{24}-BSA (ThermoFisher Scientific) overnight and incubated with 0, 1, or 10 µg/mL of rabbit IgG anti-DNP for 1 h to generate IC. 7.5×10^5 Ba/F3-FcγRI cells or 2.5×10^5 P388D1-FcγRI were placed onto the IC for 5 min at

37°C and afterwards lysed in 20 µL of hot reducing Laemmli sample buffer. Total lysates from 1.5×10^6 Ba/F3-FcγRI cells or 0.5×10^6 P388D1-FcγRI cells were subjected to SDS-PAGE and Western blotting. The following antibodies were used: phosphorylated ERK (p-ERK; clone E10; Cell Signaling), total ERK (clone 3A7; Cell Signaling), and α-tubulin (clone YL1/2; Merck Millipore). For detection of the p-ERK and total ERK antibodies, goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) was used; for detection of α-tubulin, goat anti-rat IgG-HRP (Santa Cruz Biotechnology) was used. After detection of p-ERK, the blot was washed and reprobed for α-tubulin detection. For quantification of p-ERK levels, non-saturated images were used. The p-ERK signal was divided by the α-tubulin signal of the same lane and p-ERK levels of FcγRI-WT were set to 1.

Calcium flux assay

For calcium assays, 5×10^5 Ba/F3-FcγRI were incubated for 20 min with 8 µM Fluo-3 and 10 µM Fura Red (Invitrogen) at 37 °C. Next, anti-FcγRI antibody (clone m22, own production) was added and cells were incubated for 10 min at 37 °C. Cells were washed twice and resuspended in Hank's balanced salt solution (HBSS) supplemented with 10% FCS. Cytosolic calcium levels were measured on a FACSCanto II as the ratio of Fluo-3/Fura Red (Cytosolic Ca^{2+} level). After 30 s of baseline measurement, a goat anti-mouse IgG antibody (Southern Biotech) was added and calcium flux was measured for 3.5 min. Subsequently, 2 µg/mL ionomycin (Calbiochem) was added as a loading control. Area under the curve (AUC) was calculated using FlowJo and GraphPad Prism 6 software and AUC of FcγRI-WT was set to 1.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software. Multiple comparison ANOVA was used to compare each FcγRI SNP to FcγRI-WT. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $P<0.0001$, ns: not significant. All graphs represent mean ± SD of triplicate measurements, unless indicated otherwise.

RESULTS

FcγRI SNP frequency

Data from the 1000 Genomes Project Phase 3 suggest SNP frequency data for V39I, R92*, and I338T[30]. However, in this project ~400 genomes from European citizens were sequenced, and none of them were of Dutch or German individuals[30]. In order to validate the findings in an independent Dutch cohort for SNPs located within FcγRI, we designed two PCR reactions to amplify specific regions from genomic DNA of 158 healthy donors. V39I and R92* are located in exon 3 of *FCGR1A*, while I301M and I338T are located in exon 6 of *FCGR1A* (approximate locations in the FcγRI protein are depicted in Fig. 1A). Exon 3 and exon 6 were fully sequenced and the frequency of the FcγRI SNPs is listed

Table 1. FcγRI SNP allele frequency in Dutch individuals

			Frequency Dutch individuals[c]		Frequency 1000 Genomes Project Phase 3	
SNP	Genotype[a]	Phenotype[b]	Major allele	Minor allele	Major allele	Minor allele
rs7531523	G115A	V39I	314 (99.37%)	2 (0.63%)	99.5%	0.5%
rs74315310	C274T	R92*[d]	316 (100%)	—	99.6%	0.4%
rs12078005	A903G	I301M	316 (100%)	—	No data	No data
rs142350980	T1013C	I338T	316 (100%)	—	99.7%	0.3%

A dash (-) denotes that in this study none of the tested donors had this allele.
[a]Genotype indicates major allele (left), the nt position in *FCGR1A* (middle), and the minor allele (right).
[b]Phenotype indicates the major amino acid (left), the amino acid position in the FcγRI protein (middle), and the minor amino acid (right).
[c]Frequencies are based on sequencing genomic DNA of n=158 Dutch individuals.
[d]Stop codon is indicated by an asterisk (*).

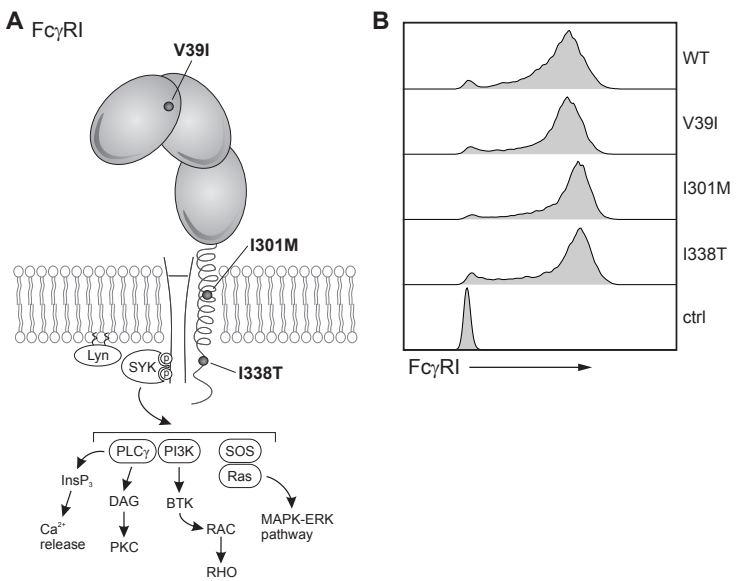

Figure 1. FcγRI SNPs location and expression on Ba/F3 cells. (A) Schematic representation of the location of FcγRI SNPs in the FcγRI protein. V39I is located in extracellular domain 1 (EC1) of FcγRI, I301M is located in the transmembrane region that associates with the Fc receptor gamma chain, and I338T is located in the intracellular domain of FcγRI. Downstream signaling after FcγRI crosslinking involves phosphorylation of the Fc receptor gamma chain and part of the further signaling machinery is depicted. (B) Ba/F3 cells were stably transduced with FcγRI, either WT or the SNP variants. FcγRI expression was measured using flow cytometry and untransduced Ba/F3 (ctrl) cells were used as control.

in Table 1. In total, we found two donors that were heterozygous for the V39I SNP (GA genotype), while the rest of the donors were all homozygous for the major alleles. In this cohort we did not observe any of the other SNPs. The minor allele frequency we found for V39I (0.63%) corresponds to the frequency found by the 1000 Genomes Project (0.5%) (Table 1). However, R92* and I338T have very similar minor allele frequencies as V39I, while we did not observe these SNPs in the Dutch cohort. Together, these data indicate, in line with data from the 1000 Genomes Project, that the frequency of FcγRI SNPs is very low within the population. Therefore, we focused on the further functional analysis of rs7531523, rs12078005, and rs142350980.

Generating Ba/F3 cells expressing FcγRI SNPs

To study the functional consequences of the nonsynonymous FcγRI SNPs, we generated plasmids for the different FcγRI SNPs using site-directed mutagenesis. We did not include the R92* SNP, since this stopcodon in EC1 does not lead to a functional protein[27]. Stable surface expression of FcγRI was obtained after transduction of Ba/F3 cells (Fig. 1B and fig. S1A), a murine pro B-cell line used before to study FcγRI function that endogenously expresses the FcRγ but does not express any murine FcγRs (Fig. S1A)[18]. However, small differences in FcγRI expression level were observed between FcγRI-WT and FcγRI-I301M or FcγRI-I338T (Fig. S1B). We isolated two single cell clones from the FcγRI-WT bulk population with different FcγRI expression (Fig. S1C) to investigate the influence of these different FcγRI expression levels. As explained in Figure S2, we corrected data for FcγRI expression when applicable throughout this study.

Monomeric IgG binding is not affected by FcγRI SNPs

Since FcγRI is a high affinity IgG receptor, monomeric IgG can efficiently bind to the receptor. We performed monomeric IgG binding assays comparing FcγRI-WT with the FcγRI SNP variants. Two different IgG isotypes, human IgG1 and mouse IgG2c, were used, of which mouse IgG2c binds with a slightly higher affinity to FcγRI than human IgG1. For both isotypes we observed a dose-dependent increase in IgG binding for all FcγRI variants. However, there were no differences in monomeric IgG binding between FcγRI-WT and any of the FcγRI SNPs (Fig. 2). This indicates that the FcγRI SNPs do not affect monomeric IgG binding of FcγRI.

FcγRI-V39I has reduced immune complex binding

As monomeric IgG binding is known not to trigger downstream signaling of FcγRI or effector functions, we next investigated immune complex binding to the SNP variants of FcγRI. First, we generated soluble IC by incubating human IgG1 with an anti-human IgG $F(ab')_2$ binding antibody and these soluble IC were then added to Ba/F3-FcγRI cells. Binding of FcγRI-I301M and FcγRI-I338T to soluble IC was comparable to FcγRI-WT. However, the binding of FcγRI-V39I, the only SNP located extracellularly, was significant-

ly reduced compared to FcγRI-WT (Fig. 3A). Next, we measured the binding of FcγRI to IgG-coated beads, which are much larger IC compared to the soluble IC and resemble opsonized bacteria or tumor cells better. Ba/F3-FcγRI cells that bound ≥5 beads were scored as rosettes (Fig. S3). Again, Ba/F3 cells expressing FcγRI-V39I had a reduced IC binding compared to FcγRI-WT as indicated by reduced rosette formation (Fig. 3B). As expected due to their transmembrane and intracellular location, the other two SNPs, I301M and I338T, had no effect on IC binding.

To test the function of these FcγRI SNPs in primary myeloid-derived cells, mouse bone marrow-derived macrophages (BMDM) from mFcγRI/III$^{-/-}$ mice were transduced with FcγRI-WT or the FcγRI SNP variants. Expression of FcγRI was confirmed by flow cytometry (data not shown) and BMDMs were incubated with IgG-coated beads to measure IC binding. While mock transduced BMDMs did not bind IC, FcγRI-WT BMDMs efficiently bound IC in a concentration-dependent manner (Fig. 3C). BMDMs expressing FcγRI-V39I had significantly lower IC binding compared to FcγRI-WT, while BMDMs expressing FcγRI-I301M or FcγRI-I338T had similar IC binding as FcγRI-WT (Fig. 3C). Together, these data indicate that the only FcγRI SNP located in the extracellular domain of FcγRI, V39I, reduces IC binding.

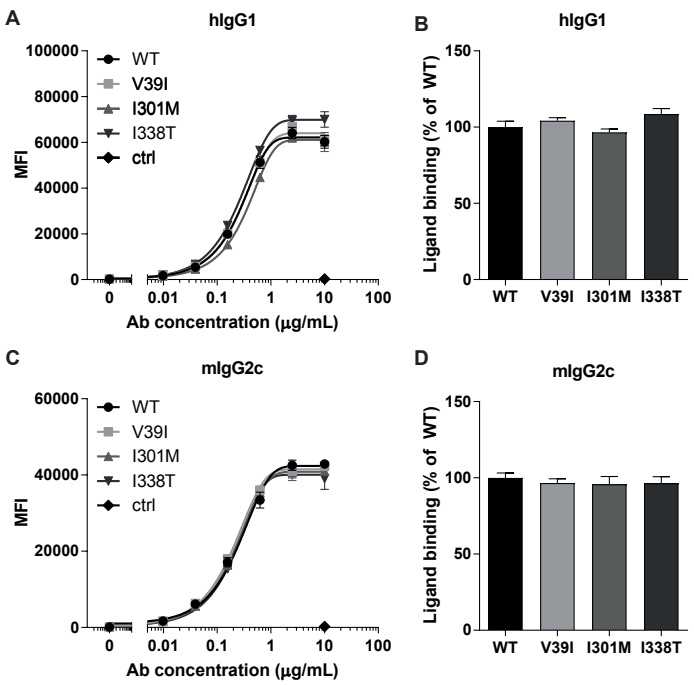

Figure 2. Monomeric IgG binding of FcγRI SNPs. Binding of monomeric IgG to untransduced Ba/F3 (ctrl) or Ba/F3-FcγRI cells was measured using flow cytometry. (A+B) Human IgG1 (hIgG1) or (C+D) mouse IgG2c (mIgG2c) was added in the indicated concentrations. Median fluorescence intensity (MFI) is depicted. (B+D) Monomeric ligand binding at 2.5 µg/mL is shown relative to FcγRI-WT, with WT set to 100% binding. The data represented here are corrected for the FcγRI expression on the Ba/F3 cells and one representative of n=4 independent experiments is shown.

Decreased intracellular calcium mobilization after crosslinking of FcγRI SNPs

Crosslinking of FcγRI induces phosphorylation of the ITAMs in FcRγ by kinases of the Src family. This leads to recruitment of SYK to the ITAMs and initiation of downstream signaling, including a rapid increase in intracellular calcium (Ca^{2+}) concentrations. This rise in Ca^{2+} levels is considered relevant for FcγR mediated phagocytosis[31]. We hypothesized that reduced IC binding of FcγRI-V39I would also lead to reduced downstream receptor signaling. In addition, SNPs located in the transmembrane and intracellular domain of FcγRI might also impact signaling. To study this, Ba/F3-FcγRI cells were loaded with calcium dyes and intracellular calcium levels were measured using flow cytometry. Within seconds after inducing in FcγRI crosslinking, the intracellular Ca^{2+} levels increased and this effect lasts for approximately 2 minutes (Fig. 4A). Quantification of multiple experi-

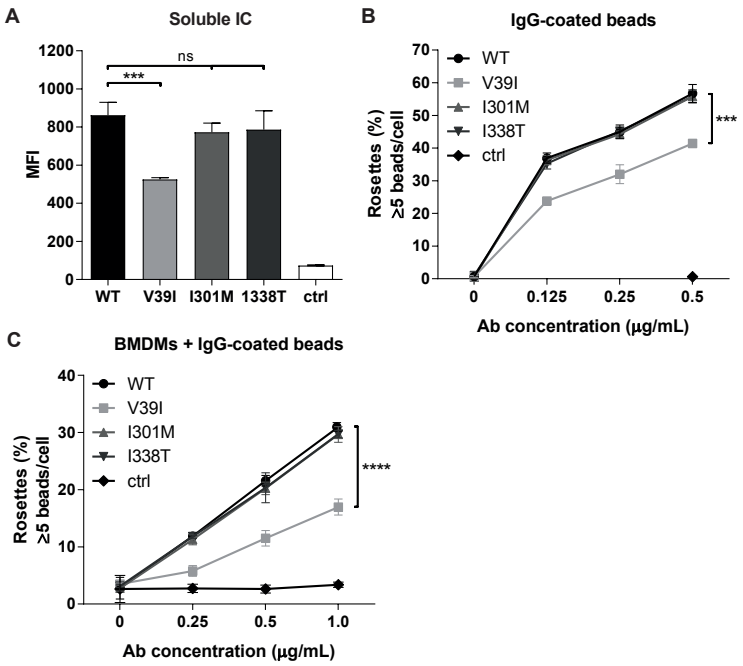

Figure 3. Immune complex binding of FcγRI SNPs. (A) Fluorescently labeled soluble immune complexes (IC) were composed of human IgG1 and anti-human IgG F(ab')$_2$ binding antibodies and added to untransduced Ba/F3 (ctrl) or Ba/F3-FcγRI cells at 0.5 µg/mL. Binding of soluble IC is depicted as median fluorescence intensity (MFI). The data represented here are corrected for the FcγRI expression on the Ba/F3 cells. (B) 4.5 µm Dynabeads were coupled to DNP_{24}-BSA and opsonized with the indicated concentrations of rabbit IgG anti-DNP, resulting in IgG-coated beads that serve as large IC. Binding of these IgG-coated beads to Ba/F3-FcγRI cells was evaluated using microscopy, and rosettes were defined as cells bound with ≥5 beads. (C) Bone marrow-derived macrophages (BMDMs) from mFcγRI/III$^{-/-}$ mice were transduced with FcγRI, either WT or the SNP variants, on day 3 and 4 of culture. On day 8, BMDMs were harvested and IC binding was measured using IgG-coated beads. Mock transduced BMDMs (ctrl) show no IC binding. One representative of n=3 independent experiments is shown. ***: p<0.001, ****: p<0.0001, ns: not significant.

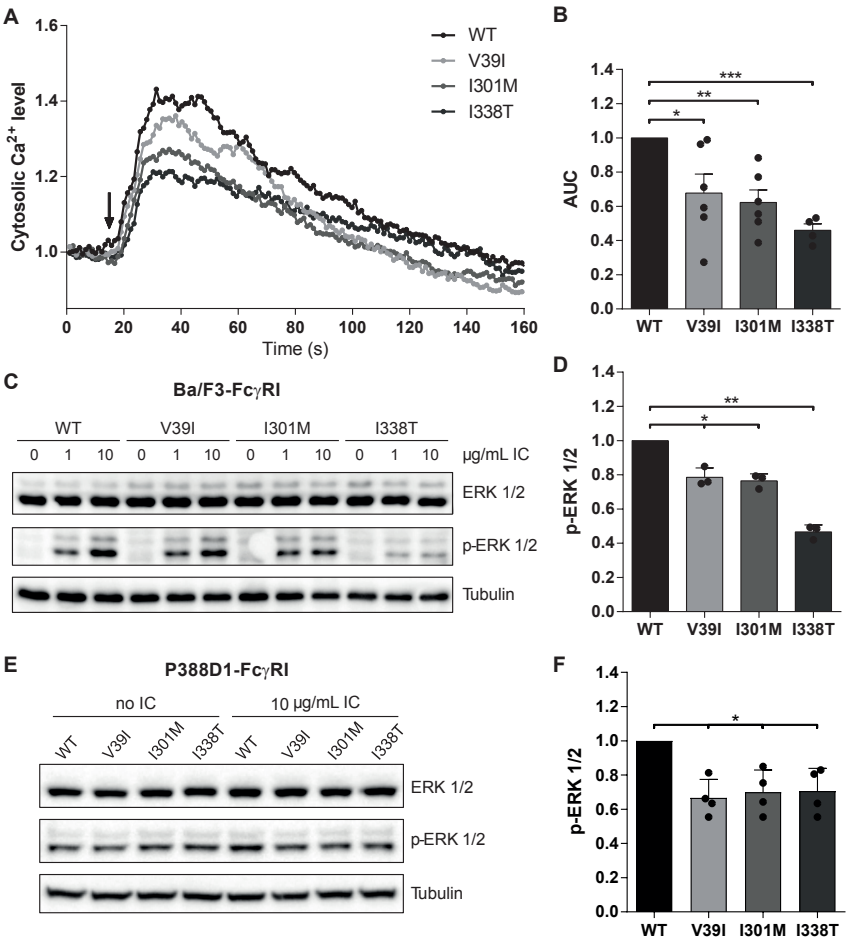

Figure 4. FcγRI crosslinking induced intracellular calcium mobilization and ERK phosphorylation. (A) Intracellular calcium mobilization kinetics in Ba/F3-FcγRI cells. Cells were loaded with calcium dyes and FcγRI dependent calcium flux was induced using an anti-FcγRI antibody (m22), crosslinked with a goat anti-mouse antibody (addition of this antibody is indicated by the arrow). (B) Summary of 4-6 independent experiments (mean ±SEM) comparing area under the curve (AUC) of the calcium flux. FcγRI-WT was set to 1. (C) Western Blot analysis of Ba/F3-FcγRI after 5 min crosslinking by plate-bound IC of the indicated concentrations. Cell lysates were analyzed for total ERK 1/2 (p42/p44), phosphorylated ERK 1/2 (p-ERK) and α-tubulin. (D) Quantification of p-ERK 1/2 levels. The p-ERK signal was divided by the α-tubulin signal of the same lane and p-ERK levels of FcγRI-WT were set to 1. Data from n=3 independent experiments were combined. (E) Western Blot analysis of P338D1-FcγRI after 5 min crosslinking by 10 µg/mL plate-bound IC. (F) Quantification of P388D1-FcγRI p-ERK 1/2 levels. Data from n=4 independent experiments were combined. *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$.

ments based on the area under the curve (AUC) indeed showed a decreased intracellular Ca2+ flux of FcγRI-V39I compared to FcγRI-WT, indicating reduced FcRγ signaling. Interestingly, while the intracellular Ca^{2+} flux of FcγRI-I301M was also less compared to FcγRI-WT (similar to FcγRI-V39I), crosslinking of FcγRI-I338T resulted in an even stronger reduction (~50%) in intracellular Ca^{2+} flux compared to FcγRI-WT (Fig. 4B), most likely due to its close proximity with the further signaling machinery (Fig. 1A). As a control, all Ba/F3-FcγRI expressing cells showed an equally strong increase in intracellular Ca^{2+} levels after addition of ionomycin (Fig. S4A).

Crosslinking of FcγRI SNPs induces less downstream signaling compared to FcγRI-WT

Besides a rise in intracellular Ca^{2+} levels, Fc receptor mediated triggering of ITAM signaling leads to activation of the MAPK-ERK pathway[31]. To determine the activation of this pathway in Ba/F3-FcγRI cells, plate bound IC were used to induce FcRγ signaling through FcγRI crosslinking on Ba/F3 cells. After 5 minutes of incubation, ERK phosphorylation was strongly induced in a concentration-dependent manner (Fig. 4C). Crosslinking of all three FcγRI SNPs resulted in decreased phospho-ERK (p-ERK) levels compared to FcγRI-WT (Fig. 4D). Similar to the intracellular Ca^{2+} flux, this decrease was most pronounced for FcγRI-I338T.

To confirm these findings in a myeloid cell line, we transduced P388D1 cells, a macrophage-like cell line, with the human FcγRI constructs and measured downstream signaling. Since these cells, like mouse BMDMs, do not induce a measurable intracellular Ca^{2+} flux upon Fc receptor crosslinking (data not shown), we studied ERK phosphorylation. Stable and equal surface expression of FcγRI WT or SNP variants was obtained after transduction of P388D1 cells (Fig. S4B). Basal p-ERK levels were higher in P388D1-FcγRI cells compared to Ba/F3-FcγRI cells. As expected, stimulation with IC increased the ERK phosphorylation of P388D1-FcγRI WT cells (Fig. 4E). However, this increased p-ERK signal was significantly less in all three FcγRI SNPs after receptor crosslinking by IC (Fig. 4E and F). Together, these data indicate that downstream signaling of FcγRI is negatively influenced by the FcγRI SNPs, both for intracellular Ca^{2+} mobilization and induction of the MAPK-ERK pathway.

DISCUSSION

Antibodies of the IgG isotype bind to FcγR on immune cells, which is essential for the induction of their cellular effector functions and efficient immune responses. Polymorphisms in these FcγR have been associated with altered avidity towards IgG, leading to changes in immune-mediated responses of antibodies. Most of the work done on FcγR SNPs focuses on the low affinity FcγR, including FcγRIIA, FcγRIIIA, and FcγRIIIB. To our knowledge, we describe in this study for the first time functional nonsynonymous SNPs for the high affinity receptor for IgG, FcγRI. The only SNP located in the extracellular domain of FcγRI, V39I, reduces IC binding of FcγRI and this leads to reduced FcRγ signaling

and intracellular calcium mobilization, suggesting that this area of FcγRI is involved in binding of IC. In line with the location of SNPs I301M and I338T, the transmembrane or intracellular domain, respectively, they have no influence on IC binding. However, these SNPs substantially reduced FcγRI signaling. This effect is most pronounced for FcγRI-I338T, suggesting that this intracellular part of FcγRI plays a key role in interacting with the intracellular signaling machinery and therefore might be interesting as a potential target in order to further enhance of inhibit FcγRI-mediated effects. Of note, there was no difference in p-ERK levels between the three SNPs in P388D1-FcγRI cells, although they were all decreased compared to FcγRI WT. The basal p-ERK levels were also higher in these cells compared to Ba/F3-FcγRI cells, indicating that these signaling pathways might be differentially regulated in these myeloid cells. However, both cell lines show that all three SNPs negatively influence FcγRI downstream signaling.

The V39I SNP is located in EC1 of FcγRI and is largely buried within this structure[26]. Furthermore, the IgG binding site is located in EC2 of FcγRI[32,33], although EC1 is positioned very close to the IgG binding site. Our data indicate that monomeric IgG binding is not affected by the change of a valine to isoleucine at position 39, which is in line with how monomeric IgG binds to FcγRI. However, IC binding is reduced in the V39I variant, possibly indicating an important role for this amino acid specifically in IC binding. It is tempting to speculate that crosslinking of multiple FcγRI by the IC requires a slightly altered FcγRI structure and that a valine at position 39 is necessary to obtain this. Interestingly, V39 appears highly conserved within the FcγR family[26], possibly implying an important role of this amino acid in structure and/or function of FcγR. Currently, it is unknown which amino acids of FcγRI interact with FcRγ. However, the reduced FcRγ signaling of the I301M variant might indicate that this isoleucine in the transmembrane region of FcγRI is involved in this interaction. It is unclear how the introduction of a threonine at position 338 in the intracellular domain of FcγRI leads to a strong reduction in FcRγ signaling. If this threonine is constitutively phosphorylated in cells, this might interfere with efficient recruitment of signaling molecules like SYK to FcRγ (Fig. 1A). Computational prediction of the phosphorylation of FcγRI-I338T indicates that several serine/threonine kinases, including MAP kinase-activated protein kinase 5 (MAPKAPK), nuclear dbf2-related kinase (NDR), and Tyrosine Kinase-like (TKL), might phosphorylate threonine 338 (data not shown).

The frequency of these FcγRI SNPs is quite low in the population. In Dutch individuals, we observed two V39I heterozygotes in 158 healthy donors (0.63%). The frequency of V39I reported by the 1000 Genomes Project Phase 3 (0.5%) corresponds with the frequency we observed. However, in this dataset the V39I SNP is only observed within the African population[30], which might indicate that the two heterozygous donors in our cohort are of African ancestry. The frequency of I301M is 0% in our cohort and no frequency data are available in the 1000 Genomes Project. The frequency of the I338T SNP is comparable to the V39I SNP according to the 1000 Genomes Project (0.3%), with the highest prevalence in the European population[30]. We did not observe the I338T SNP in our cohort, which might be explained by the smaller size (158 donors) compared to the

1000 Genomes Project (2504 donors) or the fact that no Dutch/German individuals were part of this larger project. The very low frequency of the FcγRI SNPs might indicate that having these less functional variants of FcγRI is evolutionary unfavorable. However, four individuals from one family lacking expression of FcγRI, caused by the R92* premature stopcodon, do not appear to have increased susceptibility for infections or autoimmune diseases[27]. Nonetheless, in these individuals it is likely that redundancy within the FcγR family compensates for the FcγRI deficiency.

Our data indicate that naturally occurring functional SNPs of FcγRI exist, albeit with very low prevalence. Though the impact of these individual SNPs is low due to the low frequency, this study allowed to get major insights into the functional role of different parts of FcγRI. The area around V39 in EC1 is most likely an important binding site for IC or affects folding of the extracellular parts of FcγRI, while the intracellular region containing I338 has a major role in downstream signaling of FcγRI. Both mutations are therefore most likely avoided in the general population as they are associated with a functional disadvantage. Together, these insights in FcγRI biology will allow the development of more targeted therapies to either enhance or decrease activity of FcγRI in the context of patients receiving antibodies as treatment.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

A.M.B. and J.H.W.L. designed the research and wrote the manuscript. A.M.B., E.D., T.G.C., and T.K. performed the experiments. T.B. and J.K. discussed results and critically reviewed the manuscript.

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SUPPLEMENTARY FIGURES

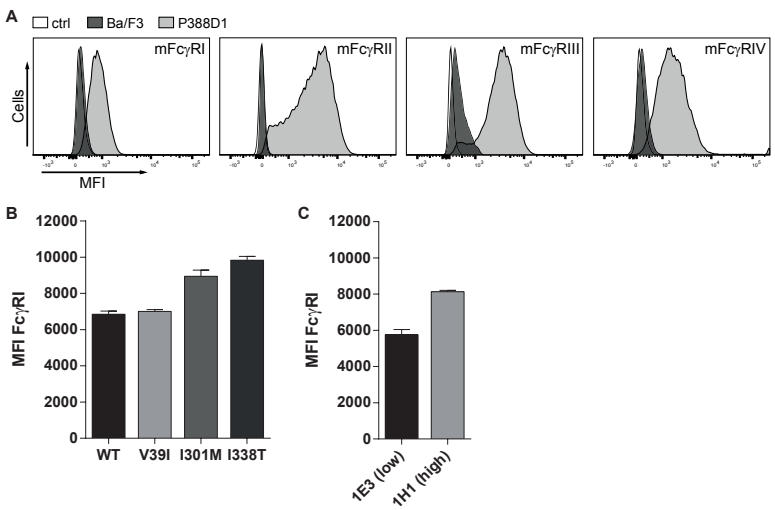

Figure S1. Expression of murine FcγR and FcγRI on Ba/F3 cells. FcγR expression was measured using flow cytometry. (A) Ba/F3 or P388D1 cells incubated with anti-murine FcγR antibodies. Ctrl indicates PBS controls for Ba/F3 cells. (B) Ba/F3 cells were stably transduced with FcγRI, either WT or one of the SNPs. (C) Two single cell clones were selected from Ba/F3-FcγRI-WT bulk cells, with 'low' (1E3) or 'high' (1H1) FcγRI expression. Mean fluorescence intensity (MFI) is depicted.

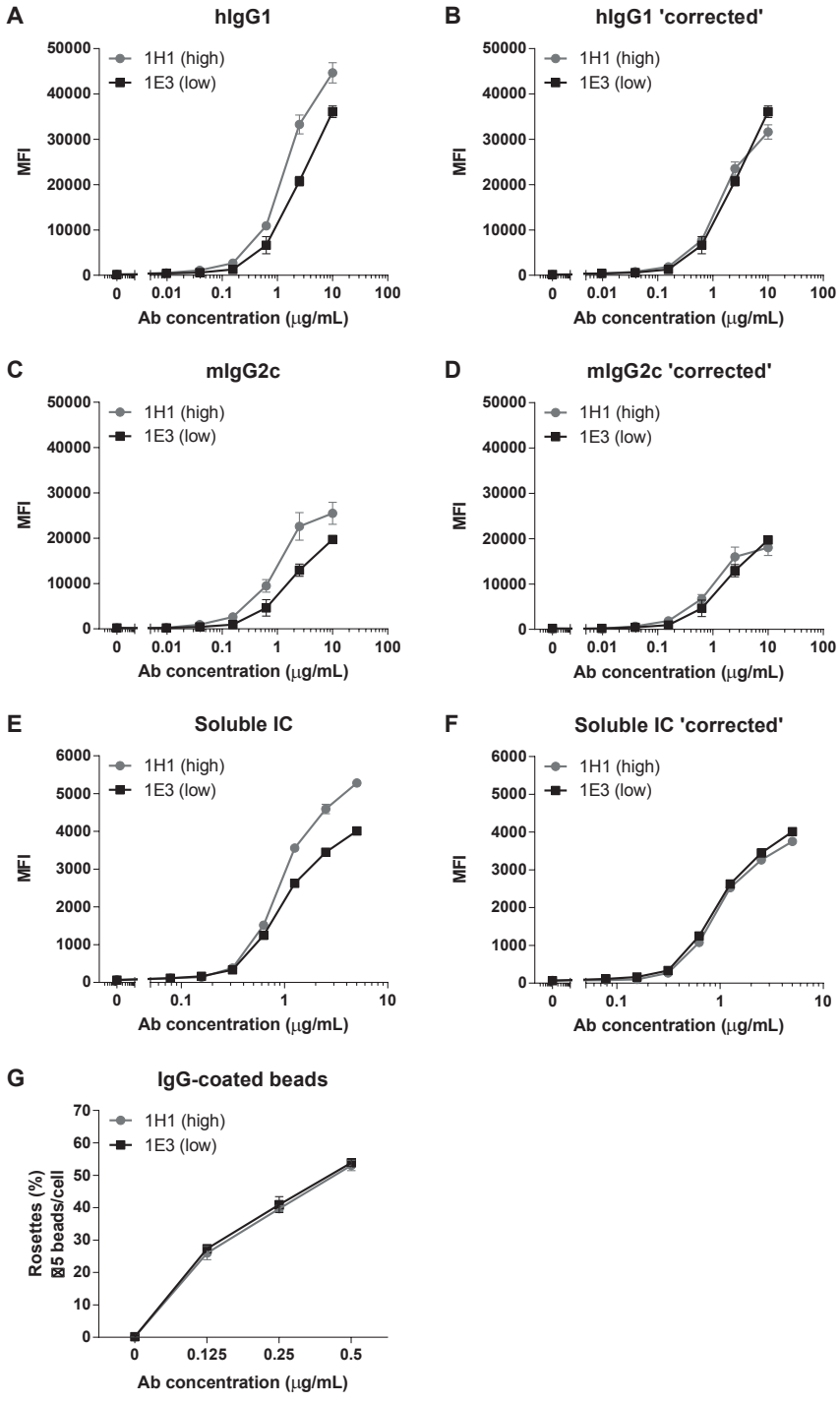
A
hIgG1
1H1 (high)
1E3 (low)
MFI
Ab concentration (μg/mL)
B
hIgG1 'corrected'
C
mIgG2c
D
mIgG2c 'corrected'
E
Soluble IC
F
Soluble IC 'corrected'
G
IgG-coated beads
Rosettes (%) ≥5 beads/cell

Figure S2. Functional analysis of Ba/F3 cells expressing high or low FcγRI. (A-D) Binding of monomeric IgG to Ba/F3-FcγRI cells was measured using flow cytometry. Median fluorescence intensity (MFI) is depicted. For both isotypes the higher expressing clone 1H1 bound more IgG than the lower expressing clone 1E3. Correcting the data for the FcγRI expression of each clone leads to similar IgG binding. (E+F) Binding of fluorescently labeled soluble immune complexes (IC) to Ba/F3-FcγRI cells, depicted as MFI. The higher expressing clone 1H1 bound more soluble IC than the lower expressing clone, and correcting the data for FcγRI expression shows similar IC binding. (G) Binding of IgG-coated beads to Ba/F3-FcγRI cells was evaluated using microscopy, and rosettes were defined as cells bound with ≥5 beads. No difference in binding between both clones was observed. Therefore, correction for the FcγRI expression was not applied here. We concluded that for monomeric IgG and soluble IC binding assays, the data obtained should be corrected for the FcγRI expression, while for the IgG-coated beads assay this is not required. This strategy was applied for all data in this study.

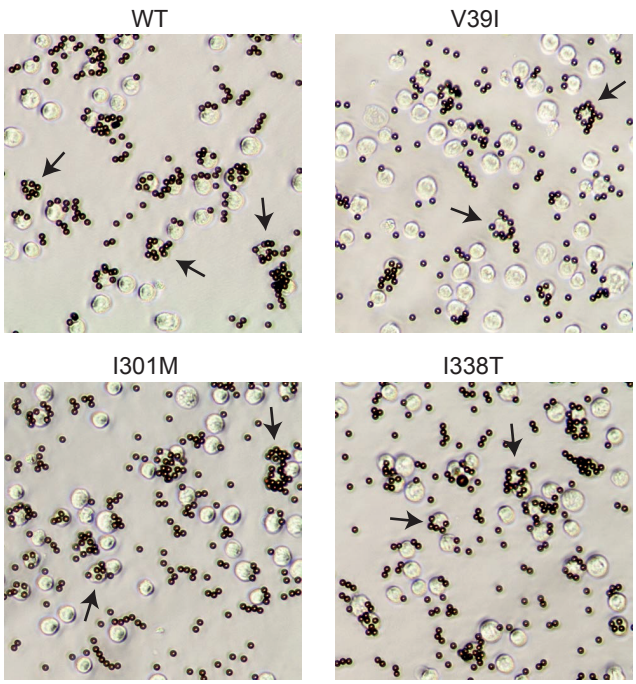

Figure S3. Example images of Ba/F3-FcγRI cells bound to IgG-coated beads. Example images of the rosette assay with IgG-coated beads are shown; arrows indicate examples of rosettes. Beads are 4.5 μm in size, Ba/F3-FcγRI cells are ~10 μm in size.

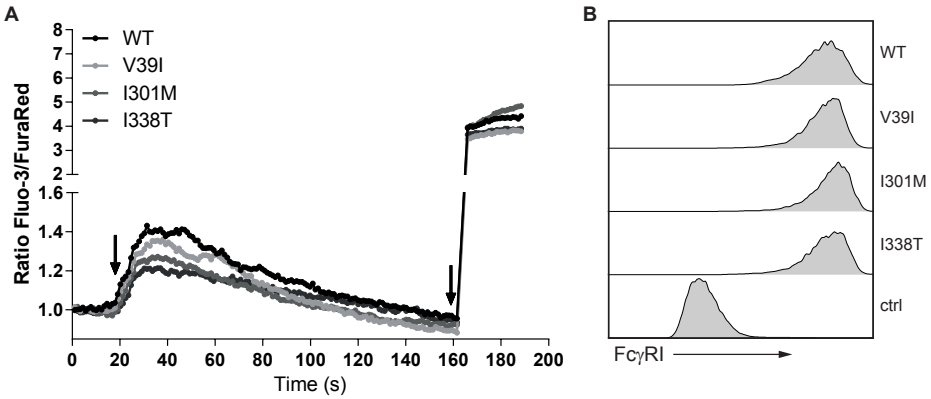

Figure S4. Intracellular calcium mobilization kinetics in Ba/F3-FcγRI cells and FcγRI expression on P388D1 cells. (A) Cells were loaded with calcium dyes and FcγRI dependent calcium flux was induced using an anti-FcγRI antibody (m22), crosslinked with a goat anti-mouse antibody (addition of this antibody is indicated by the first arrow). The second arrow indicates the addition of 2 µg/mL ionomycin; this is a loading control used to measure the maximal calcium flux. (B) P388D1 cells were stably transduced with FcγRI, either WT or the SNP variants. FcγRI expression was measured using flow cytometry and untransduced P388D1 (ctrl) cells were used as control.

CHAPTER 6

Simultaneous targeting of FcγRs and FcαRI enhances tumor cell killing

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Efficacy of anti-cancer monoclonal antibodies (mAbs) is limited by the exhaustion of effector mechanisms. IgG mAbs mediate cellular effector functions via FcγRs expressed on effector cells. We have recently shown that IgA mAbs can also induce efficient tumor killing both *in vitro* and *in vivo*. IgA mAbs recruit FcαRI-expressing effector cells, and therefore initiate different effector mechanisms in vivocompared to IgG. Here we studied killing of tumor cells co-expressing EGFR and HER2 by IgG mAbs cetuximab and trastuzumab and their IgA variants. In the presence of a heterogeneous population of effector cells (leukocytes), the combination of IgG and IgA anti-tumor mAbs against two different tumor targets (EGFR and HER2) led to enhanced cytotoxicity compared to each isotype alone. Combination of two IgGs or two IgAs or IgG and IgA against the same target did not enhance cytotoxicity. Increased cytotoxicity relied on the presence of both the peripheral blood mononuclear cell and the polymorphonuclear (PMN) fraction. Purified NK cells were only cytotoxic with IgG, whereas cytotoxicity induced by PMNs was strong with IgA and poor with IgG. Monocytes, which co-express FcγRs and FcαRI, also displayed increased cytotoxicity by the combination of IgG and IgA in an overnight killing assay. Co-injection of cetuximab and IgA2-HER2 resulted in increased anti-tumor effects compared to either mAb alone in a xenograft model with A431-luc2-HER2 cells. Thus, the combination of IgG and IgA isotypes optimally mobilizes cellular effectors for cytotoxicity, representing a promising novel strategy to improve mAb therapy.

INTRODUCTION

Antibody therapy with IgG monoclonal antibodies (mAb) represents part of the standard treatment in the clinic for the treatment of various forms of cancer. The ErbB family members EGFR (epidermal growth factor receptor) and HER2 (human epidermal growth factor receptor 2) are validated clinical targets for mAb therapy[1]. Cetuximab (Erbitux, anti-EGFR) is approved for colon and head and neck cancers, whereas trastuzumab (Herceptin, anti-HER2) is used for breast and gastric cancer treatment. EGFR mAbs have a dual mechanism of action: both Fab- and Fc-mediated anti-tumor effects were described[2]. The direct Fab-mediated effects include occlusion of the ligand binding site leading to hampering of receptor dimerization and internalization of EGFR leading to inhibition of downstream signaling[3]. The anti-tumor mechanisms of HER2 mAbs are less well understood, but also involve Fab-mediated direct effects, such as induction of receptor internalization and degradation, or induction of cell cycle arrest by inhibiting downstream signaling[4,5]. Next to direct Fab-mediated effects, the mechanisms of action of both mAbs involve Fc gamma receptor (FcγR)-dependent mechanisms. The importance of Fc-FcγR interaction is supported by clinical association studies and preclinical animal studies for both EGFR[6-8] and HER2 mAb therapy[9,10]. However, IgG mAb therapy is rarely curative and this has in part been attributed to exhaustion of cellular effector mechanisms[11].

Cetuximab and trastuzumab are, like most therapeutic mAbs, of the IgG1 subclass. IgG1 mAbs engage FcγRs on effector cells to initiate antibody-dependent cell-mediated cytotoxicity (ADCC). Each immune effector cell type has a unique expression of FcγRs. For example in humans, NK cells only express FcγRIIIa (and in some individuals FcγRIIc), polymorphonuclear cells (PMNs) express FcγRIIa and FcγRIIIb, whereas monocytes express FcγRI, FcγRIIa and FcγRIIIa.

Co-engagement of the activating FcγRs with the inhibitory FcγRIIb by IgG1 on monocytes results in inhibitory signaling and limits the cytotoxic activity of IgG1. PMNs abundantly express a GPI-linked non-signaling FcγRIIIb. Interaction of IgG1 with FcγRIIIb limits IgG1-mediated PMN cytotoxicity[12,13]. Moreover, polymorphisms in several FcγRs influence IgG1 activity: genotypes that confer to stronger IgG binding are associated with better response to mAb therapy[14].

Previously, we have shown that IgA anti-tumor mAbs can induce efficient cytotoxicity *in vitro* and *in vivo*[15,16]. IgA induces more cytotoxicity than IgG by purified PMNs and unfractionated human leukocytes[15] and induces comparable cytotoxicity to IgG by macrophages[16,17]. The cytotoxic activity of IgA is mediated by FcαRI and therefore IgA induces different effector functions than IgG, *e.g.* more efficient activation of PMNs compared to IgG[18].

EGFR and HER2 co-expression is found in a number of cancers, including pancreatic cancer, non-small cell lung cancer and breast cancer[19-21]. Moreover, EGFR and HER2 co-expression is associated with the most aggressive tumors[21]. To increase IgG activity, the combination of two anti-tumor IgG mAbs against these two targets has been investigated before[22,23]. These studies have revealed increased anti-tumor effects by the combination of two IgG mAbs, which was mainly due to the dual Fab-mediated effector functions. At

saturating target opsonization, this approach also increases the available Fc moieties for FcγR binding, however tumor cell killing is still limited by the number of available FcγRs on effector cells and/or their cytotoxic capacity.

We reasoned that the combination of IgG and IgA would induce enhanced tumor cell killing, through the recruitment and/or activation of both FcγR-bearing NK cells and FcαRI-bearing PMNs. Co-engagement of FcγRs and FcαRI on the same effector cell (monocytes/macrophages) might also result in stronger FcR signaling and tumor cell killing. To this end we studied cytotoxicity induced by the combination of IgG and IgA (cetuximab or trastuzumab as well as their IgA variants carrying the same variable region) towards tumor cells co-expressing EGFR and HER2.

MATERIALS AND METHODS

Cell lines and cell culture

The human epidermoid carcinoma cell line A431 and the human breast cancer cell line SK-BR-3 (ATCC) were authenticated by STR profiling. No reauthentication assay was performed because cell lines were directly obtained from cell banks and used at early passages after receipt or resuscitation. All cell lines were tested and validated to be *Mycoplasma* free. Cell lines were cultured in RPMI 1640 (Gibco) supplemented with 10% FCS and penicillin and streptomycin (Gibco). A431-HER2 cells were generated by retroviral transduction of A431 cells with human HER2 (pMX-puro-HER2), and positive clones were selected using puromycin. A431-luc2 cells were generated as previously described[16] and transduced with HER2. HER2 expression on A431-luc2-HER2 cells was comparable to A431-HER2 cells. HER2 expression on the transduced cell lines was regularly tested by cytofluorimetry and was found to be stable in time. The number of EGFR and HER2 molecules on the cells was determined using the Qifikit (DAKO). The number of HER2 molecules on A431-HER2 cells was similar to SK-BR-3, whereas the number of EGFR molecules was comparable to A431 and A1207 cells (Table S1).

Antibodies

The chimeric anti-human EGFR mAb cetuximab (Erbitux, Merck KGaA) and the humanized anti-human HER2 mAb trastuzumab (Herceptin, Roche) were purchased from the pharmacy of UMC Utrecht. Both cetuximab and trastuzumab are of IgG1 isotype. The generation and characterization of IgA2-EGFR has been previously described[15,24]. The generation and characterization of IgA2-HER2 mAb was described in detail elsewhere[25]. Briefly, the variable regions of trastuzumab were inserted into an expression vector containing the constant regions of the heavy and light chains of human IgA224. IgA2 was produced by transient transfection of HEK 293F cells (FreeStyle™ 293 Expression System, Invitrogen), and purified by affinity and size-exclusion chromatography.

Flow cytometry

Expression of HER2 and EGFR on tumor cells was determined using different concentrations of trastuzumab and cetuximab, respectively, and measured with flow cytometry using a PE-labeled anti-human IgG Ab (Jackson). The following mAbs were used for flow cytometry: CD3 (UCHT1, Biolegend), CD56 (HCD56, Biolegend), CD14 (HCD14, Biolegend) and CD16 (3G8, Biolegend) for isolated effector cells or CD45 (HI30, Biolegend), CD3 (OKT3, Biolegend), CD56 (HCD56, Biolegend), CD14 (G1D3, eBioscience) and CD16 (3G8, Biolegend) Abs for leukocytes. Control mouse IgG2b and mouse IgG1 mAbs did not show any background binding. BD Trucount tubes were used to determine the absolute amount of effector cells in 50 µL leukocyte suspension reconstituted to the original volume of the peripheral blood sample. During the measurement, leukocytes were identified to be $CD45^+$. Flow cytometry measurements were performed on FACS CantoII (BD Biosciences). Flow cytometry data were analyzed by FACS Diva software.

ADCC

ADCC with ^{51}Cr-labeled target cells was described previously[26]. Briefly, 1×10^6 target cells were labeled with 100 µCi (3.7MBq) ^{51}Cr for 2 hours. After extensive washing, cells were adjusted to 10^5/mL. Blood was obtained from healthy donors at the UMC Utrecht. In leukocyte ADCC, erythrocytes were lysed by incubation in water for 30 seconds and then total leukocytes were resuspended in medium. The number of leukocytes used per well corresponded to the number of leukocytes present in 50 µL of blood before lysis. Effector cells, sensitizing mAbs at various concentrations, medium and tumor cells were added to round-bottom microtiter plates (Corning Incorporated). After 4 or 20 hours (overnight) incubation at 37°C, ^{51}Cr release was measured in counts per minute (cpm). Percentage of specific lysis was calculated using the following formula: ((experimental cpm – basal cpm)/(maximal cpm – basal cpm)) × 100, with maximal lysis determined in the presence of 3% triton and basal lysis in the absence of Abs and effector cells. We only included those donors in our analysis that induced minimum 5% and maximum 80% maximal lysis with both single mAbs (Table S2).

PMN and PBMC fractions were isolated from blood by Ficoll/Histopaque separation (GE Healthcare; Sigma-Aldrich). Monocytes were isolated from the PBMC fraction using CD14 magnetic beads (Miltenyi Bioscience). NK cells were isolated from the CD14 negative fraction by negative selection using the EasySep® Human NK Cell Enrichment Kit (Stemcell Technologies). Purity of effector cells was analyzed by flow cytometry and was approximately 85% for NK cells, 70% for monocytes and close to 100% for PMNs. The effector-to-target (E:T) ratios for purified fractions were 70:1 (PBMC), 40:1 (PMN), 15-25:1 (monocytes) or 10:1 (NK cells). The E:T ratio in the leukocyte ADCC was 1:1 for both NK cells and monocytes and 12:1 (with a range of 6-18:1) for PMNs. IgG and IgA mAbs were combined at a 1:1 ratio.

In certain experiments A431-HER2 or SK-BR-3 cells were incubated for 6 hours with both PMNs (E:T=40:1) and PBMCs (E:T=70:1) and specific lysis was compared to conditions using either PMNs (E:T=80:1) or PBMCs (E:T=140:1) alone. For the experi-

ments using sequential incubation with effector cells, tumor cells were first incubated with PMNs (E:T=40:1) for 3 hours, then PBMCs (E:T=70:1) were added and incubated with the target cells for another 3 hours. As controls, tumor cells were incubated with either PMNs (E:T=80:1) or with PBMCs (140:1) twice for 3 hours. Cetuximab and IgA2-HER2 were added at 1:1 ratio at the indicated concentrations.

A431-luc2-HER2 xenograft tumor model

The *in vivo* xenograft experiment was performed as described previously[16]. FcαRI Tg or non-transgenic littermate SCID mice were injected intraperitoneally with 5×10^5 A431-luc2-HER2 cells in 100 µL PBS. Tumor outgrowth was monitored by serial bioluminescent imaging (BLI) (PhotonImager, Biospace Lab). BLI images were processed using M3Vision software (Biospace Lab) and edited in Adobe Photoshop. Mice with tumor outgrowth were randomized in different treatment groups on day 13. Cetuximab was injected once on day 14 (10 µg), whereas 10 µg IgA2-HER2 was injected daily between day 14 to 23 (in total 10 times) to compensate for its shorter half-life. The combination group was injected with both cetuximab and IgA2-HER2. Tumor growth is expressed as percentage signal compared to the beginning of the treatment. Circulating serum mAb levels were monitored in serial blood samples taken from alternating mice (2 mice / time point). Serum levels of human IgG and IgA were measured by ELISA as previously described[16]. The experiment was approved by the local Animal Ethical Committee.

Data processing and statistical analyses

Statistical analyses were performed in R (3.1.0) or in Graph Pad 6.0 software. Data are represented as mean ± SEM unless indicated otherwise. Dose-response curves for the ADCC experiments were calculated via nonlinear regression (log(agonist) vs. response) using Graph Pad 6.0 software.

Maximal specific lysis was analyzed by using the triplets of a concentration level on which maximal average specific lysis was attained in an individual ADCC experiment. Treatment effects were analyzed by using two-way full factorial linear mixed effects modeling. The model consisted of two indicator variables for drugs applied (yes or no) as fixed-effects terms, and subject and triplets nested within subject as random-effect terms. The least square mean differences in the maximal lysis among the full factorial interaction groups were estimated with Tukey's adjustment.

Inferential statistical analysis regarding the treatments' effect on the time course of tumor growth *in vivo* was carried out by using linear mixed effects modeling. The log transformed tumor size was described by a model consisted of fixed effect terms such as time since xenograft (days) as a continuous covariate, and its second and third order interactions with the cetuximab and IgA2-HER2 treatments as factors, and two random effect terms allowing individual differences in tumor size at day 1 and in tumor growth time course.

RESULTS

IgG and IgA mAbs against both EGFR and HER2 induce cytotoxicity of A431-HER2 cells

To test cytotoxicity induced by the combinations of two different isotypes, we used IgG and IgA specific for EGFR and HER2. IgA variants of the chimeric IgG1-EGFR mAb cetuximab were previously generated[15,24]. Following a similar approach, we generated IgA variants of the human HER2 mAb trastuzumab (unpublished results). The IgA2 subclass was used in our experiments because it induces stronger cytotoxicity than the IgA1 subclass[15,16].

IgA2-EGFR and IgA2-HER2 induced significantly higher cytotoxicity than cetuximab and trastuzumab, respectively, by leukocytes in a 4-hour killing assay with tumor cell lines expressing high levels of endogenous targets (Fig. S1A and B). To study the effects of different mAb combinations against EGFR and HER2, we established a target cell line with high expression of both EGFR and HER2 (A431-HER2 cells), because HER2 expression on A431 cells was too low for efficient cytotoxicity by trastuzumab (Fig. S2, Table S1, and unpublished results).

Both IgG- and IgA-mediated targeting of either EGFR or HER2 induced specific lysis of A431-HER2 cells using leukocytes as effector cells and, as expected, maximal lysis by IgA was higher than by IgG for both targets (Fig. 1A). No specific lysis was observed in

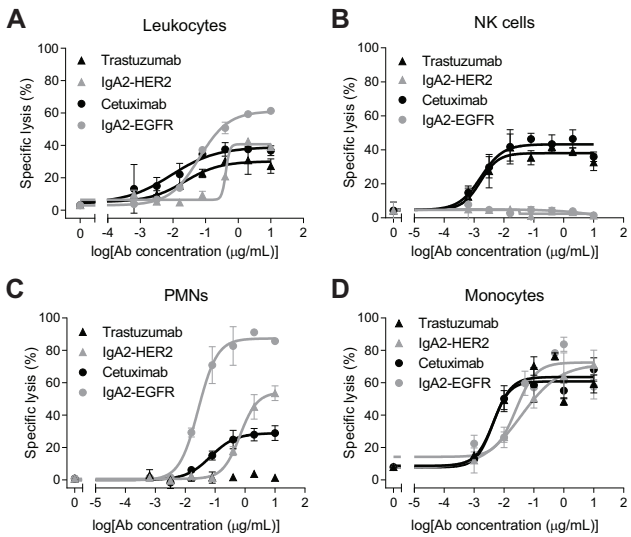

Figure 1. Cytotoxicity by IgG and IgA is mediated by distinct populations of effector cells. (A) Leukocytes corresponding to 50 μL whole blood and mAb-opsonized A431-HER2 cells were mixed and incubated for 4 hours at 37°C before ^{51}Cr-release was measured to assess specific lysis. Specific lysis of A431-HER2 cells by (B) purified NK cells (E:T=10:1), (C) PMNs (E:T=40:1) in a 4-hour assay, or by (D) monocytes (E:T=15:1) in a 20-hour killing assay. One graph each representing 9 (A), 3 (B), 5 (C) or 4 (D) independent experiments is shown, for a total of 16 (A), 3 (B), 6 (C), or 7 (D) donors.

the absence of mAbs or effector cells, nor was the lysis induced by tumor-specific mAbs, influenced by the presence of non-specific Abs (unpublished results). At low mAb concentrations, cetuximab was more effective than trastuzumab, most likely due to the higher expression of EGFR than HER2 by A431-HER2 cells.

Cytotoxicity by IgG and IgA is mediated by distinct populations of effector cells within the leukocytes

Leukocytes contain a heterogeneous population of effector cells (NK cells, monocytes and PMNs) that are all capable of inducing cytotoxicity. We therefore purified the different effector cells to determine their individual cytotoxic capacity. PMNs and monocytes expressed both FcγRs and FcαRI, whereas NK cells only expressed FcγRIIIa (Fig. S3). In accordance with their FcR expression, NK cells only induced cytotoxicity with IgG (Fig. 1B). PMNs were more efficient with IgA2-EGFR than with cetuximab, and they induced tumor cell killing with IgA2-HER2 but not with trastuzumab (Fig. 1C). Monocytes did not induce cytotoxicity after 4 hours with any of the mAbs (unpublished observation). However, monocytes induced potent cytotoxicity in an overnight killing assay with all four mAbs, which is in line with previous findings showing that monocytes require a longer incubation time for efficient cytotoxicity[16,17] (Fig. 1D). In conclusion, IgG-mediated cytotoxicity is mediated mainly by NK cells, whereas IgA-mediated cytotoxicity is mediated predominantly via PMNs. Monocytes are unlikely to directly contribute to tumor cell killing in a four hour assay, but mediate cytotoxicity with both IgG and IgA in an overnight assay.

Increased cytotoxicity by the combination of IgG and IgA targeting EGFR and HER2

We first investigated the effect of combining two IgGs (cetuximab and trastuzumab) or two IgAs (IgA2-EGFR and IgA2-HER2) on the specific lysis of A431-HER2 cells by leukocytes. These combinations did not decrease EC_{50} values or increase maximal cytotoxicity compared to the best mAb alone (Fig. 2A-D), likely because the Fc parts of both mAbs competed for the same set of FcRs. Given that IgG and IgA recruit predominantly different cellular effectors (NK cells and PMNs), we hypothesized that the combination of IgG and IgA would result in increased cytotoxicity by leukocytes containing both NK cells and PMNs. The combination of IgG and IgA directed against the same target (cetuximab and IgA2-EGFR or trastuzumab and IgA2-HER2) did not increase maximal tumor cell killing compared to the best mAb alone (Fig. 2E-H). This is most likely due to competition for the target by both IgG and IgA. IgG might decrease IgA-mediated recruitment and activation of PMNs from the leukocytes. Indeed, IgA2-EGFR-induced cytotoxicity by purified PMNs was inhibited by the addition of cetuximab (Fig. S4).

Next, we investigated the combination of IgG and IgA directed against two different targets. Cetuximab and IgA2-HER2 were mixed at varying ratios while keeping the total mAb concentration constant. The combination of cetuximab and IgA2-HER2 resulted in

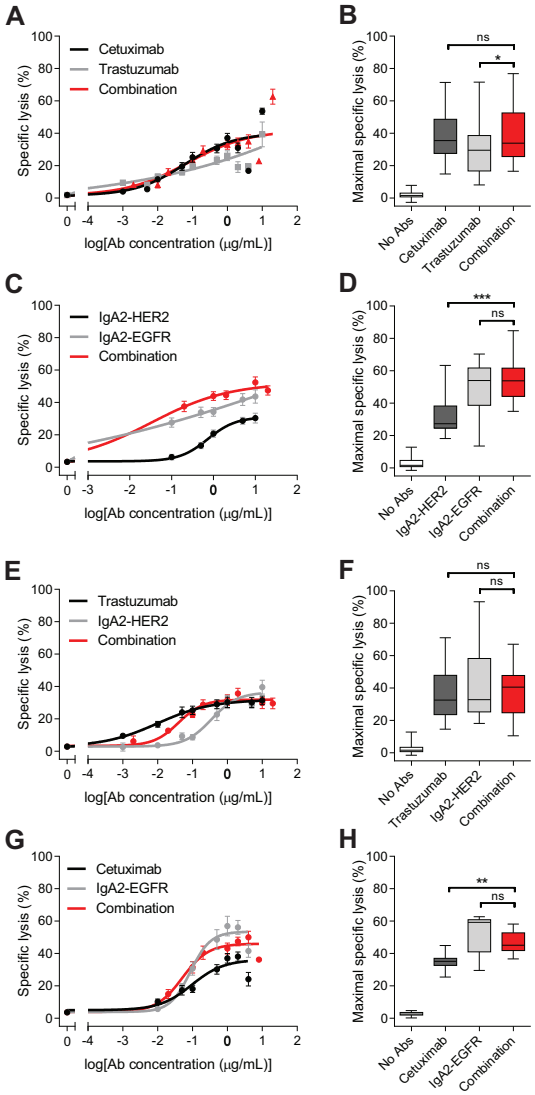

Figure 2. Leukocyte-mediated cytotoxicity by the combination of two IgGs or two IgAs against different targets or the combination of IgG and IgA against the same targets. Leukocytes and mAb-opsonized A431-HER2 cells were mixed and incubated for 4 hours at 37°C before ^{51}Cr-release was measured to assess specific lysis. Dose-response curves are calculated from pooled data from multiple experiments obtained using several donors. mAbs were mixed at a 1:1 ratio and each single mAb was used at the indicated concentration (mean ± SEM). Right, the maximal specific lysis from all donors is plotted. (A and B) cetuximab and trastuzumab (5 independent experiments, with a total of 10 donors), (C and D) IgA2-EGFR and IgA2-HER2 (4 independent experiments, with a total of 7 donors), (E and F) cetuximab and IgA2-EGFR (3 independent experiments, with a total of 6 donors), (G and H) trastuzumab and IgA2-HER2 (5 independent experiments, with a total of 9 donors). (median, box extends from the 25th to 75th percentiles, whiskers to min and max; ANOVA and Tukey's multiple comparison test, *: p<0.05; **: p<0.01; ***: p<0.001). Ab, antibody; ns, not statistically significant.

increased lysis of A431-HER2 cells compared to cetuximab or IgA2-HER2 alone (Fig. 3A). Using broad mAb concentrations with multiple donors the maximal lysis induced by the combination cetuximab and IgA2-HER2 was significantly increased compared to the maximal lysis by the best single mAb (Fig. 3B and C). The combination of trastuzumab and IgA2-EGFR did not increase maximal lysis compared to IgA2-EGFR alone (Fig. 3D and E). This might be due to the relatively high cytotoxicity induced by IgA2-EGFR alone as a result of the high EGFR expression by A431-HER2 cells. These data show that the combination of IgG and IgA against two different tumor targets can result in increased cytotoxicity by leukocytes.

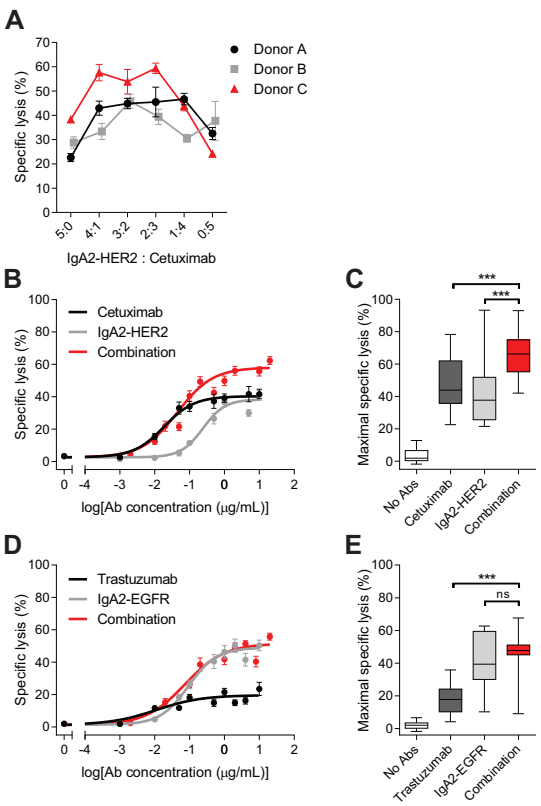

Figure 3. Increased leukocyte-mediated cytotoxicity by the combination of IgG and IgA against two targets. (A) Specific lysis of A431-HER2 cells by leukocytes (2 independent experiments, with a total of 3 donors). Cetuximab and IgA-HER2 were combined at different ratios while keeping the total amount of mAb constant at 1 µg/mL. (B and D) Pooled leukocyte ADCC data with cetuximab and IgA2-HER2 (7 independent experiments, with a total of 11 donors) and trastuzumab and IgA2-EGFR (5 independent experiments, with a total of 9 donors). mAbs were combined at 1:1 ratio, and each single mAb was used at the indicated concentration (mean ± SEM). (C and E) Maximal specific lysis (median, box extends from the 25th to 75th percentiles, whiskers to min and max; ANOVA, Tukey's multiple comparison test, ***: $p<0.001$). Ab, antibody.

Increased cytotoxicity by the combination of IgG and IgA requires multiple effector cell types

Whereas NK cells express only FcγRIIIa, human PMNs may express both FcγRs (FcγRIa, FcγRIIa, FcγRIIIb) and FcαRI, and are capable of tumor cell killing with both IgG and IgA (Fig. S3). Since trastuzumab alone did not induce cytotoxicity of A431-HER2 cells with PMNs (Fig. 1C), we only studied the combination of cetuximab and IgA2-HER2 on PMN-induced cytotoxicity. Cetuximab alone induced 5-20% maximal lysis depending on the donor. The combination of cetuximab and IgA2-HER2 did not increase cytotoxicity by purified PMNs (Fig. S5A and B). The combination of cetuximab and IgA2-HER2 resulted in increased maximal cytotoxicity of A431-HER2 cells when PBMCs and PMNs were combined compared to either fraction individually (Fig. 4A). Similar effects were observed using SK-BR-3 cells expressing both EGFR and HER2 endogenously (Fig. 4B). In addition, the sequential incubation of A431-HER2 or SK-BR-3 cells with PBMCs and PMNs in the presence of cetuximab and IgA2-HER2 resulted in increased cytotoxicity compared to incubation with either PBMCs or PMNs alone (Fig. 4C and D).

Monocytes express high levels of both FcγRs and FcαRI (Fig. S3) and induced comparable cytotoxicity by both IgG and IgA (Fig. 1D). Both combinations of IgG and IgA against different targets increased maximal cytotoxicity induced by monocytes in an over-

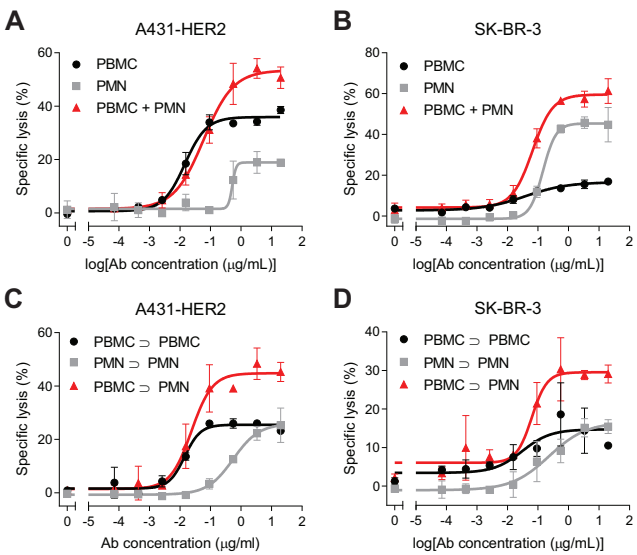

Figure 4. Increased leukocyte-mediated cytotoxicity by the combination of IgG and IgA requires the presence of multiple effector cell types. Specific lysis of A431-HER2 cells (A) or SK-BR-3 cells (B) in a 6-hour ADCC assay induced by the combination of PMNs (E:T=40:1) and PBMCs (E:T=70:1), or by PMNs (E:T=80:1), or PBMCs (E:T=140:1) alone. A431-HER2 cells (C) or SK-BR-3 cells (D) were first incubated with PBMCs (E:T=70:1) for 3 hours and subsequently incubated with PMNs (E:T=40:1) for another 3 hours (mean ± SD). Representative graphs from 5 (A and C) or 3 (B and D) independent experiments, with a total of 6 (A and C) or 3 (B and D) donors are shown. Ab, antibody.

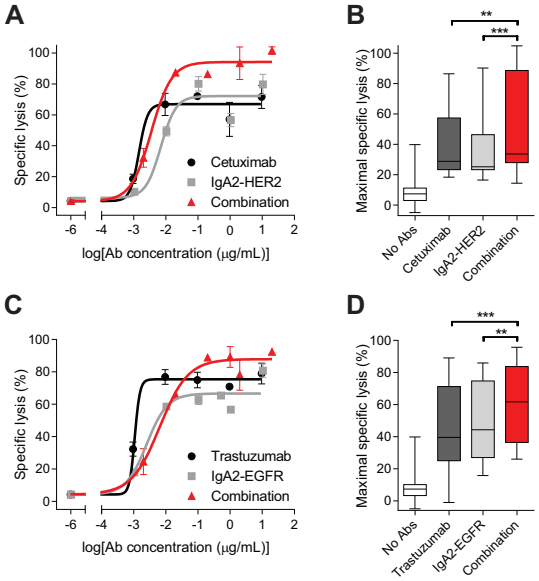

Figure 5. Increased monocyte-mediated cytotoxicity by the combination of IgG and IgA. Dose-response curves from a representative experiment in an overnight cytotoxicity assay using A431-HER2 cells and purified monocytes (E:T=25:1) with cetuximab and IgA2-HER2 (A) and trastuzumab and IgA2-EGFR (C). IgG and IgA mAbs were combined at a 1:1 ratio. (B and D) Maximal lysis was determined and plotted from 5 independent experiments, with a total of 7 donors performed with E:T=15:1 and 25:1 (median, box extends from the 25th to 75th percentiles, whiskers to min and max; ANOVA, Tukey's multiple comparison test, **: p<0.01, ***: p<0.001). Ab, antibody.

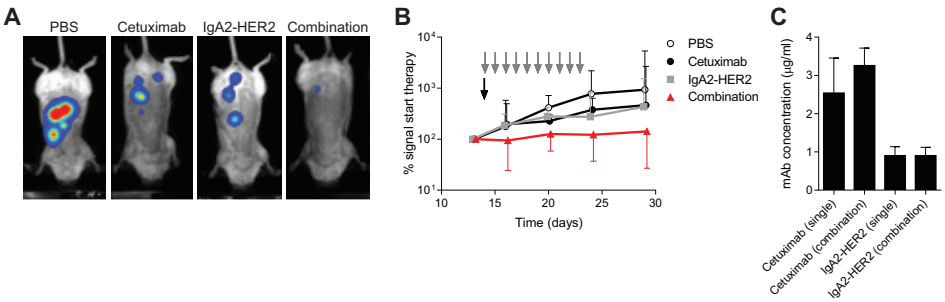

Figure. 6. Increased anti-tumor effect by the combination of IgG and IgA mAbs in a xenograft tumor model with A431-luc2-HER2 cells *in vivo*. FcαRI Tg or wild type SCID mice were injected i.p. with 1×10^5 A431-luc2-HER2 cells. On day 13, mice with tumor outgrowth were randomized in the following treatment groups: PBS, cetuximab (1×10 μg), IgA2-HER2 (10×10 μg), or combination treatment cetuximab (1×10 μg) and IgA2-HER2 (10×10 μg). Repeated injections were necessary to compensate for the shorter half-life of IgA2-HER2 compared to IgG1. Tumor growth was monitored by serial bioluminescent imaging. (A) Representative bioluminescent images taken on day 29. (B) Tumor volume as monitored by bioluminescent imaging. Signal for each individual mouse is expressed as a percentage compared with the signal measured on day 14 (median ± range). Arrows indicate mAb treatment (1×IgG, 10×IgA). (C) Serum concentration of cetuximab and IgA2-HER2 during the first 10 days of treatment (day 15-24) in mice receiving treatment with only one type of mAb (single) or both IgG and IgA (combination; 6-7 mice/group, mean ± SD).

night killing assay (Fig. 5A-D). These results show that increased cytotoxicity induced by the combination of IgG and IgA by leukocytes in a 4-hour assay requires the presence of at least two effector cell types, most likely NK cells and PMNs. Furthermore, the combination of IgG and IgA can also increase cytotoxicity induced by monocytes alone.

Enhanced anti-tumor effect by the combination of IgG and IgA in an *in vivo* xenograft tumor model

Using an *in vivo* xenograft tumor model in human FcαRI transgenic SCID mice we have earlier shown that administration of cetuximab and IgA2-EGFR was able to decrease intraperitoneal outgrowth of A431-luc2-HER2 cells[16]. PMNs and macrophages expressing mouse FcγRs as well as human FcαRI were both recruited to the peritoneal cavity and could participate in tumor killing[16]. We now show that treatment of established tumors with either cetuximab or IgA2-HER2 alone reduced tumor growth compared to the control group. Mice that received both cetuximab and IgA2-HER2 displayed a reduced tumor volume compared to treatment with single mAbs (Fig. 6A and B). The concentration of circulating cetuximab (~3 μg/mL) in serum during the first 10 days after treatment was higher than IgA2-HER2 (~1 μg/mL) (Fig. 6C). At these mAb concentrations maximal lysis was achieved *in vitro* with both mouse PMNs and macrophages with both mAbs (unpublished results; [16]). These data demonstrate that the combination of IgG and IgA enhances anti-tumor effects of mAbs *in vivo*.

DISCUSSION

Intense efforts are being undertaken to increase efficacy of anti-tumor IgG mAbs. However, *in vivo* activity of IgG is limited by the number of available FcγRs, their biological distribution and the cytotoxic capacity of the recruited effector cells. Here we present evidence that the combination of IgG and IgA isotypes can increase mAb-induced tumor cell killing by more optimally harnessing the cytotoxic potential of heterogeneous population of immune effector cells.

Increased maximal killing in the leukocyte ADCC assay results from the recruitment of both NK cells and PMNs that – as purified effector cells – only, or preferentially mediate cytotoxicity by either IgG or IgA. The different anti-tumor effector mechanisms employed by NK cells and PMNs could also contribute to the increased killing. NK cells induce apoptosis in tumor cells through the release of granzymes and perforin. Cytotoxicity mediated by PMNs is less well understood, but it was reported that PMNs can mediate frustrated phagocytosis or induce autophagy in tumor cells by IgA[27].

Purified PMNs preferentially induced cytotoxicity by IgA and only poorly with IgG due to the high expression of the non-signaling FcγRIIIb, which scavenges IgG away from FcγRIIa and therefore decreases IgG activity[12,13,28]. The combination of IgG and IgA did not increase cytotoxicity using purified PMNs. In contrast, purified monocytes induced comparable cytotoxicity by both IgG and IgA and mediated increased cytotoxicity by the combination of IgG and IgA. As both FcγRs and FcαRI signal through the same FcR

gamma chain ITAM, the combination of IgG and IgA could result in stronger cellular signaling in monocytes resulting in increased cytotoxicity.

IgG-opsonized tumor cells in leukocyte ADCC recruit NK cells, eliciting efficient cytotoxicity, but also recruit PMNs and monocytes, leading to less efficient cytotoxicity in a 4-hour assay. Although monocytes alone did not induce cytotoxicity within 4 hours, they could have indirectly contributed to tumor cell killing, *e.g.* through stimulation of NK cells[29]. Triggering of FcRs on monocytes could result in the release of cytokines or other mediators, which may enhance FcαRI- or FcγRI-mediated killing by PMNs (or by monocytes) through activation via inside-out signaling. Cross-talk between effector cells is suggested by the fact that when purified effector cells were used at E:T ratios present in leukocyte ADCC, they were only poorly cytotoxic.

The combination of IgG and IgA led to increased cytotoxicity only when directed against two different tumor targets. The combination of IgG and IgA against the same target did not increase tumor cell killing possibly due to competition for tumor target binding by IgG and IgA. The combination of two IgGs or two IgAs against two different targets also did not increase tumor cell killing, which could be due to saturation of the corresponding receptors. In our *in vitro* leukocyte ADCC assays, we have found that IgA-EGFR alone already induced relatively high maximal tumor cell lysis, and this was not further enhanced by adding trastuzumab. The combination of trastuzumab and IgA-EGFR, however, enhanced cytotoxicity by monocytes, a major cellular effector population of both IgG and IgA, suggesting that this combination can also have a beneficial effect *in vivo*.

Previously, the combination of EGFR and HER2 IgG1 mAbs has been described to lead to synergistic increase of anti-tumor effects, which was mainly attributed to increased direct Fab-mediated anti-tumor effects[22,23]. Although Fab-mediated anti-tumor effects are readily induced by both IgG and IgA, in this study we focused on Fc-mediated effector functions since we do not expect differences mediated by the Fab portion of the mAbs within the time frame of our *in vitro* experiments. However, we expect that combination therapy with IgG and IgA against EGFR and HER2 in patients might also result in enhanced Fab-mediated anti-tumor effects. Despite that maximal specific killing by the combination of IgG and IgA in a 4-hour *in vitro* assay does not reach 100%, the measured increase (~20%) might still have profound consequences *in vivo* and may lead to therapeutic synergy.

At the site of the tumor, the magnitude of tumor lysis will be determined by the number of available Fc moieties (influenced by the target expression, orientation of the Fc part, clustering of target in the cell membrane), the available FcRs (influenced by the numbers and types of effector cells present) and the killing capacity of the effector cells. Studies using engineered mAbs that have a higher affinity for activating FcRs suggest that increasing cellular signaling above a certain threshold does not further increase cytotoxicity[30]. Cytotoxicity can thus be limited by both saturation of the target and by the saturation of the FcRs. This could explain why cytotoxicity by leukocytes was enhanced only by the combination of IgG and IgA against two targets and not by the combination of two IgGs,

or two IgAs against the same target or by the combination of IgG and IgA against the same target. Monocytes express high levels of FcαRI and have a favorable expression pattern of FcγRs (high expression of FcγRI and no expression of FcγRIIIb). This may explain why cytotoxicity was also enhanced by the combination of IgG and IgA against two targets when monocytes alone were used as effector cells.

Individual donors showed variability in the extend by which the combination of IgG and IgA increased cytotoxicity, which could be a result of polymorphisms in FcγRs. Analysis of a number of SNP and CNV polymorphisms as tested by multiplex ligation-dependent probe amplification (MLPA)[31] in various FcγR genes did not reveal a clear correlation with increased cytotoxicity, although the numbers analyzed were small (data not shown).

Intimate contact between effector and target cells is required for efficient cytotoxicity[32]. Our preliminary data suggest that the combination of trastuzumab and IgA2-EGFR, but not cetuximab and IgA2-EGFR or cetuximab and trastuzumab, increased the formation of conjugates between A431-HER2 cells and monocytes or PMNs. Thus, it appears that for PMNs increased binding does not directly translate to more efficient killing, most likely reflecting a contribution of the non-signaling FcγRIIIb to the formation of conjugates.

In the xenograft tumor model both PMNs and macrophages were recruited to the tumor site[16]. In FcαRI Tg mice, both PMNs and macrophages express FcγRs and FcαRI. In pre-clinical studies, macrophages as well as PMNs were found to mediate effects of IgG *in vivo*[26,33,34], whereas in a different model we previously identified macrophages as primary effectors of IgA[16]. However, since PMNs as well as macrophages are effective *in vitro*, it is reasonable to assume that both effector cell types contribute to cytotoxicity *in vivo*. In our mouse model, the increased tumor killing by the combination of IgG and IgA likely results from the recruitment of PMNs and macrophages and increased FcR-triggering on both cell types.

Compared to IgG mAbs, the biology of IgA is less well understood and the production and purification processes are not well-established. However, replacing IgA with a bispecific mAb targeting both a tumor target and FcαRI might be an alternative strategy to overcome this.

In conclusion, we have presented evidence that the simultaneous targeting of both FcγRs and FcαRI appears to more optimally mobilize the cytotoxic resources of effector cells and therefore represents a conceptually new approach to improve the efficacy of mAb therapy.

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the excellent animal care.

AUTHOR CONTRIBUTIONS

A.M.B., T.t.B., L.A.P.M.M., G.v.T., R.U., G.R., S.L., T.V., J.H.W.L and P.B. designed research; A.M.B., T.t.B., M.N., J.H.M.J, L.A.P.M.M., G.v.T., S.Q.N., M.A.B.B., and P.B. performed research; S.L., and T.V., contributed new reagents / analytic tools; A.M.B., T.t.B., L.A.P.M.M., G.v.T., R.U., G.R., I.N., S.L., T.V., J.H.W.L., and P.B. analyzed data; and A.M.B., T.t.B., J.H.W.L., and P.B. wrote the paper.

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SUPPLEMENTARY MATERIAL

Table S1. Number of target molecules on the different cell lines

	Molecules / cell ($\times 10^4$)	
Cell line	EGFR	HE
A1207	114.7 ± 4.4	1.1
A431	92.6 ± 0.6	1.9
A431-HER2	101.1 ± 1.3	69.
SK-BR-3	5.1 ± 0.2	78.
BxPC3	23.8 ± 0.5	1.1

Table S2. Overview of ADCC assays

			Maximum lysis		
Effectors	Antibodies		<5%	5-80%	>80%
Leukocytes	Cetuximab	IgA2-HER2	1/11 (9%)	10/11 (91%)	-
Leukocytes	Trastuzumab	IgA2-EGFR	-	5/9 (55.6%)	4/9 (44.4%)
Leukocytes	Trastuzumab	IgA2-HER2	-	9/9 (100%)	-
Leukocytes	Cetuximab	IgA2-EGFR	-	4/6 (66.7%)	2/6 (33.3%)
Leukocytes	Cetuximab	Trastuzumab	-	10/10 (100%)	-
Leukocytes	IgA2-EGFR	IgA2-HER2	-	7/7 (100%)	-
PMN	Trastuzumab	IgA2-EGFR	2/2 (100%)	-	-
PMN	Cetuximab	IgA2-HER2	1/9 (11.1%)	7/9 (77.7%)	1/9 (11.1%)
Monocytes	Trastuzumab	IgA2-EGFR	-	7/7 (100%)	-
Monocytes	Cetuximab	IgA2-HER2	-	7/7 (100%)	-

For each effector cell type and mAb combination the donors are distributed into the following categories based on the maximum lysis by the single mAbs: maximum lysis <5%, between 5% and 80%, >80%. Only the donors where both single mAbs resulted in a maximal lysis between 5 and 80% were analyzed further.

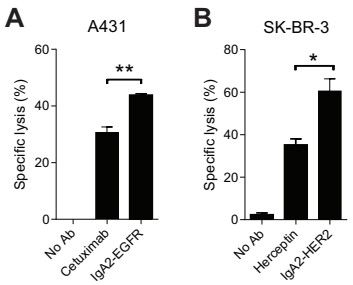

Figure S1. Leukocyte-mediated cytotoxicity with IgG and IgA. 4-hour ^{51}Cr-release assay by leukocytes using (A) A431 cells and (B) SK-BR-3 cells and 1 μg/mL mAb. Representative results of 2 (A) and 4 (B) experiments performed with different donors (mean ± SEM, *p<0.05, **p<0.05, Student's t-test).

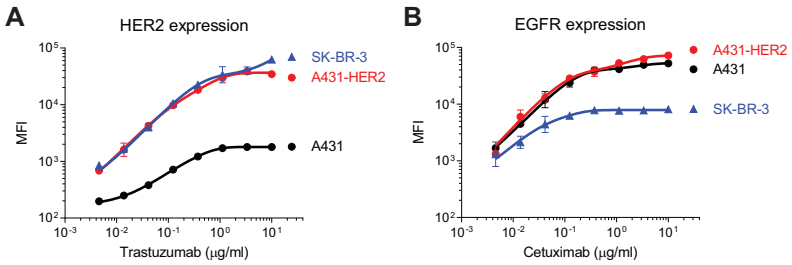

Figure S2. Binding of cetuximab and trastuzumab to different tumor cell lines. To study cytotoxicity of both EGFR and HER2 mAbs we established A431-HER2 cells by retrovirally transducing A431 cells with human HER2. HER2 expression on A431-HER2 cells was comparable to SK-BR-3 cells, whereas EGFR expression did not change compared to parental A431 line. A431, A431-HER2 and SK-BR-3 cells were incubated with trastuzumab (A) or cetuximab (B) followed by staining with FITC-labeled anti-human IgG mAb.

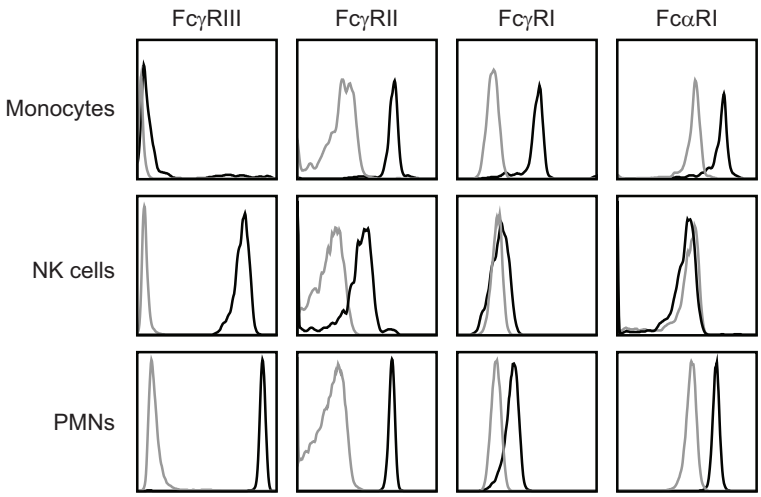

Figure S3. FcR expression on effector cells. Expression of FcγR and FcαRI expression was measured by flow cytometry using mAbs specific for FcγRI (CD64, clone 10.1), FcγRIIa (CD32, clone IV.3), FcγRIII (CD16, clone 3G8) and FcαRI (CD89, clone A77). Effector cells were identified in leukocytes by the following surface markers: monocytes: $CD14^{+}CD16^{+}$, PMNs: $CD16^{+}$ and NK cells: $CD3^{-}CD56^{+}$.

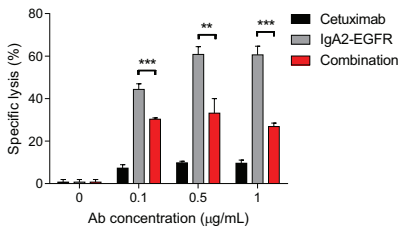

Figure S4. Cetuximab inhibits IgA2-EGFR-induced cytotoxicity by purified PMNs. PMNs were isolated by Ficoll/Histopaque separation and used for ADCC assay at E:T=40:1 against A431-HER2 cells. mAbs were mixed at a 1:1 ratio and each single mAb was used at the indicated concentration (mean ± SD; ***: $p<0.001$; **: $p<0.01$; Student's t-test).

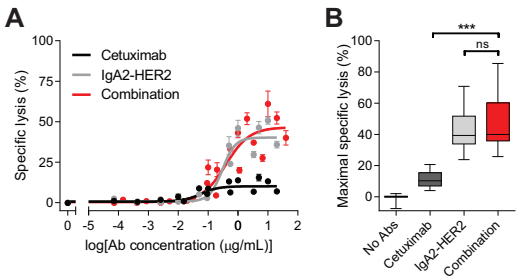

Figure S5. PMN-mediated cytotoxicity is not increased by the combination of IgG and IgA against two targets. (A) Pooled data (8 donors) from ADCC assays were performed using purified PMNs isolated by Ficoll/Histopaque separation and A431-HER2 target cells either with cetuximab, or IgA2-HER2 or both mAbs (mean ± SEM). (B) Maximal specific lysis plotted from 8 donors (median, box extends from the 25th to 75th percentiles, whiskers to min and max; ***: p<0.001; ANOVA, Tukey's multiple comparison test).

CHAPTER 7

Clarifying the confusion between cytokine and Fc receptor "common gamma chain"

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The terms for the common cytokine receptor gamma chain (γ_c or CD132) and the Fc receptor gamma chain (FcRγ) have often been mixed or inconsistently used. This is problematic, because they are clearly distinct molecules in sequence, function, and chromosome location. However, they also share some signaling pathways and can be expressed on the same leukocytes. This makes the term "common gamma chain" potentially misleading, especially to those not in the field. To avoid future confusion, we are proposing the use of a consistent nomenclature to distinguish between the two molecules.

Cytokines are important soluble mediators of the immune system that can interact with cytokine receptors on many cells of the immune system. In this way, cytokines can regulate the activation, differentiation, and responsiveness of immune cells. The common cytokine receptor gamma chain (γ_c) was first identified as the third chain of the IL-2 receptor complex and therefore was known as the IL-2R γ[1]. However, the same subunit was identified as part of several other cytokine receptors complexes: IL-4, IL-7, IL-9, IL-15, and IL-21, and therefore renamed to γ_c (common cytokine receptor gamma chain)[2, 3]. The γ_c is essential for the signal transduction of these cytokine receptors, but is also required for ligand binding. All γ_c cytokines have different functions in the immune system, although they use the same γ_c.

Binding of a cytokine to its receptor activates the associated Janus kinase (JAK)-family protein tyrosine kinases JAK1 and JAK3, and triggers the transphosphorylation of JAK1 and JAK3 on tyrosines. JAK1 is associated with the unique α or β chain and JAK3 with the γ_c of the receptor. The phosphorylated JAKs can in turn activate the signal transducers and activators of transcription (STAT) proteins, which together form the JAK/STAT signaling pathway. The phosphorylation of STATs causes dimerization of STATs, which now adopt a high-affinity DNA-binding activity and translocate to the nucleus. Here, they act as transcription factors inducing the transcription of target genes.

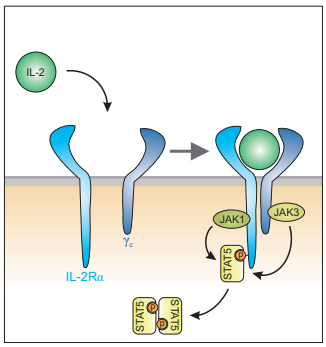
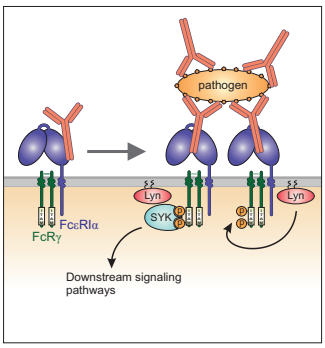
The γ_c gene (*IL2RG*) is located on chromosome Xq13 and mutations in the γ_c gene cause the most common form of SCID, X-linked severe combined immunodeficiency diseases (XSCID). XSCID patients lack or have strongly reduced numbers of T cells and NK cells, while B cell numbers are normal. However, the B cells are not functional, resulting in a severe immunodeficiency. Defective cytokine signaling underlies the cause of XSCID and also mutations in JAK3 result in the same phenotype as XSCID[4,5].

Fc receptors (FcR) on immune cells bind to the crystallizable fragment (Fc) of immunoglobulins (antibodies), which is an essential step in the activation of cellular effector functions by complexes of antibody and antigen. In normal immunity this Fc:FcR recognition of opsonized pathogens and non-self antigens leads to their elimination; in pathological immunity the same interactions can lead to destructive inflammation as seen in systemic lupus erythematosus or IgE dependent allergy. FcRγ was first discovered as the third subunit of FcεRI and therefore termed FcεRIγ[6] where it is essential for FcεRI signaling via an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic tail. Later it became clear that this subunit is shared by other activating FcR (FcγRI, FcγRIIIa, and FcαRI), but also many other immune receptors, including GPVI, OSCAR, and TREM (Table S1). There is considerable confusion on the terminology of FcRγ, being

referred to in many ways: FcεRIγ, common γ chain subunit, FcR common γ-chain, and even common gamma-chain. The last term is especially confounding, since the most used name for γ_c is also common gamma chain. This is even more confusing as FcRγ has been reported to associate with at least one cytokine receptor, IL-3R[7].

FcRγ is usually a homodimer of two chains linked via a disulfide bride and each chain contains one ITAM that has a $YxxLx_{(7)}YxxL$ sequence. FcRγ, which contains only five extracellular residues, is not involved in ligand binding of the receptor. It also forms heterodimers with the CD3ζ chain. After crosslinking of activating FcR by immune complexes, kinases of the SRC family phosphorylate the tyrosine residues of the FcRγ ITAM that provide a docking site for kinases of the SYK family. Via the activation of several downstream targets, SYK activation results in activation of the RAS-MAPK pathway, increased intracellular calcium levels and ultimately activation of NF-κB transcription factors. These signals can activate the immune cell and lead to *e.g.* phagocytosis, oxidative burst, and cytokine release.

Table S1. Comparing the common cytokine receptor gamma chain and the Fc receptor gamma chain

	Common cytokine receptor gamma chain (γ_c)	**Fc receptor gamma chain (FcRγ)**
Illustration		
Gene	*IL2RG*	*FCER1G*
Chromosome	X	1
Polypeptide length	369 aa	86 aa (monomer)
Associates with	IL-2R, IL-4R, IL-7R, IL-9R, IL-15R, IL-21R	FcεRI, FcγRI, FcγRIIIa, FcαRI, GPVI, OSCAR, TREM, PIR-A, Dectin-2, active γδTCR, IL-3R
Cellular distribution	T cells, B cells, NK cells, dendritic cells, mast cells, basophils	Monocytes, macrophages, neutrophils, B cells, NK cells, dendritic cells, mast cells, eosinophils, basophils
Associated signaling molecules	JAK3, STATs	Src family kinases (Lyn), SYK
Associated diseases	XSCID	N.F.

N.F. = Not found, indicating that to date no diseases have been directly linked to mutations in FcRγ. Abbreviations: aa = amino acid; XSCID: X-linked severe combined immunodeficiency diseases.

To date, no mutations in the FcRγ gene (*FCER1G*), located on chromosome 1 of humans and mice, have been described that lead to pathogenicity. However, Fcer1g$^{-/-}$ mice have shown the importance of FcRγ for the function of activating FcR in anti-tumor therapy, antigen presentation, and bacterial clearance[8]. FcRγ is also required for optimal expression of the associated FcR, thus mice deficient in FcRγ also lack or have reduced activating FcR expression. Another mouse model harboring signaling incompetent FcRγ (NOTAM mice) with normal FcR expression confirmed the necessity of FcRγ signaling for CD20 therapeutic antibodies activity and antigen cross-presentation[9].

Although both gamma chains are essential for the signal transduction of many immune cell receptors, they are very different (Table S1). To make nomenclature more confused, the CD3 complex on T cells contains an invariant gamma chain and the TCR of gamma delta (γδ) T cells also contains a gamma chain. In addition, FcRγ has been reported as part of at least one cytokine receptor, IL- 3R. Furthurmore, γδ T cells express several members of the γ_c cytokine receptor family and after activation the γδTCR actually incorporates FcRγ into the complex[10]. Other immune cells can also express both γ_c- and FcRγ-containing receptors, like NK cells, mast cells, and basophils (Table S1). Thus, it should be clear when using "common gamma chain" in literature which gamma chain is meant! We propose the consistent use of the following nomenclature for these two subunits, as is also used in this review throughout: for the *IL2RG* encoded protein, common cytokine receptor gamma chain (γ_c or CD132) or "common gamma chain", and for the *FCER1G* encoded protein, Fc receptor gamma chain (FcRγ). While our understanding of the immune system increases, the complexity and number of proteins involved rapidly expands as well. It is therefore important to be precise and consistent with the nomenclature of proteins.

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CHAPTER 8
General discussion

Fc receptors (FcR) are required for the cellular effector functions of antibodies, including protection against bacteria, phagocytosis, cytokine release, reactive oxygen species (ROS) production, and antigen presentation. Since FcR are mainly expressed on innate immune cells, they facilitate the link between the specific adaptive immune system and potent innate immune responses. FcR activation is tightly regulated to prevent immune responses by non-antigen-bound antibodies or in the absence of other 'danger signals'. Most Fc gamma receptors (FcγR) recognize antibodies of the IgG subclass with low affinity, but bind immune complexes (IC) such as opsonized pathogens with high affinity. FcγRI is the only human receptor capable of binding monomeric IgG with high affinity. Therefore, FcγRI is saturated with monomeric IgG *in vivo*, but also after isolation or extravasation of immune cells. Because of this, FcγRI has been far less studied than the low affinity FcγR. However, an important role for FcγRI during inflammation, autoimmune responses, and antigen presentation has been established[1-4]. FcR-mediated effector functions are exploited by therapeutic antibodies, including those approved for the treatment of cancer. Although monoclonal antibody (mAb) therapy can be effective in treating malignancies, patients are rarely cured by mAb therapy alone. Therefore, a better understanding of FcR regulation and activation is needed, to device strategies to enhance mAb therapy efficacy.

FC GAMMA RECEPTOR I

High affinity binding of FcγRI

FcγRI is the only receptor with a high affinity for monomeric IgG (K_D of 10^{-8}-10^{-9} M). The high affinity has been a major defining but also puzzling feature of FcγRI. In an environment with very high IgG concentrations, like peripheral blood, FcγRI is bound by monomeric IgG and this leaves little opportunity for activation by IC. In tissues, FcγRI is most likely still occupied by monomeric IgG. The high affinity of FcγRI is also a unique feature within the FcγR family and much research has focused on how this high affinity is achieved. FcγRI is the only FcγR with three extracellular domains, of which the outer two domains (EC1 and EC2) are homologues to the other FcγR while EC3 is only present in FcγRI. Therefore, it has been postulated that EC3 is required for this high affinity binding[5,6]. Indeed, initial studies showed that EC3 is required but not sufficient for high affinity IgG binding[7,8]. However, when chimeric receptors were generated by exchanging EC domains between FcγRI and FcγRIIa, it was shown that EC2 of FcγRI is essential in mediating the high affinity binding[9]. The fusion of FcγRI EC3 to EC1 and EC2 of FcγRIIa did not induce measurable monomeric IgG binding, indicating that FcγRI EC3 indeed is not sufficient for high affinity binding.

Although the crystal structures of the low affinity FcγR in complex with IgG were already resolved several years ago, for FcγRI this only happened quite recently. The first structure of FcγRI in complex with the Fc domain of IgG1 revealed that the binding of FcγRI to IgG is very similar as for the other FcγR[10,11]. This involves an asymmetrical binding interface common for the binding of Fc to all FcγR, where each chain of the Fc homodimer uses a different set of residues to interact with the receptor. FcγRI EC2

interacts with the lower hinge region of the Fc homodimer, while EC3 is not in direct contact with the Fc. The FG loop of FcγRI EC2 was identified to interact with the glycan structure at Asn^{297} of the Fc homodimer[10]. The characteristics of the FcγRI FG loop are unique compared to the other FcγR, since it one amino acid shorter (six instead of seven residues) and positively charged, enabling it to move closer to the Fc glycan and adopting a glycan binding conformation. Indeed, placing the FG loop of FcγRI into FcγRIIIa resulted in a drastic increase in IgG binding affinity, although this chimeric receptor bound IgG fivefold weaker than FcγRI[12]. Moreover, the characteristics of the FG loop of FcγRI are conserved in mammalian species[12].

Two other crystal structures of FcγRI in complex with the Fc domain of IgG1 were published. While all three structures depict a similar overall interaction of FcγRI with the Fc domain, some differences were observed. First of all, Kiyoshi *et al.* do not observe the interaction of the FC loop with the Fc glycan. Furthermore, they describe a different conformation of one of the Fc chains for the FcγRI-Fc interaction in which Leu^{235} inserts into a unique hydrophobic pocket of FcγRI[13]. This changes the orientation of the Fab domains relative to the membrane, since the lower hinge region of the Fc homodimer adopts a more linear conformation. EC3 of FcγRI is postulated to function as a spacer, allowing the Fab domains to face the plasma membrane. Oganesyan *et al.* describe a crystal structure with an even higher resolution than the previous two structures and describe an FcγRI-Fc interaction very similar to that of Kiyoshi *et al.* No direct contribution of Fc glycans to the FcγRI-Fc interaction was observed and Leu^{235} was again found to be positioned inside a hydrophobic pocket of FcγRI[14]. In this study, chimeric receptors were generated by exchanging EC domains of FcγRI with FcγRIIIa. Removing EC3 from FcγRI results in only a slightly lower affinity for IgG. Concurrently, the addition of FcγRI EC3 to FcγRIIIa results in a slightly increased IgG binding[14]. While EC1 is interchangeable between both receptors, exchanging EC2 of FcγRI for EC2 of FcγRIIIa severely impacts the binding of monomeric IgG.

To conclude, there is a common binding mode for the Fc homodimer to FcγR in which one receptor interacts with the lower hinge region of two identical Fc domains through EC2. For FcγRI, the FG loop of EC2 is, at least partly, responsible for the high affinity interaction with IgG. To what extent the interaction of FcγRI with the Fc glycans is contributing to the high affinity remains to be resolved. Although unique to FcγRI, EC3 is not directly involved in the binding of IgG or solely responsible for the high affinity of FcγRI. EC3 might have an indirect role, for example by inducing a more favorable orientation of EC1 and EC2 relative to the plasma membrane. Possibly, EC3 might be involved in the enhanced IC binding after FcγRI inside-out signaling. As the V39I SNP indicates (**chapter 5**), monomeric IgG binding and IC binding somehow use different residues or conformations of FcγRI. The major limitation of crystallography studies is that only soluble complexes can be crystalized, while for these research questions the structure of FcγRI within the plasma membrane with or without IC would be highly informative.

Despite the fact that FcγRI is the only FcγR that can bind monomeric IgG, it is important to note the difference between binding and retaining the interaction. Compared to the

other high affinity FcR FcεRI, FcγRI has a much higher dissociation constant leading to a half-life of only several minutes for the interaction of FcγRI with monomeric IgG1, whereas the interaction of FcεRI with IgE has a half-life of >19 hours[15]. This indicates that monomeric IgG rapidly associates and dissociates from FcγRI under physiological conditions. In this way, FcγRI on immune cells can bind and represent the IgG repertoire in the immediate microenvironment. Furthermore, the high dissociation rate might actually be beneficial for IC binding. If IC are present in the surroundings of FcγRI-expressing cells, the rapid release of monomeric IgG from FcγRI might give IC the opportunity to bind FcγRI with higher avidity. Despite the higher dissociation constant, the high IgG titers in serum will most likely saturate FcγRI *in vivo*. It must be noted that the serum IgG titers should be sufficient to saturate the low affinity FcγR as well. Together, this highlights the importance of cytokine-induced inside-out signaling of FcR, which induces a rapid increase in IC binding and allows immune cells to respond to their environment even in the presence of high antibody concentrations.

Regulating FcγRI function

Crosslinking of FcγRI by IC initiates immunoreceptor tyrosine-based activation motif (ITAM)-dependent signaling pathways essential for the effector functions of FcγRI[16,17]. These signaling pathways and downstream effector functions are similar for FcγRI and the other activating FcγR. First, kinases of the SRC family phosphorylate the tyrosine residues of the FcRγ ITAM that provide a docking site for kinases of the SYK family. Via the activation of several downstream targets, SYK activation results in activation of the RAS-MAPK pathway, increased intracellular calcium levels, and ultimately activation of NF-κB transcription factors[18]. These signals can activate the immune cell and lead to *e.g.* phagocytosis, oxidative burst, cytokine release, and antigen presentation.

Interestingly, binding of monomeric IgG does not trigger downstream signaling of FcγRI[17,19]. Binding of monomeric IgG does, however, induce FcγRI internalization followed by a rapid recycling to the cell surface[19]. This recycling is tyrosine kinase independent, indicating that the ITAM signaling is not involved. Crosslinking of FcγRI diverts FcγRI-IgG-antigen complexes from the recycling pathway to the lysosomal degradation pathway[19]. This leads to the degradation of proteins, including the antigen bound by IgG, and allows the presentation of antigen peptides. It was speculated that the recycling of FcγRI is a mechanism to sample the environment for antigens that need to be presented to T and B cells. However, FcγRI crosslinking is required for the induction of antigen presentation and it has been shown that binding of monomeric IgG induces distinct signaling compared to receptor clustering[4,17,19]. Recycling of FcγRI to the cell surface occurs with IgG still bound to FcγRI, and not 'free' FcγRI, making this sampling hypothesis not very likely. Thus, the function of monomeric IgG bound-FcγRI endocytosis and recycling remains unclear, although this does not contribute to the long half-life of IgG, for which the neonatal Fc receptor (FcRn) is essential. Studies with high-resolution imaging techniques might provide more insight, since most of the work on FcγRI biology was performed two decades ago when imaging techniques, for example, were not as advanced as they are

today. Indeed, as shown in **chapter 3**, FcγRI nanoclusters in the plasma membrane cannot be observed with confocal microscopy but are clearly detected by super-resolution imaging. Moreover, it would be interesting to study the internalization and recycling dynamics in primary human cells expressing FcγRI, like monocytes and macrophages, since previous studies mainly used FcγRI-expressing cell lines.

FcγRI expression and activation is regulated by cytokines. Certain cytokines, like IFNγ and IL-10, can upregulate FcγRI expression on innate immune cells by inducing transfer of intracellular FcγRI to the plasma membrane as well as inducing *de novo* expression[20-22]. The FcγRI promotor has an IFNγ response region and exposure to IFNγ has been shown to significantly enhance FcγRI expression on monocytes, macrophages, and myeloid cells[21,23,24]. Furthermore, FcγRI expression can be induced on other immune cells, especially neutrophils, but also mast cells[20,25]. Induction of FcγRI on neutrophils correlates well with the expression of proinflammatory cytokines and expression of FcγRI on neutrophils has been used as a biomarker for sepsis with quite good sensitivity and specificity[26,27]. Both IL-10 and granulocyte colony-stimulating factor (G-CSF) increase FcγRI expression on monocytes and dendritic cells (DCs)[22,28]. IL-4, on the other hand, decreases FcγRI expression on human monocytes and can counteract the IFNγ-induced FcγRI expression increase[29]. IL-10, in turn, can reverse the IL-4 mediated downregulation of FcγRI on monocytes[22]. Together, these data indicate that several cytokines can act in concert to regulate FcγRI expression.

Cytokines can also induce inside-out signaling of FcγRI and thereby enhance the binding capacity of FcγRI towards IC. This is a rapid process occurring within minutes after addition of cytokines and precedes the possible increase in FcγRI expression. Previously, it was shown that IL-3 stimulation of Ba/F3-FcγRI cells resulted in a slight increase in monomeric IgG binding and a strong increase in IC binding, even in the presence of monomeric IgG[30]. In **chapter 3**, we sought to elucidate the mechanisms underlying FcγRI inside-out signaling. Using high resolution imaging techniques, we established that FcγRI is organized in small clusters in the plasma membrane and that FcγRI clustering increases after cytokine stimulation. These FcγRI clusters remain mobile within the plasma membrane, since the diffusion of FcγRI is not altered after inside-out signaling. This bears resemblance to the other high affinity FcR, FcεRI, where small FcεRI clusters remain mobile and signaling competent in the plasma membrane[31]. Addition of multivalent antigen to FcγRI reduced receptor mobility under all conditions, consistent with the formation of crosslinking-induced receptor aggregates, as was previously shown for FcεRI[32], and the increased FcγRI cluster size.

Recently, the inhibitory receptor SIRPα was shown to colocalize with nanoclusters of FcγRI in the plasma membrane of human macrophages[33]. SIRPα binds to CD47 on target cells and this interaction induces a 'don't eat me' signal via immunoreceptor tyrosine-based inhibitory motif (ITIM) signaling of SIRPα, thereby recruiting the phosphatase SHP-1[34]. Upon crosslinking of FcγRI with human IgG, FcγRI and SIRPα segregate into separate nanoclusters[33]. Like for the increased FcγRI clustering after inside-out signaling, actin polymerization was required for this altered FcγRI organization in the membrane. The cluster sizes of FcγRI in resting macrophages in this study were comparable to the

cluster sizes we observed in **chapter 3**. The authors propose a model where FcγRI crosslinking induces segregation of SIRPα and FcγRI to keep SHP-1, recruited to the ITIM of SIRPα, away from FcγRI to allow ITAM signaling of FcγRI. When SIRPα is activated via CD47, the segregation is prevented and SHP-1 can now counteract FcγRI signaling. One question arises with this model: how is FcγRI signaling initiated? Because FcγRI signaling is most likely inhibited until FcγRI and SIRPα clusters segregate, but FcγRI signaling is seemingly also necessary to initiate this segregation. The crosslinking of FcγRI might increase FcγRI clustering, as we observed as well, and this mechanical force might initiate the segregation from SIRPα clusters, although this theory requires additional studies. Furthermore, it would be interesting to study the dynamics of FcγRI and SIRPα nanoclusters interactions, whether these clusters are mobile or fixed within the plasma membrane.

Enhanced FcγRI clustering most likely skews cells towards the binding of IC over monomeric IgG by increasing the receptor avidity. However, monomeric IgG binding is also slightly enhanced after cytokine stimulation, indicating that FcγRI affinity is increased, possibly by a conformational change. In **chapter 4**, we set out to generate conformation-specific antibodies that would recognize either 'active' or 'inactive' FcγRI. After immunizing mice with FcγRI-expressing cells, we could detect more antibodies in the serum that bound Ba/F3-FcγRI cells stimulated with IL-3 compared to antibodies that bound unstimulated (no IL-3) cells. Furthermore, crystallography data indicate that the third ectodomain (EC3) of FcγRI undergoes a conformational change after monomeric IgG binding[13]. These data support the hypothesis that FcγRI can adopt a higher affinity conformation. Further research might lead to the generation of conformation-specific antibodies for FcγRI and provide structural insights into FcγRI inside-out signaling.

The cytoplasmic (CY) domain of FcγRI is essential for the increased IC binding after cytokine stimulation (**chapter 3**), although the CY domain is not required for monomeric IgG binding[35]. While the Fc receptor γ-chain (FcRγ) is required for stable surface expression and intracellular signaling after FcγRI crosslinking, the α-chain of FcγRI has been shown to have several functions itself. Deletion of the CY domain reduces phagocytosis, slows receptor endocytosis kinetics, and abrogates IL-6 production[36]. Additionally, even though internalization of IC was unaffected, the CY domain deletion resulted in more recycling of FcγRI-IC complexes and decreased antigen presentation[4]. The four serines in the CY domain of FcγRI are constitutively phosphorylated and have to be dephosphorylated to allow efficient FcRγ signaling[37]. The serine/threonine phosphatase PP1 is involved in FcγRI inside-out signaling and we therefore hypothesized that the serines and/or threonines in the CY domain of FcγRI were targeted by PP1. However, our data indicate that these residues are not involved in FcγRI inside-out signaling and that PP1 dephosphorylates other proteins in order for FcγRI activation to occur.

Since deletion of the CY domain abrogates FcγRI inside-out signaling, the question remains what the role of FcγRI CY is in this process. We propose two, not mutually exclusive, hypotheses: (1) the CY domain interacts directly or indirectly with the actin cytoskeleton and (2) other post-translational modifications, like ubiquitination, occur on the CY domain leading to altered receptor localization or internalization/recycling. The

CY domain of FcγRI was shown to interact with the actin-binding proteins filamin A and periplakin[38,39], and an intact actin cytoskeleton is required for FcγRI inside-out signaling (**chapter 3**). Crosslinking of FcγRI reduces the colocalization of FcγRI and filamin A, possibly releasing FcγRI from the actin cytoskeleton and allowing efficient phagocytosis[38,40]. During inside-out signaling, the actin cytoskeleton is most likely involved in arranging FcγRI in larger clusters. It should be tested whether FcγRI without CY domain, or FcγRI mutants only lacking the filamin A or periplakin binding site, indeed does not increase its cluster size after inside-out signaling. To follow up on the second hypothesis, ubiquitination of a protein can lead to its proteasomal degradation, but might also alter its localization or protein interactions. Since FcγRI is continuously internalized and recycled after monomeric IgG binding, ubiquitination of FcγRI might play a role in the internalization and the decision between recycling or degradation of FcγRI. During inside-out signaling, FcγRI ubiquitination or other post-translational modifications might induce receptor internalization. Indeed, inhibiting endocytosis with Dynasore or nystatin reduces the increased IC binding induced by cytokines (own unpublished observations). This indicates a role for FcγRI endocytosis during FcγRI inside-out signaling, although it is puzzling how receptor internalization can increase FcγRI IC binding. Possibly, clustering of FcγRI occurs on endosomes after internalization and these preformed clusters are then recycled to the plasma membrane. However, this premise requires further investigation.

Besides cytokines, receptor function is regulated by single nucleotide polymorphisms (SNPs) of FcγRI (**chapter 5**). The only SNP located in the extracellular domain of FcγRI, V39I, reduces IC binding of FcγRI and this leads to less FcRγ signaling and intracellular calcium mobilization. Interestingly, this amino acid change has no impact on monomeric IgG binding, only on IC binding. Because of this, it would be interesting to study FcγRI inside-out signaling is affected by the V39I SNP. Furthermore, this indicates that the interaction of FcγRI with monomeric IgG or IC has different structural requirements. In line with the location of SNPs I301M and I338T, the transmembrane (TM) or CY domain, respectively, they have no influence on IC binding but have substantially reduced FcγRI signaling. The amino acids of the FcγRI TM domain involved in the interaction with FcRγ have not been identified yet. This is contrast to FcαRI, where a positively charged arginine (Arg^{209}) associates with a negatively charged aspartic acid (Asp^{11}) in the TM domain of FcRγ[41,42]. Recently, the interaction of FcγRIIIa with the FcRγ was described in more detail, in which a network of polar and aromatic residues on one side of the TM domain interacts with FcRγ[43]. Three key amino acids of the FcγRIIIa TM domain were described to be very important for the interface with FcRγ. The FcγRI TM domain contains a very comparable motif that forms a similar interface with FcRγ as FcγRIIIa. Interestingly, FcγRI Ile^{301} in the TM domain is very close to this motif and therefore we hypothesize that the I301M SNP interferes with the interaction between FcγRI and FcRγ, leading to reduced intracellular signaling after receptor crosslinking. However, the association of FcγRI with FcRγ was shown to increase the affinity of FcγRI for monomeric IgG, indicating that this interaction might induce an optimal conformation of FcγRI for IgG binding[9], while monomeric IgG binding was not affected by the I301M SNP. The addition of a threonine (I338T) in the

CY domain of FcγRI also reduces intracellular signaling. Especially when phosphorylated, this threonine might interfere with efficient recruitment of signaling molecules. It would be interesting to study if the combination of all three SNPs completely abrogates intracellular signaling, although this combination is not likely to occur within the population due to the low SNP frequency.

In conclusion, FcγRI functions are regulated on multiple levels: (1) the interaction of FcγRI with monomeric or multivalent ligands induces different responses, (2) short term exposure to cytokines can rapidly increase IC binding and subsequent effector functions of FcγRI, while long term exposure also induces increased FcγRI expression, (3) the association with FcRγ influences the FcγRI ligand binding and downstream signaling, and (4) FcγRI SNPs can modulate the effector functions of FcγRI (Fig. 1). Aberrant regulation of FcγRI might lead to autoimmune diseases or excessive inflammation if FcγRI function is increased or higher susceptibility to infections and less efficient mAb therapy if FcγRI function is decreased. Indeed, a role for FcγRI in these IgG-mediated immune responses and diseases has been demonstrated, as discussed below.

FcγRI *in vivo*

Because of its high affinity for monomeric IgG, for a long time FcγRI was believed to be incapable of participating in antibody responses. However, studies with genetically manipulated mice have shown considerable roles for FcγRI in different immunological processes and diseases. In humans, FcγRI is the only high affinity IgG receptor, whereas in mice both mFcγRI and mFcγRIV are high affinity IgG receptors for mIgG2a and mIgG2a/b, respectively. Based on sequence similarity, mFcγRI is the homolog of human FcγRI, although the intracellular domain differs substantially between human and mouse FcγRI[44]. mFcγRIV, on the other hand, is not related to human FcγRI based on amino acid sequence but is proposed to be a functional homolog of human FcγRI (and human FcγRIIIa)[45].

The generation of mFcγRI$^{-/-}$ mice revealed that FcγRI substantially contributes to the severity of arthritis and hypersensitivity responses and is required for protection against bacterial infections[2]. Furthermore, a reduction in phagocytosis, cellular cytotoxicity, cytokine production, and antigen presentation was observed in cells from mFcγRI$^{-/-}$ mice[1,2]. An unexpected role of FcγRI in inhibiting angiogenesis was discovered more recently[46,47]. Using mFcγRIV deficient mice or specific blocking antibodies against mFcγRIV, it was demonstrated that this receptor is involved in autoimmune anemia, thrombocytopenia, arthritis, and systemic anaphylaxis[48,49].

For mAb therapy of melanoma lung metastases, mFcγRI was shown to play a central role in one study[50], while another study concluded that mFcγRIV and not mFcγRI was essential in this process[48]. In a liver metastases model of melanoma, mFcγRI and mFcγRIV seem to have redundant roles because only the inhibition of both receptors abrogated mAb therapy[51]. It can be concluded that high affinity IgG receptors are involved in mAb therapy for cancer, but that dependent on the exact experimental model and use of receptor knock-out mice or blocking antibodies different, partly contradictory, out-

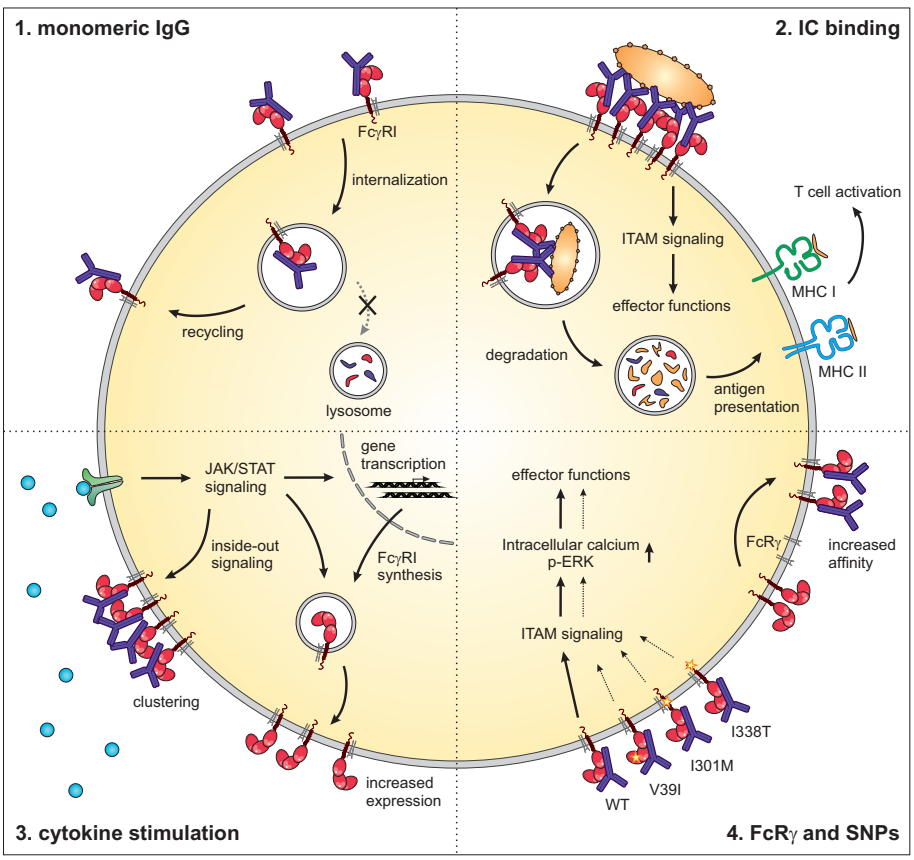

Figure 1. FcγRI functions are regulated on multiple levels. (1) The interaction of FcγRI with monomeric IgG induces rapid internalization and recycling of FcγRI bound to IgG to the plasma membrane. (2) Crosslinking of FcγRI by multivalent antigen induces immunoreceptor tyrosine-based activation motif (ITAM) signaling via FcRγ and internalization and degradation of the receptor-antigen complex in the lysosome. The generated peptides can be presented on MHC class I (MHC I) or MHC class II (MHC II) and this can lead to T cell activation. Following ITAM signaling, other cellular effector functions besides antigen presentation like phagocytosis, cytokine release, and reactive oxygen species production are induced. (3) Cytokines activate JAK/STAT signaling via cytokine receptors. This induces inside-out signaling of FcγRI leading to enhanced clustering of FcγRI in the membrane and this process is dependent on PP1, the actin cytoskeleton and the intracellular domain of FcγRI. Cytokines also regulate the expression of FcγRI. IFNγ and IL-10 upregulate FcγRI surface expression by inducing transfer of intracellular FcγRI to the plasma membrane as well as inducing *de novo* expression. (4) Association of FcγRI with FcRγ in the membrane increases its affinity for monomeric IgG and is required for stable surface expression and ITAM signaling. Single nucleotide polymorphisms (SNPs) of FcγRI lead to reduced ITAM signaling compared to FcγRI WT, thereby reducing the intracellular calcium rise and phosphorylation of ERK (p-ERK). Together, this leads to less FcγRI-mediated effector functions.

comes can be obtained. This underlines the importance of using multiple experimental models and indicates possible redundancy between FcγR.

Using human FcγRI transgenic mice, it was shown that FcγRI can mediate antigen (cross) presentation *in vivo*[52,53]. Furthermore, direct targeting to human FcγRI by bispecific antibodies (bsAbs) in these mice results in tumor cell killing and increased antigen presentation[54]. For IgG1-mediated protection against malaria, the transgenic expression of FcγRI was shown to be essential[55]. Targeting FcγRI and *Candida albicans* with a bsAb protected FcγRI transgenic mice against infection and induced a potent immune response[56]. One important difference in FcγRI expression in the constitutive expression of human FcγRI on mouse neutrophils, while human neutrophils have inducible FcγRI expression[52,57]. Besides that, mFcγRI and mFcγRIV are still present in these human FcγRI transgenic mice. The influence of these two factors on the interpretation of the role of human FcγRI *in vivo* is unclear.

More recently, the human FcγRI transgenic mice were crossed with mice deficient for multiple endogenous FcR (FcγRI/II/III and FcεRI/II). In these mice, human FcγRI was sufficient to mediate autoimmune arthritis, thrombocytopenia, IC-induced airway inflammation, and both active and passive systemic anaphylaxis[3]. In addition, human FcγRI was sufficient for mAb therapy of metastatic melanoma[3]. Since mFcγRIV was still expressed in these mice, the function of this receptor was blocked with a specific blocking antibody. Using the mouse FcγRI/II/III and FcεRI/II knock-out mice as mFcγRIV^{only} mice more firmly established the role mFcγRIV in autoimmune thrombocytopenia, arthritis, IgG-mediated airway inflammation, and systemic anaphylaxis[58-60]. Interestingly, the role of mFcγRIV in mAb therapy of metastatic melanoma could not be confirmed in these mice. mFcγRI^{only} mice were recently described to mediate autoimmune thrombocytopenia and anti-CD20 mAb therapy, but arthritis, systemic anaphylaxis, or airway inflammation could not be induced[61]. Taken together, multiple roles of the high affinity human FcγRI, mFcγRI, and mFcγRIV have been demonstrated using mouse models, although these studies also reveal the difficulty of dissecting the roles of individual FcγR *in vivo*.

Directly demonstrating the role of FcγRI in humans is more challenging, since there are no specific blocking antibodies available for human, nor mouse, FcγRI. The identified SNPs of human FcγRI reduce the function of FcγRI and in this way the role of FcγRI in humans might be demonstrated, similar as for the low affinity FcγR. Due to the low frequency of these SNPs, large patient cohorts are required to be able to correlate FcγRI SNPs to prevalence of, for example, autoimmune diseases or mAb therapy outcome. However, with multi-center studies and the low costs of DNA sequencing, this might be feasible in the near future if sequencing of the *FCGR1A* gene is included in the study design.

FCεRI, FCγRIIA, FCγRIIA, AND FCαRI

Besides FcγRI, the other high affinity FcR in humans is FcεRI. This receptor is expressed on mast cells and basophils, but also on Langerhans cells, eosinophils, platelets, monocytes, and DCs, and plays a major role in allergic responses. FcRγ was first discovered as the third subunit of the FcεRI receptor complex and termed FcεRIγ, although later

it became clear that this subunit is shared by other receptors (**chapter 7**). Besides the FcεRI α-chain and FcRγ, the receptor complex consists of a β-subunit that functions as a signaling amplifier by enhancing ITAM phosphorylation of FcRγ[62]. FcεRI crosslinking by foreign allergens leads to degranulation of mast cells and basophils and the release of inflammatory mediators. The nanoscale organization and mobility of FcεRI has been studied extensively and this has given unique insights into FcεRI biology. Actin structures confine FcεRI in the plasma membrane where they remain mobile[32]. Upon receptor crosslinking by multivalent antigens, FcεRI signaling is induced and this coincides with receptor immobilization. Interestingly, low concentrations of multivalent antigen were able to induce FcεRI signaling without immobilization, indicating that FcεRI immobilization is not critically required for ITAM phosphorylation[31]. Since IgE is being investigated as isotype for therapeutic antibodies, the extensive knowledge on FcεRI will aid in devising optimal strategies for IgE mAb therapy.

The serum concentration of IgE is very low compared to IgG and because of the high affinity of FcεRI most of the IgE is bound to FcεRI under physiological conditions. This indicates that antigens mainly bind to IgE-FcεRI on the surface of mast cells and basophils, instead of forming IgE-opsonized IC. For this to be efficient, the IgE repertoire cannot be to broad, because multiple IgE directed against the same antigen must be present on the same cell. Indeed, several studies indicate that the IgE repertoire is limited, although the techniques and study designs used to determine this were not optimal[63-65]. The IgG repertoire is much broader compared to IgE, although the IgG repertoire might not be as variable as initially thought and theoretically possible[65,66]. The current estimate for the IgG repertoire is <20,000 distinct clonotypes for an adult[66]. Combining the fact that IgG-opsonized pathogens can bind to FcγRI, the broad IgG repertoire, and the high dissociation constant of FcγRI together makes it likely that most of the time preformed IC bind to FcγRI. However, if multiple IgG antibodies directed against the same antigen are bound to FcγRI on the same cell, antigens might directly interact with IgG-FγRI on the surface of cells. This might occur in tissues, where local plasma cells can generate high concentrations of single antibody clones. We exploited this way of antigen-IgG-FcγRI interaction for our experiments to study the mechanisms of FcγRI inside-out signaling (**chapter 3**).

FcγRIIa contains two extracellular domains and an ITAM motif in its intracellular domain. FcγRIIa has a broad expression pattern, since expression is found on platelets, monocytes/macrophages, neutrophils, DCs, basophils, eosinophils, and mast cells. Crosslinking of FcγRIIa can result in many different effector functions, including internalization, phagocytosis, cytokine production, antigen presentation, and ADCC. Cytokine stimulation enhances IC binding of FcγRIIa without changing surface expression levels, indicating that FcγRIIa is under inside-out control[67]. In resting neutrophils, FcγRIIa is in a low avidity state but after neutrophil activation FcγRIIa is converted to a higher avidity state[68]. The exact mechanism of inside-out signaling of FcγRIIa is unknown, although a conformation-specific antibody has been generated that recognizes the active form of FcγRIIa[69-71]. This indicates that a conformational change, possibly induced by (altered) homodimerization, is part of the mechanism of activation[72]. The activation state of FcγRIIa is correlated with infections and autoimmune diseases, indicating an important

role for this receptor during these immunological processes. A SNP at position 131 changing a histidine to arginine (131H/R) of FcγRIIa has underlined the importance of this FcR in protection against opsonized bacteria but also in mAb therapy[73]. FcγRIIa-131HH has a higher binding capacity for IgG2 than FcγRIIa-131RR[74]. It would be interesting to study the nanoscale organization and mobility of this receptor, especially before and after cytokine stimulation and comparing the 131H/R variants. This can provide more insight into the mechanism of inside-out signaling and FcγRIIa biology.

FcγRIIIa also contains two extracellular domains but associates with FcRγ for ITAM signal transduction. Interestingly, FcγRIIIa can also associate with CD247 on NK cells, a signaling adaptor molecule very homologues to FcRγ that normally is incorporated in the T cell receptor complex[75]. Expression of FcγRIIIa is restricted to monocytes/macrophages and NK cells, the latter on which FcγRIIIa is the only FcR expressed. Crosslinking of FcγRIIIa on NK cells induces efficient ADCC and leads to cytokine release. For FcγRIIIa, 158VV has a higher avidity for IgG1, IgG3, and IgG4 compared to 158FF[76]. The FcγRIIIa-158F/V SNP is associated with clinical outcome of mAb treatment against cancer[77-80]. To date, FcγRIIIa has not been described to be under inside-out control. We have obtained preliminary data that IC binding of FcγRIIIa might be enhanced after cytokine stimulation, but only for FcγRIIIa-158V and not FcγRIIIa-158F (own unpublished observations). Further research is required to investigate if this is true inside-out activation and how this SNP might change the susceptibility for cytokine-induced activation. Interestingly, the CY domain of FcγRIIIa itself can regulate the extent and type of effector functions after FcγRIIIa crosslinking. PKC can phosphorylate a consensus motif (RSSTR) in the FcγRIIIa CY domain, leading to a stronger intracellular calcium flux, more tyrosine phosphorylation of Syk, and higher proinflammatory cytokine production[81]. Non-phosphorylated FcγRIIIa on the other hand, is more effective at inducing degranulation. This demonstrates another level of regulating the effector functions of FcγR.

The receptor for monomeric IgA, FcαRI, is expressed on monocytes/macrophages, neutrophils, eosinophils, Kupffer cells, alveolar macrophages, and some DC subsets. While FcαRI is part of the immunoglobulin (Ig) gene superfamily, it is only distantly related to other FcR and the FCAR1 gene is located on a distinct chromosome[82]. Interestingly, there is no homologue of FcαRI in mice. The molecular basis of the FcαRI-IgA interaction mediated by the extracellular domains is clearly distinct from other FcR and their ligands. For instance, the binding site for IgA on FcαRI is located on EC1 instead of EC2. In addition, a stoichiometry where IgA can bind two FcαRI simultaneously has been described[83]. One non-synonymous SNP of FcαRI has been reported and involves the change of a serine at position 248 to glycine (248S/G) in the CY domain and FcαRI-G248 is correlated with systemic lupus erythematosus84. Cytokine-induced inside-out activation of FcαRI has been shown quite extensively[67,85,86]. The intracellular signaling events required for FcαRI inside-out signaling are discussed in **chapter 2**. The phosphorylation status of serine 263 (S263) is crucial for the activation of FcαRI[86]. Similar as for FcγRI inside-out signaling, the actin cytoskeleton is involved in FcαRI inside-out signaling[86]. Although some mechanisms are shared between FcγRI and FcαRI inside-out signaling,

they are distinct processes involving different molecules.

There is clear evidence that several FcR (FcγRI, FcγRIIa, FcαRI) can be activated by cytokine-induced inside-out signaling, indicating that this is a key concept in the regulation of FcR. Whether all FcR are under inside-out control for the regulation of their binding affinity is still unknown. Given the sequence homology between the FcR, *e.g.* FcγRIIa, FcγRIIb, and FcγRIIc, it is tempting to speculate that other FcR are also regulated via inside-out signaling.

ANTIBODY THERAPY

mAb therapy against cancer

Many mAbs have been developed for therapeutic use and a significant portion of these are for the treatment of cancer. mAb therapy is one of the most important strategies for the treatment of patients with solid tumors or hematological malignancies. These mAbs are directed against antigens (over)expressed on tumor cells, like epidermal growth factor receptor (EGFR, colorectal cancer), human epidermal growth factor receptor 2 (HER2, breast cancer), CD20 (B-cell malignancies), CD38 (multiple myeloma), and GD2 (neuroblastoma). In addition, several mAbs have now been approved that modulate the immune system by blocking immune checkpoints. These immune checkpoint inhibitors can block the inhibitory signals of the immune system and thereby potentiate anti-cancer responses. The field of immune checkpoint inhibitors is rapidly growing and will discussed in more detail below. Besides for cancer therapy, mAbs are used and developed for the treatment of chronic inflammatory of autoimmune diseases.

As discussed in **chapter 1**, mAbs have several mechanisms of action of which FcR-mediated effector functions are generally recognized as major contributors to tumor cell killing. The main FcR-expressing effector cells able to induce ADCC are NK cells, monocytes/macrophages, and neutrophils. NK cells only express FcγRIIIa (and in some individuals FcγRIIc), monocytes express FcγRI, FcγRIIa and FcγRIIIa, whereas neutrophils express FcγRIIa and FcγRIIIb. Besides the FcγR, monocytes and neutrophils express FcαRI. These different effector cell populations have different anti-tumor effector mechanisms. NK cells induce apoptosis in tumor cells through the release of granzymes and perforin. Monocytes/macrophages can phagocytose the tumor cells, thereby eliminating them. Cytotoxicity mediated by neutrophils is less well understood, but it was reported that neutrophils can mediate frustrated phagocytosis or induce autophagy in tumor cells[87]. Efforts to reproduce the finding that neutrophils induce autophagy in tumor cells were unsuccessful (own unpublished observations). More recently, excessive trogocytosis was shown to be the mechanism by which neutrophils kill tumor cells[88].

Despite their demonstrated clinical efficacy, currently the anti-cancer mAbs are not effective enough to eliminate all tumor cells and cure cancer. In humans, each antibody isotype elicits different types of immune activation, providing a fine-tuning of the immune response. For instance, IgA antibodies are very efficient in recruiting neutrophils, a populous immune cell type in blood. Currently, all antibodies in the clinic are of the IgG

isotype. We hypothesized that the combination of antibodies of different isotypes can lead to improved anti-tumor responses (**chapter 6)**. We found that the combination of IgG and IgA antibodies increased tumor cell killing when each isotype binds to a different target antigen (EGFR and HER2) on the same tumor cell. If both antibodies bind to the same target, they compete for tumor cell binding and no increased killing was observed. Furthermore, we have shown that in mice with growing tumors the combination of IgG and IgA antibodies inhibited tumor growth better than either isotype alone. Thus, combining antibodies of different isotypes is a new and promising approach to improve anti-tumor responses. The combination of IgG and IgA isotypes recruits multiple types of immune cells (NK cells and neutrophils) for tumor killing. Both effector cells will attempt to kill the tumor cell, and we believe that this combination will give a 'double-hit', increasing the effectiveness of killing. Monocytes express high levels of FcαRI and have a favorable expression pattern of FcγRs (high expression of FcγRI and no expression of FcγRIIIb). This may explain why cytotoxicity was also enhanced by the combination of IgG and IgA against two targets when monocytes alone were used as effector cells.

Isolated monocytes do not induce tumor cell lysis after 4 hours incubation with antibodies and tumor cells (data not shown). Only after longer incubation times, for example 20 hours, do isolated monocytes induce high levels of ADCC. After 2 hours of incubation on ice, monocytes, like neutrophils, can efficiently bind to tumor cells when specific mAbs are present (own unpublished observations). This indicates that monocytes can rapidly interact with tumor cells through antibody-FcR interactions, but that phagocytosis of tumor cells requires some time (more than 4 hours). Furthermore, monocytes might still contribute to tumor cell lysis in a 4-hour assay with all blood leukocytes. Monocytes were shown to produce cytokines, including TNFα, after interaction with antibody-opsonized tumor cells in a 4 hour assay[89]. This might lead to the induction of inside-out signaling of FcR, including FcγRI and FcαRI, on neutrophils, thereby increasing the binding and most likely the killing of tumor cells by neutrophils. Indeed, TNFα can increase the ADCC mediated by neutrophils (**chapter 3**). NK cells also release cytokines, such as IFNγ, TNFα, and GM-CSF, upon FcγRIIIa ligation by IgG-opsonized target cells. Again, these proinflammatory cytokines can induce inside-out activation of FcR but activate effector cells in other ways as well, for example by promoting monocyte/macrophage differentiation. Interestingly, IFNγ and TNFα can induce upregulation of intercellular adhesion molecule 1 (ICAM-1) on NK cells, thereby increasing the conjugate formation between NK cells and tumor cells[90,91]. IFNγ also accelerates dendritic cell (DC) maturation and these mature DCs have enhanced antigen presentation and are essential in producing an immunological memory response[92,93]. This might not occur within a 4 hour assay, this is a very important process for in vivo anti-tumor responses. Other immune cells present in the total leukocyte pool, like T and B cells, might likewise be activated by these proinflammatory cytokines, although these cells most likely require specific T or B cell receptor stimulation to respond. Taken together, assays with all blood leukocytes involve complex interactions between different leukocyte populations where the release of cytokines are essential signal mediators. These assays most likely represent the *in vivo* setting better than

experiments with isolated effector cell populations.

Enhancing mAb therapy

The focus of many studies is to enhance the effectiveness of mAb therapy. To improve tumor cell killing by NK cells, the interaction between IgG1 and FcγRIIIa is enhanced via afucosylation of the Asn[297] glycan of IgG1. Indeed, this leads to enhanced NK cell-mediated ADCC[94,95]. Other modifications on IgG have been described to enhance antibody-dependent cellular phagocytosis (ADCP) or complement-dependent cytotoxicity (CDC), either by increasing the affinity for FcγRIIa or C1q, respectively[96-99]. Aglycosylated IgG engineered to bind specifically to FcγRI induced enhanced FcγRI-mediated ADCC by monocyte-derived DCs[100].

We observed improved tumor cell killing by the combination of IgG and IgA antibodies (**chapter 6**). Other strategies to engage both FcγR and FcαRI have been described before. Combining IgG1 and IgA1 antibodies against the tumor-associated antigen Ep-CAM did not increase tumor cell lysis with unstimulated neutrophils or peripheral blood mononuclear cells (PBMCs). This was attributed to the binding of IgG1 to the inhibitory FcγRIIb on neutrophils, which would inhibit FcαRI-mediated killing by neutrophils[101]. With neutrophils from G-CSF stimulated donors the combination of IgG1 and IgA1 antibodies was superior to either antibody alone, indicating an important role for FcγRI in IgG1-mediated anti-tumor effects by neutrophils. These data are in line with our observation that the combination of IgG1 and IgA antibodies against the same target antigen does not lead to enhanced ADCC, most likely by competition for the target by both isotypes. Importantly, it was shown that FcRγ was not the limiting factor for ADCC with neutrophils when FcγR and FcαRI are targeted simultaneously[102]. In this study, bsAbs were used that target either FcγRI and HLA class II (FcγRI×HLAII), or FcαRI and CD20 (FcαRI×CD20). bsAbs represent one of the strategies to enhance mAb therapy, since they have two different variable regions that allow them to recognize two distinct antigens, as well as engaging with FcγR via the Fc domain. The combination of both bsAbs significantly enhanced tumor cell lysis by neutrophils compared to either bsAb alone[102].

More recently, two other antibody formats to combine FcγR and FcαRI effector functions were described. First, an IgG-IgA cross-isotype (IgGA) was engineered that combines properties of the IgG and IgA Fc regions on a single Fc domain[103]. Second, a tandem IgG1/IgA2 antibody was generated, where the Fc domain of IgA2 was fused to the C-terminus of the anti-HER2 IgG1 antibody trastuzumab[104]. Both molecules induce IgG- and IgA-mediated effector functions, since they engage FcγR and FcαRI but also retain C1q binding. However, the IgGA molecule does not bind FcγRIIIa or the neonatal FcR (FcRn). The tandem IgG1/IgA2 does bind these receptors and could induce better neutrophil-mediated tumor cell lysis than either IgG1 or IgA2 parental antibody[104]. However, the authors do not compare the efficacy of the tandem IgG1/IgA2 with the combination of both parental antibodies in their study. Although it is speculated that one tandem antibody can engage both FcγR and FcαRI at the same time, sterically this seems highly unlikely. Therefore, it would be interesting to see crystal structures of this tandem antibody in complex with both FcγR

and FcαRI and to compare its anti-tumor efficacy to the combination of IgG1 and IgA2.

Combination therapy with cytokines

Besides strategies to engage both FcγR and FcαRI for enhancement of mAb therapy, other combination therapies are often used. Based on the potent proinflammatory effects and induction of FcR inside-out signaling, the combination of mAb therapy with cytokines has great potential. The combination of G-CSF with anti-CD20 mAb rituximab for non-Hodgkin lymphoma (NHL) patientsachieved good responses and combining GM-CSF with rituximab and chemotherapy gave high response rates for chronic lymphatic lymphoma and NHL patients[105,106.] G-CSF combined with the anti-CD52 mAb alemtuzumab gave good, albeit short, responses in acute lymphoblastic leukemia patients[107]. The activity of the anti-GD2 mAb dinutuximab against neuroblastoma was enhanced by GM-CSF and IL-2 in preclinical experiments[108,109]. In the clinic, dinutuximab is combined with administration of GM-CSF, IL-2, and chemotherapy, which significantly improves the outcome of patients compared to chemotherapy alone[110]. Cytotoxicity of the anti-EGFR mAb cetuximab was enhanced by combining it with IL-2 or IL-15[111]. In other studies, the use of mAbs fused to cytokines (termed immunocytokines) are evaluated[112]. While this has the benefit of only activating immune cells at the tumor site, these immunocytokines most likely have reduced receptor-ligand interactions because of sterical hindrance.

Only a few clinical trials are available investigating cancer treatment with a combination of mAb and cytokines without addition of chemotherapy. Often control groups receiving monotherapy (the mAb or cytokine alone) are not included in these studies, for good ethical reasons. However, this makes it difficult to determine the impact of cytokine treatment on mAb therapy. Evaluating the influence of FcR inside-out signaling on mAb therapy is even more difficult, since the activation status of FcR is hard to determine and often FcR expression is not measured in clinical trials. Furthermore, inside-out signaling is a rapid process occurring within minutes after cytokine administration, while clinical trials focus on the long-term effects of mAb therapy.

When devising strategies to enhance mAb therapy and for the interpretation of clinical trials with mAbs for cancer treatment, several important factors have to be taken into account: (1) cancer is a heterogeneous disease and even within a patient metastatic tumors can have different antigen expression patterns than the primary tumor, (2) patients receiving novel mAb therapies during clinical trials often have failed multiple other treatments and usually have poor a prognosis, and (3) chemotherapy is often given simultaneously with mAb therapy, while chemotherapy also has a great impact on the immune system. These factors make the translation of preclinical data on novel mAb therapies to clinical response more difficult and may partly explain why many mAb in clinical trials do not perform as outstanding as expected. Chemotherapy reduces the number of neutrophils in the patient. However, these are important effector cells for the FcR-mediated functions of mAbs. Simultaneous administration of GM-CSF or G-CSF can to some extent counteract this reduction. Another option would be to change the timing: first administer mAbs and several days later start with additional chemotherapy, or treat patients first with

chemotherapy and after a recovery period for the immune system start mAb treatment. Indeed, administering chemotherapy six days after mAb therapy significantly inhibited tumor growth more compared to simultaneous administration in a neuroblastoma xenograft model[113]. On the other hand, administration of chemotherapy after therapy with anti-HER2 mAb reduced the induced anti-tumor memory response while administration of chemotherapy before mAb therapy preserved this[114]. Thus, simple alterations in the timing of administering drugs could have major impact on the clinical outcome. Careful monitoring of the immune system, including FcR expression and activation status, before and during mAb therapy might provide valuable information in clinical trials with mAbs.

Targeting immune checkpoints

With the discovery of mAbs that block immune checkpoints, substantial research has focused on these immune checkpoint inhibitors and the potential combinations that can be made to enhance anti-tumor activity. The first approved immune checkpoint inhibitor is ipilimumab, an anti-cytotoxic T lymphocyte antigen 4 (CTLA4) mAb. CTLA4 is a powerful inhibitor present on the surface of T cells and interaction of CTLA4 with CD80 or CD86 on antigen presenting cells (APCs) inhibits the proliferation of activated T cells. Interestingly, anti-CTLA4 mAb is now in clinical trials in combination with nivolumab, another immune checkpoint inhibitor against PD-1. These mAbs can induce strong anti-tumor responses[115-117]. Many immune checkpoint inhibitors, including those currently approved for cancer therapy, are directed against T cell checkpoints and function by 'releasing the brakes' on these cells. However, this is not a very targeted approach specific for tumor cell killing, but rather a general T cell activation strategy. Therefore, it makes sense to combine these immune checkpoint mAbs with a more specific therapy, like mAbs against tumor antigens. Indeed, the combination of PD-1 blockade with rituximab is studied to enhance follicular lymphoma treatment and this seems to give high overall and complete response rates[118,119]. 4-1BB (CD137) is a broadly expressed activating checkpoint and agonistic 4-1BB mAbs enhance anti-CD20 mAb therapy against B cell lymphoma[120]. Furthermore, blocking the inhibitory killer-cell immunoglobulin-like receptor (KIR) on NK cells enhances anti-CD38-mediated lysis of multiple myeloma cells[121].

Of particular interest for combination therapy with tumor-antigen specific mAbs is inhibiting myeloid immune checkpoints, since monocytes/macrophages and neutrophils are essential in inducing ADCC. As mentioned above, the SIRPα-CD47 interaction serves as a myeloid checkpoint by inducing a 'don't eat me' signal. Blocking this interaction with an anti-CD47 mAb or by CD47 knockdown enhances ADCC of anti-HER2 mAb opsonized breast cancer cells[122]. Furthermore, anti-CD47 mAb synergized with rituximab in mouse models of NHL[123,124]. This anti-CD47 mAb is now further tested in clinical trials in patients with acute myeloid leukemia and solid tumors. The inhibitory CD200 receptor (CD200R), highly expressed on myeloid cells but also NK cells, T cells, and B cells, is another promising target to enhance mAb therapy. Indeed, expression of CD200, the ligand of CD200R, on tumor cells directly inhibited NK cell-mediated cytotoxicity and cytokine production[125]. Blocking these myeloid immune checkpoints might be even more

interesting for IgA anti-tumor mAbs, since IgA mAbs induce higher ADCC with neutrophils than IgG mAbs[126,127]. Thus, inhibiting immune checkpoints, especially on myeloid cells, is an effective strategy to enhance mAb therapy. Future preclinical and clinical studies will give insight into the most potent combinations for anti-cancer therapy.

CONCLUSIONS AND FUTURE DIRECTIONS

FcR are a diverse family of receptors that are required for the cellular effector functions of antibodies. FcγRI is unique in its ability to bind monomeric IgG and the regulation of FcγRI activation occurs on multiple levels. Inside-out signaling of FcγRI is an important mechanism by which IC can bind with high avidity to FcγRI. Increased clustering of FcγRI in the membrane, an intact actin cytoskeleton, PP1 activity, and the CY domain of FcγRI are essential for FcγRI inside-out signaling. However, some details about the underlying mechanism are still unclear, including the target of PP1 and the exact role of the actin cytoskeleton. Further insights in this mechanism will aid us in manipulating immune responses using cytokines.

Although this thesis focuses mainly on FcγRI, the other FcγR are important in IgG-mediated responses as well. Each FcγR has some unique and some shared functions. The expression pattern, activation status, organization within the plasma membrane, and SNPs of FcγR on immune cells together dictate the exact outcome of the cellular response towards opsonized particles or cells. However, it is important to study the single FcR to learn the specific characteristics and regulatory mechanisms of each receptor. This is also key in better understanding the mechanisms of action of mAbs against cancer and other diseases. Dissecting the contributions of the different FcR during mAb therapy can be used to optimize the development of novel mAbs.

In my opinion, the future of mAb therapy lies in combination therapies. Promising strategies include the combination of different isotypes (like IgG and IgA), the combination of mAbs with cytokines, or the combination of mAbs against tumor antigens with immune checkpoint inhibitors. Preferably, the tumor cell will get multiple hits to prevent tumor cell escape and specific parts of the immune system are activated for increased anti-tumor responses. This might also lead to adaptive immune responses, possibly leading to anti-tumor memory cells. The optimal treatment regimen might, however, differ between patients and mAbs. Ideally, the combination of therapies that is most effective for each patient is determined beforehand. This should be based on the unique mutations of the tumor, patient characteristics, FcR expression, and genetic polymorphisms, and possibly high throughput screenings before the start of therapy.

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8

APPENDICES

Summary

Nederlandse samenvatting

Acknowledgments

List of publications

Curriculum vitae

SUMMARY

Antibodies consist of two identical Fab (antigen binding) domains with hypervariable regions at the N-terminus which determine the specificity to an antigen and a constant Fc (crystallizable) domain which mediates the effector functions of antibodies. Fc receptors (FcR) are transmembrane proteins that bind to the Fc tail of antibodies and are widely expressed on leukocytes. Antibody-opsonized pathogens can cross-link FcR, leading to phagocytosis and pathogen killing by phagocytic leukocytes. In the case of antibody-opsonized cells (*e.g.* virus-infected cells, malignant cells), processes termed antibody-dependent cellular cytotoxicity/phagocytosis (ADCC/ADCP) are triggered. Other cellular processes elicited by the interaction of FcR with antibodies include cytokine release, reactive oxygen species (ROS) production, and antigen presentation. These processes emphasize the central role of FcR in bridging the humoral and cellular responses of the immune system. FcR-mediated effector functions are exploited by therapeutic monoclonal antibodies (mAbs), including those approved for the treatment of cancer. The research described in this thesis focuses on the regulation and activation of Fc receptors and how this can be used in antibody therapy for cancer.

The high affinity receptor for IgG, FcγRI, is saturated with monomeric IgG *in vivo*, but also after isolation or extravasation of immune cells. Because of this, FcγRI has been far less studied than the low affinity FcγR. However, an important role for FcγRI during inflammation, autoimmune responses, and monoclonal antibody immunotherapy in tumor models has been established. It was demonstrated that FcγRI, saturated with prebound IgG, was capable of effective IC binding after cytokine stimulation. This phenomenon was termed inside-out signaling because ligand binding of the receptor is rapidly enhanced after intracellular signaling without altering its expression levels. In **chapter 3** we found that cytokine stimulation enhanced the clustering of FcγRI in the plasma membrane both before and after immune complex (IC) addition using super-resolution imaging. This increased clustering does not change receptor mobility, but is dependent on an intact actin cytoskeleton and the phosphatase PP1. Furthermore, cytokine-stimulated neutrophils demonstrated a stronger ADCC of CD20-expressing tumor cells. These results indicate a role for cytokine-induced inside-out signaling in altering the nanoscale organization of FcγRI which enhances FcγRI effector functions. Whether FcγRI also undergoes a conformation change after inside-out signaling to enhance IC binding is still unclear. To study this hypothesis, we set out to generate conformation-specific antibodies in **chapter 4**. This research is still ongoing and a phage display library will be used to screen FcγRI-specific antibodies in an 'Fc-free' system. However, after immunization we could detect more antibodies in the serum binding to Ba/F3-FcγRI cells stimulated with IL-3 compared to unstimulated cells, possibly supporting the hypothesis of a conformation change after IL-3 stimulation. FcγRI functions are also regulated by three nonsynonymous single nucleotide polymorphisms (SNPs), since all three SNPs lead to reduced downstream signaling of FcγRI (**chapter 5**). Although the frequency of these SNPs is low within the population, these SNPs have the potential to alter efficacy of therapeutic mAbs.

Antibody therapy with IgG mAbs is part of the standard treatment of several forms of cancer. IgG mAbs engage FcγRs on effector cells to initiate ADCC. However, IgG mAb therapy is rarely curative and this has in part been attributed to exhaustion of cellular effector mechanisms. Co-engagement of activating FcγRs with the inhibitory FcγRIIb on monocytes or the non-signaling FcγRIIIb on neutrophils also limits the cytotoxic activity of IgG mAbs. Engaging FcαRI with IgA anti-tumor mAbs results in more efficient activation of neutrophils compared to IgG and induces different effector functions. In **chapter 6**, we show that in the presence of a heterogeneous population of effector cells (leukocytes), the combination of IgG and IgA anti-tumor mAbs against two different tumor targets (EGFR and HER2) led to enhanced cytotoxicity compared to each isotype alone. Co-injection of cetuximab and IgA2-HER2 resulted in increased anti-tumor effects compared to either mAb alone in a xenograft model with A431-luc2-HER2 cells. Thus, the combination of IgG and IgA isotypes optimally mobilizes cellular effectors for cytotoxicity, representing a promising novel strategy to improve mAb therapy.

In the final chapter of this thesis, **chapter 8**, the above mentioned observations are further discussed and some future perspectives on FcγRI research and using cytokines or other strategies to enhance mAb therapy of cancer are provided. In conclusion, the findings in this thesis give insight into the regulatory mechanisms of FcγRI and provide a basis for future research to improve mAb anti-tumor therapy.

&

NEDERLANDSE SAMENVATTING

Ons lichaam wordt constant blootgesteld aan ziekteverwekkers zoals virussen en bacteriën. Het immuunsysteem kan meestal goed omgaan met deze ziekteverwekkers en ze snel elimineren. Het immuunsysteem bestaat uit het aangeboren (innate) en het verworven (adaptive) immuunsysteem. Het aangeboren immuunsysteem kan snel reageren op ziekteverwekkers door immuuncellen zoals natuurlijke killer cellen, macrofagen en neutrofielen te rekruteren om lichaamsvreemde organismen te verwijderen en door het complementsysteem te activeren, dat kan leiden tot zowel directe eliminatie van pathogenen als activatie van de aanwezige immuuncellen. Het verworven immuunsysteem, ook wel het specifieke immuunsysteem genoemd, heeft als karakteristieke eigenschap dat na een eerste besmetting met een ziekteverwekker een specifieke respons wordt geïnduceerd waardoor mensen de rest van hun leven meestal immuun zijn voor deze specifieke ziekteverwekker (immunologisch geheugen). Dit komt doordat bepaalde immuuncellen, zoals macrofagen, ziekteverwekkers kunnen opnemen ('fagocyteren') en daarna in de cel kunnen afbreken tot kleine stukjes, peptiden genaamd. Deze peptiden kunnen dan op het oppervlakte van de cel worden gepresenteerd aan cellen van het verworven immuunsysteem, B en T cellen. Deze cellen kunnen deze 'vreemde' peptiden specifiek herkennen en als dit gebeurt wordt de immuuncel geactiveerd. B cel activatie leidt tot de productie van antistoffen die met hoge specificiteit een antigeen van de ziekteverwekker kunnen herkennen. Deze antistoffen komen in de bloedbaan terecht waar ze, bij een tweede infectie, aan de ziekteverwekker kunnen binden en daarmee de cellen van het aangeboren immuunsysteem kunnen activeren.

Antistoffen bestaan uit twee Fab-fragmenten, die voor de specificiteit van de antistof zorgen, en uit een Fc-fragment, wat voor de functionaliteit van de antistof zorgt. Het Fc-fragment wordt ook wel het constante deel genoemd en kan uit vijf types bestaan, die het isotype van de antistof bepalen: IgA, IgD, IgE, IgG en IgM. Voor elk van deze isotypes zijn er receptoren (eiwitten op het oppervlakte van cellen) die specifiek kunnen binden aan het Fc-fragment, waarbij bijvoorbeeld Fcγ receptoren aan IgG binden en Fcα receptoren aan IgA. Cellen van het aangeboren afweersysteem hebben veel van deze Fc receptoren op hun oppervlakte, en kunnen daardoor snel en specifiek reageren op antigenen die gebonden zijn door antistoffen, ook wel immuuncomplexen genoemd. De interactie van Fc receptoren met immuuncomplexen leidt tot de activatie van de immuuncel, waardoor verschillende processen geïnitieerd kunnen worden: fagocytose van het immuuncomplex, het vrijkomen van inflammatoire signaalmoleculen zoals cytokines, of antistof-gemedieerde celdood via immuuncellen ('antibody-dependent cell-mediated cytotoxicity' of ADCC, bijvoorbeeld van virus-geïnfecteerde cellen). Daarnaast kan via het Fc-fragment van antistoffen een cascade van eiwitten in de bloedbaan geactiveerd worden, het complementsysteem genaamd, dat ook kan leiden tot eliminatie van een immuuncomplex.

Kanker is een groep van ziektes waarbij cellen abnormaal gaan delen doordat er meerdere genetische mutaties zijn ontstaan in het DNA van de kankercel. Afhankelijk van het type kanker worden patiënten behandeld met bestraling, chirurgie en chemother-

apie. Deze behandelingen zijn gericht op het aanpakken van de tumorcellen, maar vaak worden ernstige bijwerkingen veroorzaakt doordat ook het gezonde weefsel wordt aangetast. Daarom is er een grote vraag naar meer specifieke behandelmethodes, zodat alleen de tumorcellen worden aangepakt. Dit wordt gedaan met behulp van antistof immuuntherapie, waarbij antistoffen specifiek voor de tumorcel aan de patiënt worden gegeven waarna het eigen immuunsysteem de tumorcellen gaat opruimen. Antistoffen tegen tumorcellen kunnen meerdere effecten hebben: (1) het direct binden van de antistof aan tumor-geassocieerde antigenen kan leiden tot bijvoorbeeld de blokkade van signaaltransductiewegen die essentieel zijn voor het delen van de tumorcel, (2) via het Fc-fragment van de antistof kunnen cellen van het aangeboren immuunsysteem geactiveerd worden om de tumorcellen te doden, en (3) de activatie van het complementsysteem dat ook kan leiden tot tumorceldood. Alle antistoffen die momenteel in de kliniek worden gebruikt bij de behandeling van kanker zijn van het IgG isotype, en binden dus aan Fcγ receptoren. Behandeling van tumoren met antistoffen geeft goede resultaten, maar patiënten kunnen vaak niet genezen door behandeling met alleen antistoftherapie. Daarom wordt er veel onderzoek gedaan naar hoe de antistoftherapie verbeterd kan worden. Daarnaast heeft de komst van antistoftherapie het onderzoek naar de receptoren van antistoffen, Fc receptoren, gestimuleerd. Beter inzicht in hoe deze receptoren werken en geactiveerd kunnen worden, zou potentieel gebruikt kunnen worden om de effectiviteit van antistoftherapie te verhogen.

Veel van de Fc receptoren kunnen geactiveerd worden door stimulatie van de immuuncel met cytokines, waardoor de Fc receptoren op het oppervlakte van de immuuncel beter kunnen binden aan immuuncomplexen. Dit proces wordt 'inside-out signaling' genoemd. Bij dit proces veranderd het aantal receptoren op het oppervlakte niet na stimulatie met cytokines, maar wel de affiniteit voor immuuncomplexen. **Hoofdstuk 2** beschrijft de inside-out signaling van Fc receptoren en hoe dit gebruikt kan worden om antistoftherapie te optimaliseren. Er is een Fc receptor voor IgG met een hoge affiniteit voor monomeer IgG, namelijk FcγRI. Omdat er in het bloed hoge concentraties van antistoffen, zeker IgG, aanwezig zijn, is FcγRI altijd bezet met monomeer IgG. De andere Fc receptoren voor IgG hebben allemaal een lage affiniteit voor monomeer IgG, waardoor ze niet binden aan 'losse' antistoffen in de bloedbaan maar alleen aan immuuncomplexen, waar meerdere antistoffen vlak bij elkaar zitten. Daarom werd lang gedacht dat FcγRI geen belangrijke rol zou spelen in het binden en opruimen van immuuncomplexen, omdat deze receptor toch al bezet was. Maar onderzoek heeft aangetoond dat FcγRI wel degelijk betrokken is bij afweerreacties en dat zeker na stimulatie met cytokines FcγRI veel beter kan binden aan immuuncomplexen. In **hoofdstuk 3** beschrijven wij de mechanismen die ten grondslag liggen aan FcγRI inside-out signaling. Met hoge-resolutie microscopie hebben we gekeken naar de verdeling van FcγRI in de membraan en gevonden dat FcγRI altijd al in kleine clusters bij elkaar zit, maar dat deze clusters groter worden als de cel wordt gestimuleerd met cytokines. Hierdoor kunnen immuuncomplexen beter binden aan de FcγRI clusters. Het actine cytoskelet van de cel, eiwitten die lange filamenten vormen en daardoor zorgen dat de cel een bepaalde vorm heeft en kan bewegen, is essentieel voor het vergroten van

deze FcγRI clusters. We hebben ook een fosfatase eiwit, PP1, geïdentificeerd als essentieel voor het induceren van deze clusters en van de verhoogde binding voor immuuncomplexen. Deze kennis hebben we toegepast in een ADCC experiment, waarbij we meten hoeveel tumorceldood er geïnduceerd wordt door immuuncellen in combinatie met een antistof specifiek voor de tumorcel. Als we de immuuncellen eerst stimuleren met cytokines, kunnen we inderdaad een verhoging van de ADCC meten. Een ander mechanisme waardoor FcγRI beter zou kunnen binden aan immuuncomplexen is een conformatieverandering van de receptor. Dit zou met behulp van antistoffen specifiek voor een actieve of inactieve conformatie van FcγRI bewezen kunnen worden. **Hoofdstuk 4** beschrijft hoe zulke antistoffen gemaakt kunnen worden.

In eerdere studies is onderzocht of er varianten van Fcγ receptoren in de populatie bestaan die beter of slechter aan IgG kunnen binden. Dit gaat om single nucleotide polymorphisms (SNPs), wat betekent dat er in het genoom bij verschillende mensen één andere nucleotide aanwezig is in het gen voor een Fcγ receptor. Als deze nucleotideverandering leidt tot een ander aminozuur in het eiwit, kan dit invloed hebben op de functie van dit eiwit. Voor FcγRIIIa, een van de lage affiniteit Fcγ receptoren, is inderdaad een SNP beschreven die leidt tot een ander aminozuur, dat ervoor zorgt dat de binding van IgG hoger (bij de FcγRIIIa-158V variant) of lager is (bij de FcγRIIIa-158F variant). Als er dan in kankerpatiënten die behandeld worden met antistoffen gekeken wordt naar de effectiviteit van de behandeling, dan blijkt dat patiënten die de SNP met de hoge IgG binding hebben beter reageren op de therapie dan de patiënten die de SNP met de lage IgG binding hebben. Voor FcγRI was er nog niet gekeken naar zulke SNPs en of deze invloed hebben op de functie van FcγRI. In **hoofdstuk 5** beschrijven wij drie SNPs op verschillende locaties in FcγRI die allemaal een negatieve invloed hebben op de functie van FcγRI. Deze SNPs komen niet vaak voor in de populatie (1 op de 80 mensen of minder), maar zouden toch potentieel invloed kunnen hebben op de effectiviteit van antistoftherapie.

Zoals eerder genoemd kan therapie met IgG antistoffen zelden voor genezing van de patiënt zorgen en dit wordt vaak toegeschreven aan het uitputten van de immuuncellen die nodig zijn voor de therapie. Daarnaast hebben macrofagen ook inhibitoire receptoren voor IgG antistoffen op het oppervlakte (FcγRIIb) en neutrofielen een niet-signalerende receptor (FcγRIIIb). Dit is niet het geval voor IgA antistoffen, omdat macrofagen en neutrofielen alleen de activerende FcαRI op het oppervlakte hebben. IgA is eerder getest als alternatief voor IgG voor kankertherapie. De eerste resultaten in tumormodellen bij muizen zijn veelbelovend, zeker omdat IgA antistoffen erg effectief neutrofielen kunnen aanzetten tot het doden van tumorcellen. In **hoofdstuk 6** laten we zien dat de combinatie van IgG en IgA antistoffen tot hogere tumorceldood kan leiden als er een mix van immuuncellen wordt gebruikt. Belangrijk hierbij is dat een hogere concentratie van een van beide antistoffen alleen geen extra effect geeft. Ook in een tumormodel in muizen bleek de combinatie van antistoffen effectiever dan therapie met alleen IgG of IgA. Deze resultaten laten de potentie van de combinatie van IgG en IgA antistoffen tegen kanker zien.

Veel Fc receptoren maken gebruik van een gedeelde Fc receptor γ-keten waarmee ze een interactie aangaan in de membraan en die essentieel is voor de functies van Fc recep-

toren. Maar ook cytokine receptoren hebben subunits die γ-keten worden genoemd, en in de literatuur worden de namen van deze ketens vaak door elkaar gehaald. In **hoofdstuk 7** geven wij een overzicht van de overeenkomsten en verschillen tussen de cytokine receptor γ-keten en de Fc receptor γ-keten.

Samenvattend, dit proefschrift geeft inzicht in hoe Fc receptoren, voornamelijk FcγRI, gereguleerd worden en hoe dit gebruikt kan worden om antistoftherapie tegen kanker te verbeteren, zoals ook beschreven in de algemene discussie van **hoofdstuk 8**. Cytokines kunnen FcγRI activeren en dit leidt tot betere binding van immuuncomplexen en uiteindelijk tot meer tumorceldood via antistoffen. Meer inzicht in de precieze mechanismen van activatie en regulatie van FcγRI kan ons helpen om FcγRI specifiek te activeren tijdens antistoftherapie. Daarnaast is de combinatie van IgG en IgA antistoffen een veelbelovende strategie om betere resultaten te krijgen met antistoftherapie tegen kanker.

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CURRICULUM VITAE

Arianne Brandsma was born on the 22nd of September 1990 in Vlaardingen, the Netherlands. In 2008 she completed her secondary education at the Farel College in Amersfoort. In the same year she started with the bachelor Biomedical Sciences at Utrecht University. After graduation, she enrolled the master program Infection and Immunity at Utrecht University. As part of this program she did her nine month internship at the department of Immunology under supervision of Dr. Jeanette Leusen and Dr. Peter Boross. During this internship she focused on the mechanisms of neutrophil-mediated tumor cell killing with IgA antibodies and found that the combination of IgG and IgA antibodies can increase tumor cell killing with whole blood leukocytes. Hereafter, she did her six month internship at the Max Planck Institute for Infection Biology, Berlin, under supervision of Prof.dr. Kai Matuschewski. During this internship she studied the critical factors of the malaria parasite Plasmodium required for liver stage development in the mammalian host. After obtaining her master's degree in August 2013, which was conferred *cum laude*, she started in November of that year as a PhD candidate under supervision of Dr. Jeanette Leusen (copromotor) and Prof.dr. Jürgen Kuball (promotor) at the Laboratory for Translational Immunology, UMC Utrecht. The results of her research are described in this thesis, entitled 'Fc receptors: regulatory mechanisms and function in antibody therapy'.

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