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Review

Targeting the high affinity receptor, FcγRI, in autoimmune disease, neuropathy, and cancer

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Abbreviations: ADCC/ADCP: Antibody-dependent cellular cytotoxicity/phagocytosis; AIA: Antigen-induced arthritis; ATG: Anti-thymocyte globulin; bsAbs: Bispecific antibodies; CDC: Complement-dependent cytotoxicity; CIA: Collagen-induced arthritis; CY: Cytoplasmic; DRG: Dorsal root ganglion; EC: Extracellular domain; Fab: Antigen-binding fragment; FcγR: Fc gamma receptor; G-CSF: Granulocyte colony-stimulating factor; IC: Immune complex; INF-γ: Interferon gamma; ITAM/ITIM: Immunoreceptor tyrosine-based activation/inhibition motif; ITP: Immune Thrombocytopenic Purpura; IVIg: Intravenous immunoglobulin; LN: Lupus nephritis; mAb: Monoclonal antibody; MCP-1: Monocyte chemoattractant protein 1; NK: Natural killer cell; PMN: Polymorphonuclear; PP1: Protein phosphatase 1; RA: Rheumatoid Arthritis; SLE: Systemic lupus erythematosus; TNF-α: Tumor necrosis factor alpha.

Introduction

Fc gamma receptors (FcγRs) are transmembrane proteins that bind to the Fc tail of IgG antibodies. They are required for cellular effector functions of antibodies, including direct neutralization of pathogens, recruitment of the complement system to directly lyse pathogens or infected cells, and cellular effector functions including phagocytosis and antibody-dependent cellular cytotoxicity/phagocytosis (ADCC/ADCP) [1, 2]. As many FcRs are mostly expressed on innate immune cells [3], they serve as a vital connection between the cellular and humoral parts of the immune system, connecting antigen-specific interactions of antibodies to non-specific effector mechanisms of FcR-bearing cells.

FcγR activation is tightly regulated to prevent immune responses by non-antigen-bound antibodies, or in the absence of other 'danger signals' (e.g. cytokines). For example, most FcγRs recognize IgG with low affinity, but can bind immune complexes (ICs), like opsonized pathogens, with high avidity [4]. FcγRI is the only FcγR with a high affinity for IgG. Because of its high affinity, FcγRI is constitutively saturated with monomeric IgG, even after isolation or extravasation of immune cells, but it does not lead to intracellular signaling and subsequent effector functions [1]. This led to the dogma that this receptor played no role in immune responses, resulting in FcγRI to be much less studied than the low affinity FcγRs. However, several studies demonstrated that FcγRI does play a significant part in inflammation, autoimmunity, and neuropathy, as well in antibody-therapy in tumor models [5–9].

FcγRI saturated with pre-bound IgG is able to effectively bind ICs after cytokine stimulation [10]. This process is called 'inside-out signaling', as the ligand binding capacity of the receptor is rapidly enhanced after intracellular signaling without affecting the receptor expression. This process is also described for integrins [11], as well as for FcαRI and FcγRIIa [12, 13]. Stimulation with cytokines strongly enhances IC binding, resulting in stronger cellular effector functions such as ADCC [10, 14]. Moreover, therapeutic antibodies that bind to FcγRI can also benefit from cytokine stimulation, leading to improved tumor killing [12, 15, 16].

In this review, we will discuss FcγRI interactions with IgG and ICs, as well as how to enhance FcγRI cellular effector functions via inside-out signaling. Additionally, this review will focus on how therapeutic antibodies targeting FcγRI, possibly in combination with inside-out signaling, can be turned into novel therapeutic strategies to treat human autoimmune and malignant diseases.

The high affinity IgG receptor FcγRI

The affinities and specificities for the different IgG subclasses vary considerably within the FcγR family (e.g. FcγRI, FcγRIIa, FcγRIIb, and FcγRIII). Only FcγRI binds with high affinity to human IgG, and binds exclusively to IgG1, IgG3, and IgG4, not IgG2 [4, 17]. This high affinity binding is cross-species, as also rabbit IgG and mouse IgG2a and IgG2c can bind to human FcγRI with high affinity [18–20]. Likewise, mouse FcγRI is also a high affinity receptor for mouse IgG2a, which is a functional homologue of human IgG1 [21], as well as for human and rabbit IgG [19, 22].

FcγRI consists of three extracellular domains, a transmembrane part, and an intracellular tail (FcγRI-CY) (Fig. 1A). Extracellular domains one (EC1) and two (EC2) are homologous to those found in other FcγRs, but EC3, which bridges EC2 with the transmembrane part of the receptor, is unique to FcγRI [23]. The transmembrane part is responsible for FcR γ-chain association, and the α-chain of the receptor has an intracellular tail [24]. The interaction between IgG and FcγRI lies within two binding sites of the EC2 domain (Fig. 2A). The binding of the Fc-tail to these two sites occurs in an asymmetric manner: to engage the receptor, the Fc-tail

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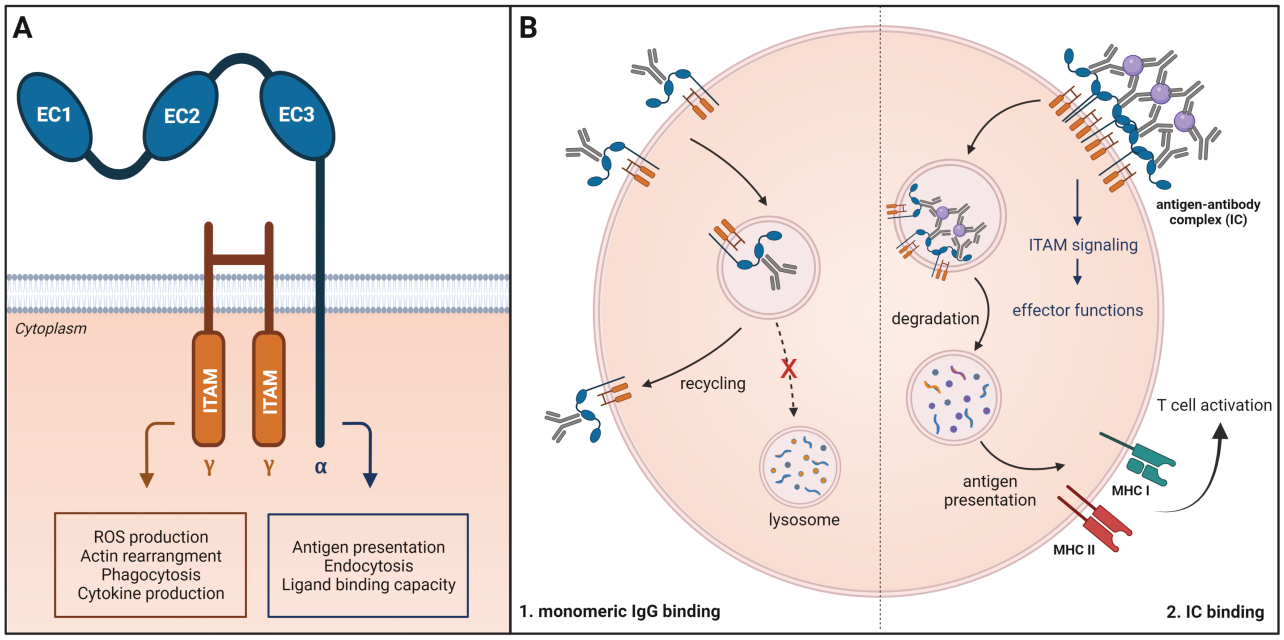

Fig. 1. Schematic representation of FcγRI and FcγRI functions. (A) Schematic representation of FcγRI with three extracellular domains; EC1, EC2, and EC3, the transmembrane part and intracellular tail (FcγRI-CY). The γ-chain and α-chain are responsible for different actions of the receptor. (B) FcγRI functions are regulated on multiple levels [1]. Interaction of FcγRI with monomeric IgG leads to rapid internalization and recycling of the receptor-IgG complex to the plasma membrane [2]. Crosslinking of FcγRI by ICs induces immunoreceptor tyrosine-based activation motif (ITAM) signaling via FcRγ and internalization and degradation of the antigen-receptor complex in the lysosome. The degraded peptides can be presented on MHC class I (MHC I) or MHC class II (MHC II), which leads to T cell activation.

must use a different set of residues from each chain of the homodimer [17]. Binding to site 1 is governed by the insertion of Leucine235 (Fig. 2B), while binding to site 2 involves a relatively flat interaction surface and does not involve a local conformational change (Fig. 2C). Leucine235, as well as the critical residues 233 and 234, on IgG-Fc has been identified to be crucial for binding to FcγRI [17, 25]. These critical residues are changed in IgG2, explaining why this antibody cannot bind to FcγRI [17].

It was proposed that the high affinity of FcγRI was due to the extra EC3 domain [23], which was supported by studies that switched the extracellular domains of FcγRI with extracellular domains of low affinity FcγRs [23, 26]. However, the crystal structure of FcγRI indicated that EC3 is located distally from the IgG binding site in EC2 [27]. As a result, EC3 is unlikely to come into direct contact with IgG, although it may promote high affinity binding by stabilizing the IgG binding conformation or extending the receptor into the extracellular space [17, 27]. Also, studies on the affinity of recombinant FcγRI lacking EC3 show that the effect of EC3 on high affinity binding is relatively minor [23].

Several studies now show that the EC2 domain converts the high affinity binding of FcγRI [17, 27]. Because of a deletion of one amino acid, the FG-loop, which is present in one of the two binding sites of EC2, is shorter in FcγRI than in the other FcγRs. This deletion may reduce steric hindrance for IgG. This deletion also revealed a unique hydrophobic pocket in EC2 of FcγRI, which perfectly suited the residue Leu235 of the Fc-tail, explaining the high affinity [17] (Fig. 2B). Additionally, the FG-loop within EC2 has been demonstrated to interact with glycans on the Fc-tail of IgG, which is unique for FcγRI [28].

Binding of monomeric IgG to FcγRI does not lead to intracellular signaling and subsequent FcγRI activation, but it does facilitate receptor-mediated endocytosis and recycling [23, 29, 30] (Fig. 1B). Monomeric IgG binding to FcγRI likely creates a threshold for IC, allowing only large ICs or many smaller complexes to displace monomeric IgG. Studies with FcγRI knock-out mice, as well as biochemical studies investigating IC binding, show that FcγRI does significantly bind and respond to ICs of various sizes [5, 20, 31]. FcγRI effector responses include bacterial clearance, inflammation, anaphylaxis, endocytosis, phagocytosis, antigen presentation, release of B-cell stimulating factors, and anti-tumor responses [5, 6, 20, 32].

FcγRI expression and signaling

FcγRI is constitutively expressed on most myeloid cells, including macrophages, monocytes, and dendritic cells, and can be induced by cytokines on neutrophils, mast cells, and eosinophils [3]. FcγRI is also inducible on endothelial cells [33] and neurons [34]. Besides FcγRI, effector cells express other FcγRs that can bind to ICs as well.

FcγRI expression can be upregulated by cytokines. For example, FcγRI expression on dendritic cells and monocytes can be increased by pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) [10, 35]. Anti-inflammatory cytokines, like transforming growth factor beta (TGF-β), IL-4, and IL-10 can decrease FcγRI expression while increasing expression of the inhibitory FcγRIIb. By altering expression levels of FcγRI, the immune response can be regulated, as an increased number of receptors can bind more ligand.

FcγRI signals via the immunoreceptor tyrosine-based activation motif (ITAM), which is located on the γ-chain (Fig. 1). The ligand-binding domain of FcγRI can interact with ICs, such as antibody-opsonized pathogens or tumor cells

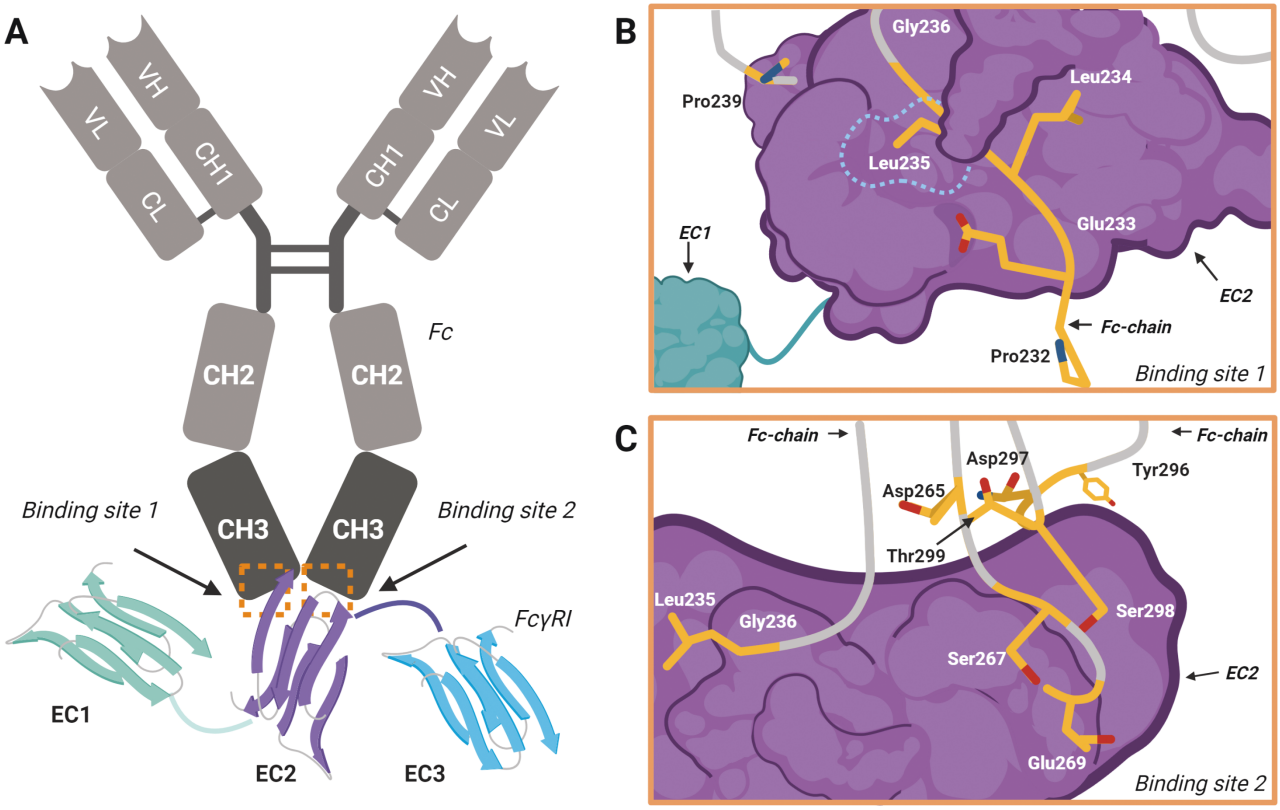

Fig. 2. Schematic representation of Fc-FcγRI interaction. (A) Extracellular domains EC1, EC2, and EC3 are depicted in cyan, purple, and blue, respectively. The Fc-tail of the antibody is depicted in grey. CH stands for constant heavy chain, CL for constant light chain, VL for variable light chain, and VH for variable heavy chain. Orange dashed boxes indicate binding sites 1 and 2. (B) Detailed depiction of binding site 1 (FcγRI and Fc-chain). The surface of EC2 is shown in purple and the surface of EC1 in cyan. The residues of the Fc-chain involved in the interaction of FcγRI are represented with lines (grey = Fc-chain, yellow = carbon, red = oxygen, and blue = nitrogen). The blue dashed line represents the hydrophobic pocket where Leu235 can bind. (C) Detailed depiction of binding site 2. The same color scheme as in (B) is used. Here the interaction surface lies relatively flat. For additional details see review by Kiyoshi *et al.* [17].

[20, 31, 36]. After cross-linking the ligand-binding EC2 by ICs, the tyrosine residues of the ITAM can be phosphorylated by kinases of the SRC family [1]. These phosphotyrosines serve as a docking site for SYK kinases, which bind via their SH2 domains and are activated upon binding [5]. SYK activation results in the activation of the RAS-MAPK pathway, increased intracellular calcium levels, and eventually activation of NF-κB transcription factors through induction of multiple downstream targets [37]. These signals activate immune cells, resulting in phagocytosis, oxidative burst, and cytokine release [24, 37]. However, cross-linking of the inhibitory FcγRIIb causes LYN to phosphorylate the immunoreceptor tyrosine-based inhibitory motif (ITIM), which recruits and activates the SH2-containing phosphatases SHIP and SHP1 [38, 39]. Subsequently, the kinases downstream of ITAM signaling will be dephosphorylated by these phosphatases. This can suppress FcγRI effector functions by balancing the activating signaling cascades.

Immunomodulatory effects are mainly regulated via the single inhibitory receptor, FcγRIIb. However, recent studies have shown that the activating FcγRI and FcγRIIa can also produce inhibitory signals [40–43]. FcγRI engagement along with phagocytic signaling may facilitate inhibitory functions, including secretion of IL-10, reducing pro-inflammatory cytokine production, and reducing T-cell proliferation [44]. Upon IC binding to FcγRI, it is possible that IL-10 secretion affects the macrophage response, leading to both augmenting FcγRI and altering the status of the macrophage and subsequently the outcome of the FcγRI engagement. IC-mediated signaling by FcγRI can also inhibit IFN-γ signaling events [45]. This points to an IC-mediated inhibition of IFN-γ signaling that involves the ITAM-containing FcγRI as well as the ITIM-dependent phosphatase SHP-1, thereby effectively suppressing STAT1 phosphorylation. Studies using various FcR-blocking antibodies in FcR γ-chain knockout mice and FcγRI$^{-/-}$ mice confirmed that the FcγRI mediates these suppressive effects [5, 6, 46]. However, more research is needed to better understand the possible inhibitory signals of FcγRI.

Inside-out signaling on FcγRI regulation

Since triggering of FcR leads to strong effector responses and potential cytokine storms, these receptors need to be tightly regulated. An example of regulation is that only clusters of immunoglobulins can activate low affinity receptors. For FcγRI this is regulated differently; the receptor function is always blocked by its ligand IgG, and the receptor can only be activated with a proper second signal. FcγRI, like integrins, is under ‘ inside-out’ signaling, which means that binding of a cytokine to a cytokine receptor can change FcγRI so that it can bind and respond to ICs even when saturated with monomeric IgG [10, 12, 14] (Fig. 3.I). TNF-α and IFN-γ, can rapidly enhance the IC binding capacity and subsequent effector

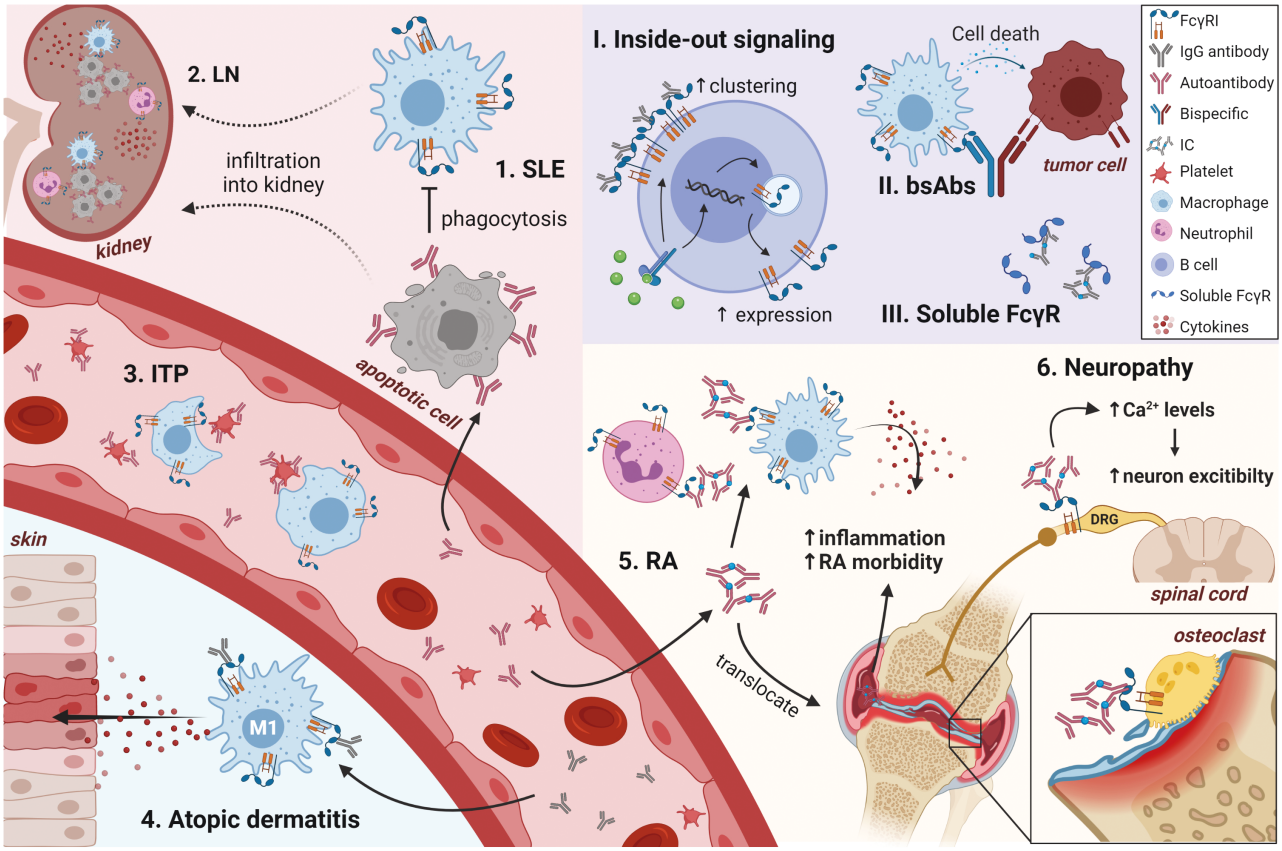

Fig. 3. Role of FcγRI in autoimmune diseases and possible clinical applications for targeting FcγRI. Numbers depict autoimmune diseases and Roman numerals the possible intervention techniques [1]. In SLE, IgG autoantibodies are produced against self-antigens, which opsonize late apoptotic cells. Blocking FcγRI (not depicted) increases phagocytosis of apoptotic cells [2, 47]. ICs, formed by autoantibodies and self-antigens, reside in the kidney, causing the infiltration of FcγRI-expressing monocytes and macrophages, which release pro-inflammatory cytokines and chemokines, leading to inflammation and subsequently LN [3]. In ITP, IgG autoantibody-coated platelets get cleared by FcγRI-expressing macrophages and blocking FcγRI reduced clearance by 50% [20] (not depicted) [4]. In atopic dermatitis, FcγRI expression is increased on M1 macrophages, making them readily bind to the increased total and antigen-specific serum IgG4 [48]. FcγRI-targeted immunotoxins alter the polarization towards M2 phenotype [5, 49]. In RA, IgG autoantibody ICs can bind macrophages and neutrophils causing the release of pro-inflammatory cytokines which increase inflammation and RA morbidity. The IgG-ICs also translocate to the joints, causing further inflammation. They can also directly bind to osteoclasts. Capturing the IgG-ICs with recombinant soluble FcγRs (III) reduces cartilage degradation (not depicted) [6, 50, 51]. Activation of FcγRI on DRG neurons caused increased neuron excitability and subsequent pain. Blocking the receptor relieved pain in mice [52, 53]. (I) Cytokines cause inside-out signaling, leading to enhanced clustering of FcγRI in the membrane. Cytokines also regulate expression of FcγRI by inducing transfer of intracellular FcγRI to the plasma membrane as well as *de novo* expression. (II) bsAbs can be engineered to bind FcγRI-expressing target cells and a tumor-target, thereby inducing tumor-killing [54–56]. (III) Recombinant soluble FcγR can 'capture' circulating ICs. SLE = systemic lupus erythematous, LN = lupus nephritis, ITP = immune thrombocytopenic purpura, RA = rheumatoid arthritis, and bsAbs = bispecific antibodies.

functions, without altering FcγRI surface expression levels, and affecting affinity for monomeric IgG [10].

Two mechanisms have been proposed by which cytokine stimulation enhances the binding capacity of FcγRI: increased clustering of the receptor and/or a conformational change of FcγRI. First, FcγRI, which is normally found in lipid rafts [57], becomes more clustered in the plasma membrane in response to cytokine stimulation, increasing its avidity [23, 58]. This was demonstrated by super resolution imaging, which revealed that cytokines influence the cluster size of FcγRI in Ba/F3-FcγRI cells [14]. This process is dependent on an intact actin cytoskeleton, and the involvement of the serine/threonine phosphatase PP1 (protein phosphatase 1), while the phosphorylation of FcγRI-CY itself is unaffected [14]. This in contrast to FcγRI crosslinking (outside-in signaling), where all four serines in the CY domain of FcγRI need to be dephosphorylated upon crosslinking to lead to phagocytosis [59]. Interestingly, okadaic acid, which is a phosphatase inhibitor of PP2 at low concentrations and PP1 at higher concentrations, prevents the dephosporylation of these serines in outside-in signaling, while it does not directly influence FcγRI phosphorylation in inside-out signaling [14, 60]. Second, a change in FcγRI conformation may increase its binding capacity and affinity for IC. In outside-in signaling, dephosphorylation changes the charge of FcγRI-CY, resulting in a conformational change. This is accompanied by a conformational change in the transmembrane domain and, ultimately, in one or more extracellular domains [61]. FcγRI crystallization revealed that when IgG binds, the EC1 and EC2 domains rotate 19 degrees with respect to the EC3 domain [10]. This rotation could lead to a conformation change of the receptor that alters the affinity of FcγRI for ICs. If the rotation tilts the EC2 domain, there may be more space for an IC to bind to multiple FcγRI molecules. Future research may reveal if increased FcγRI clustering after cytokine stimulation correlates with a conformational change of the receptor.

The implications of FcR inside-out signaling in the treatment of patients with therapeutic antibodies is still under investigation. Activating inside-out signaling could be advantageous to increase the clinical efficacy of therapeutic antibodies. However, FcRs also play a role in the clearance of target–antibody complexes via endocytosis and subsequent degradation in the lysosomal compartment [12]. In these cases, it might be beneficial to inhibit FcRs with antagonistic cytokines to preserve sufficient therapeutic doses of the antibodies, ultimately allowing for lower antibody administration or maintenance doses [62]. Taking advantage of FcR inside-out signaling will ultimately depend on the requirements of the effector cells needed, which FcR they express, and the mode(s) of action of the therapeutic antibodies [62]. This complexity should be taken into account to optimize the therapeutic effectiveness of existing and new antibodies.

Clinical applications of FcγRI

Antibody therapy

There is an increasing evidence that FcRs play a significant role in the induction and maintenance of pathological inflammatory responses induced by ICs, especially autoantibody ICs [47, 63]. The balance between 'activating' and 'inhibitory' roles of FcRs modulates FcR-dependent antibody effector responses in normal immunity [64]. Deviation towards activating functions of FcγRs, as seen in some autoimmune diseases, will reduce the threshold for IC-mediated activation of inflammatory cells, leading to inflammation and tissue damage [2, 64]. Murine FcγRI has been implicated in a number of monoclonal antibody (mAb)-mediated disease models [6, 7, 65] and mAb therapy showed the potency of FcγRI in eliciting mAb-mediated effector responses [36] (Fig. 3).

Antibodies are of interest in immunotherapy due to their high target specificity. Binding of specific antigens is possible through the variable region located in the antigen-binding fragment (Fab), while the non-variable Fc tail can simultaneously bind to FcγRI. The Fab domain of FcγRI can interact with ICs, such as antibody-opsonized pathogens or tumor cells, resulting in antigen internalization or blockade of signal transduction pathways [20, 31, 36]. Strategies for targeting FcγRI, inducing direct Fc blocking, and overcoming IC-mediated autoimmune disorders, are all part of the advancement of therapeutics for the treatment of inflammation [5, 66].

Autoimmune diseases

The pathogenic effects of systemic autoimmune diseases are thought to be triggered by the development of autoantibodies and subsequent IC deposition in tissues [67]. Autoantibodies cause inflammation in antibody-dependent autoimmunity syndromes including immune thrombocytopenic purpura (ITP), systemic lupus erythematosus (SLE), and arthritis by binding to FcγRs [67–70]. In several chronic inflammatory diseases increased expression of FcγRI on M1 macrophages has been observed. Targeting FcγRI with mAbs that block the receptor showed promising results in inducing elimination of the disease-causing M1 macrophage population, while leaving the M2 anti-inflammatory population intact, even though both express FcγRI [49].

Atopic dermatitis is an allergic skin disease characterized by increased levels of total and antigen-specific serum IgE and IgG4 [48]. In both the acute and chronically inflamed skin, FcγRI expression levels are increased, which probably results from upregulation of the receptors on macrophages [48]. *In vitro* and *in vivo* studies showed that FcγRI-targeted immunotoxins can effectively eliminate murine and human M1 macrophages with high specificity [49]. This elimination alters the micro-environment, favoring polarization towards the M2 phenotype. FcγRI-targeted therapeutics can thus be a powerful tool for both identifying M1 macrophages *in vivo* and reversing M1-associated chronic disease (Fig. 3.4).

ITP is an autoimmune disease characterized by low platelet counts caused by autoantibody-mediated clearance of platelets, which results in easy bruising and increased bleeding. Here, FcγRI-expressing macrophages seem to play an essential role in this chronic disease, where they contribute to platelet clearance (Fig. 3.3). Using an anti-FcγRI antibody, platelet clearance could be reduced by 50% [20]. In mouse models, ITP could only be prevented by combining blocking antibodies for FcγRI and FcγRIV, the latter being a murine specific Fc-receptor. Nonetheless, the use of an anti-FcγRI mAb (clone 197) in a chronic ITP patient resulted in clinical improvement by preventing FcγRI mediated destruction of IgG-coated platelets [71].

SLE is a non-organ specific autoimmune disease in which IgG autoantibodies are produced against a wide range of self-antigens [68]. One pathogenic factor in SLE is the opsonization of late apoptotic cells by autoantibodies, resulting in decreased FcγRI-mediated clearance of apoptotic cells by phagocytes [47]. IgG autoantibodies may bind to molecules on apoptotic cells that are required for recognition and facilitation of macrophage uptake, such as phosphatidylserine or C1q [72–74]. Intracellular autoantigens may become exposed, in altered or unaltered form, at the outer surface of apoptotic cells during the apoptotic process [75, 76]. Decreased clearance of apoptotic cells results in prolonged exposure of these cell surface-expressed autoantigens to the immune system, which may explain the development of autoantibodies against these intracellular antigens. In part, this is due to increased signaling caused by IgG binding to FcγRs, which may affect the ability of monocyte-derived macrophages to internalize apoptotic cells [47, 77]. Fc receptor blockade, with the partial blocking anti-FcγRI antibody 10.1 (see Limitations section), significantly reduced phagocytosis inhibition [47]. This effect was amplified when anti-FcγRI antibody was combined with anti-FcγRIII blocking antibody (3G8) on macrophages, and it abolished the inhibitory effect of SLE autoantibodies [47].

The formation of ICs between autoantibodies and self-antigens has also been linked to the development of lupus nephritis (LN) [68, 78]. In the kidneys, the presence of ICs activates monocytes and macrophages by interacting with FcγRI and FcγRIII, triggering an inflammatory cascade of cytokines and chemokines. Monocytes secrete monocyte chemoattractant protein 1 (MCP-1) [79], which recruits macrophages but may also promote a further influx of monocytes into the kidney. The greater ability of monocytes to migrate and secrete MCP-1 is linked to increased FcγRI expression on the cell surface, especially in LN patients [79], and also with markers of impaired renal function. This could lead to a vicious cycle of renal inflammation and facilitate the infiltration of monocytes to sites of IC deposition in the kidney [80], which eventually could result in permanent tissue damage [81]. FcγRI is critical for the development of LN, as mice with gamma chain-deficient FcγRI forming and depositing ICs were surprisingly protected from severe nephritis [67]. Taken

together, these data suggest an important role for FcγRI in the pathogenesis of both SLE and LN and blocking FcγRI seems to increase the clearance of apoptotic cells, but this warrants further research (Fig. 3.1 and 3.2).

The most common form of autoimmune inflammatory arthritis is rheumatoid arthritis (RA), which affects up to 1% of the human population [82]. The pathogenesis of RA has been linked to IgG-IC/FcγRI signaling [50–52, 66, 83] and FcγRI expression levels correlate with disease progression [6]. When compared to total serum IgG1, RA-specific autoantibodies show differences in Fc-linked galactosylation and fucosylation. These IgG1 changes resulted in decreased affinity for FcγRIIIa and FcγRIIb, but not for FcγRI, which could increase the availability of RA-specific autoantibodies to bind and activate FcγRI [84]. In collagen-induced arthritis (CIA) and antigen-induced arthritis (AIA) models, FcγRI-deficient mice showed reduced arthritic symptoms [50, 66, 83], and treatment with a FcγRI-directed immunotoxin reduced inflammation and bone degradation in human FcγRI–transgenic rats with joint inflammation [85]. In another AIA model, deletion of the FcγRI α-chain was partially protective, but it also impaired their ability to clear infection with *Bordetella pertussis* [6]. Additionally, IgG autoantibody ICs can bind to neutrophils, macrophages, and monocytes, causing pro-inflammatory cytokine release and aggravating inflammation, contributing to RA morbidity. In RA patients, IgG-IC was present in high concentrations in the serum and infected joints, making it an important pathological characteristic of the disease [50, 86]. IgG-autoantibodies can directly bind to pre-osteoclasts and induce differentiation, as well as activate mature osteoclasts [69]. Interestingly, in AIA and CIA models, capturing IgG-IC with recombinant soluble FcγRI reduced cartilage degradation [50, 51]. Given this, it is of interest to investigate the effect of osteoclast specific anti-FcγRI blocking antibodies in combination with anti-inflammatory treatment options (Fig. 3.5).

Besides being a direct target, FcγRI is also widely used as early biomarker for disease detection and prediction of disease outcome for sepsis, HIV, pediatric and adult Crohn's Disease, and tuberculosis [87–92]. The FcγRI expression levels were significantly elevated on neutrophils in patients [87–90] and macrophages in *in vivo* models [91]. These data suggest an important role for FcγRI and it could be interesting to examine if blocking FcγRI in these settings could improve the disease outcome.

Neuropathy and pain

Chronic pain is a widespread condition that affects 20% of adults in Western countries [93, 94]. Some chronic pain disorders, such as arthritis, are characterized by persistent peripheral nociceptive feedback linked to peripheral inflammation, while others, such as neuropathic pain, are the result of irregular nervous system functioning due to injury or disease [95].

IgG-IC/FcγRI signaling has been linked to both the pathogenesis [50, 66, 83] and disease-associated pain [8, 52, 53] in RA. Joint pain is a prominent clinical characteristic of RA, which is caused in part by synovitis and joint destruction [96]. While RA pain is commonly thought to be caused by inflammation, it often continues even after inflammation has been controlled with available therapies, implying the involvement of non-inflammatory mechanisms. FcγRI, but not FcγRII or FcγRIII, has been shown to be expressed in subsets of nociceptive dorsal root ganglion (DRG) neurons in rats and mice [97, 98]. Activation of neuronal FcγRI in rat DRG neurons by ICs, raises intracellular calcium levels by activating the non-selective cation channel TRPC3 through the Syk-PLC-IP3 pathway, thereby increasing neuronal excitability [97, 99]. Hence, IgG-IC accumulation in the inflamed joint is sufficient to directly stimulate and sensitize joint sensory neurons through neuronal FcγRI, resulting in joint pain. Direct FcγRI blockade with neutralizing antibodies and FcγRI genetic knockout substantially reduced pain-related behaviors in the AIA mouse model [52, 53]. These findings suggest that FcγRI can contribute to arthritis pain through a non-inflammatory mechanism, making it a promising therapeutic target in RA patients with pain refractory to current anti-inflammatory treatments (Fig. 3.6).

Tumor targeting with antibodies

Therapeutic mAbs are designed to recognize antigens on tumor cells and mediate anti-tumor effects via various direct Fab-mediated and indirect Fc-mediated mechanisms. Direct mechanisms include inducing receptor internalization and degradation, directly inducing pro-apoptotic signals, and blocking the ligand binding site of growth factor receptors [100–102]. Indirect effects include IgG-mediated activation of the classical complement pathway, which results in complement-dependent cytotoxicity (CDC), and FcγR engagement, which results in ADCC of tumor cells [103–105]. The recruitment of cytotoxic effector cells, such as natural killer (NK) cells, monocytes/macrophages, and polymorphonuclear (PMN) cells mediates ADCC. The Fab-arms of mAbs can recognize and bind to only one unique epitope, whereas bispecific antibodies (bsAbs) can simultaneously bind two different and unique antigens, attributing dual functionalities.

Some therapeutic bsAbs were developed to act as a bridge between cytotoxic effector cells expressing FcγRI and tumor-target-overexpressing malignant cells [106, 107] (Fig. 3.II). FcγRI expression is restricted mainly to cytotoxic immune cells such as monocytes, macrophages, and cytokine-stimulated PMNs. As a result, tumor cells bound with bsAbs can be selectively detected by effector cells with cytotoxic potential. Multiple studies have shown the potential of FcγRI in anti-cancer therapy by using bsAbs and fusion proteins to recruit FcγRI on immune cells to tumor-associated-antigens on various tumor cell types including acute myeloid leukemia, acute, and chronic myelomonocytic leukemia and melanoma [9, 16, 54, 108, 109].

FcγRIs are highly potent triggering molecules to activated PMNs and, in the presence of mAbs that bind FcγRI outside the ligand binding domain, it can mediate lysis of various tumors [106]. A humanized mAb that can bind outside of the Fc-region of FcγRI is H22. As a result, serum IgG has no effect on H22 binding to FcγRI [110]. In the presence of human IgG or serum, bispecific molecules derived from H22 and coupled to tumor-specific antibodies like HER2/neu and TAG-72 or other ligands will trigger lysis of target cells [54–56]. Interestingly, the ADCC activity of the bsAb is significantly higher than that of the mAb alone [56, 106]. Experiments in murine tumor models using bsAbs that target tumor cells and FcγRI also demonstrate the potent effector functions of FcγRI [16, 106]. In some cases, FcγRI-based bsAb could establish long-term T-cell immunity [106]. Since these bsAbs can bind FcγRI in the presence of saturating concentrations of IgG [16, 110], they can effectively exploit the cytotoxic

potential of FcγRI under physiological conditions [16, 54]. It should be noted that most of these studies into FcγRI bsAbs were performed more than two decades ago. Only recently research into FcγRI targeting has revived, as the role of FcγRI in several diseases is becoming increasingly clear. Moreover, the recent developments regarding designing and producing bsAbs resulted in better and more stable bsAbs [107], making it easier to study FcγRI tumor targeting. After selecting the appropriate tumor target and anti-FcγRI mAb when developing novel bsAbs, they could become valuable tools in effective tumor destruction [54–56, 106, 111].

Additionally, cytokine stimulation could be a promising addition to anti-tumor therapeutic antibodies to enhance the activity of FcγRI-expressing immune cells [12] (Fig. 3.I). The efficacy of various therapeutic antibodies was increased when combined with cytokine stimulation both *in vitro* and *in vivo* [15, 112, 113]. Some cytokines, like granulocyte colony-stimulating factor (G-CSF), are associated with increased FcγRI expression on neutrophils and increased recruitment of effector cells [54, 114]. However, these processes take several hours or even days. Inside-out signaling of FcγRI to enhance FcγRI-IC binding occurs rapidly, within minutes [14]. Cytokine stimulation combined with antibody therapy may increase the binding capacity of FcγRI-expressing cells to tumor cells immediately after cytokine administration (minutes), while increasing FcγRI expression and recruiting more effector cells from the bone marrow occurs later (hours or days). This may result in more effective anti-tumor responses, especially when timing of cytokine administration correctly. Understanding the regulatory mechanisms of FcγRI activation may aid in the manipulation of immune responses using cytokines during infections, autoimmune diseases, and antibody-based immunotherapy.

Limitations

The use of mAbs directed against the IgG binding site of FcγRI, which block IC binding and subsequent cell activation, is the most direct way to inhibit or modulate FcγRI signaling. Mouse anti-human FcγRI antibodies to inhibit FcγRI ligand binding have been developed [19], but these antibodies may not be FcγRI-specific blocking antibodies as they bind via their Fc domain rather than their Fab domain or outside of the ligand binding domain of FcγRI [23, 52, 115, 116]. The current commercially available FcγRI blocking antibodies include clones 10.1 (mIgG1), m22 (mIgG1), and 197 (mIgG2a). Clone 10.1 is the most commonly used commercial antibody [19, 23], but it is not directly a ligand binding blocking antibody as the binding site of 10.1 is located in EC3 of FcγRI [23], while the IgG binding site is located in EC2 (17) (Fig. 2). Our own unpublished data indicates that 10.1 can achieve ~40% IgG blocking at saturating antibody concentrations, indicating it can probably sterically hinder IgG binding to FcγRI to some extent. Hence, there is a need to develop specific FcγRI blocking antibodies. However, this is difficult as the Fc portion of IgG antibodies will readily bind with high affinity to FcγRI, making screening for potential candidates very challenging. To our knowledge, no specific and high affinity Fab-mediated FcγRI blocking antibodies are currently available. Alternative strategies to block FcγRI are using an IgG Fc-fragment preparation or intravenous administration of pooled human immunoglobulin (IVIg) [64, 117]. Indeed, in pediatric ITP patients, IVIg results in rapid recovery of platelet counts [118].

Another promising strategy in autoimmune diseases is to target the ICs rather than FcγRI. Recombinant soluble FcγRs can be used to 'capture' circulating ICs and subsequently prevent binding to surface-bound FcγRs [50, 51] (Fig. 3.III). Normally, recombinant soluble ectodomains of FcγRI will be expected to be rapidly and fully occupied by circulating IgG. Surprisingly, *in vivo* mouse experiments revealed that soluble FcγRI is effective at alleviating IC-induced inflammation in antibody-dependent models of tissue damage, including CIA [50, 51]. The 'on' and 'off' rates of monomeric IgG are fast enough to enable recombinant soluble FcγRI to interact with high concentrations of oligomeric ICs in tissues, which may retain soluble FcγRI locally long enough to effectively inhibit inflammatory cell activation through IC binding to cell surface-bound FcγRI [4]. However, since FcγRI can bind to more than one IgG subclass, it would act more as a universal inhibitor of ICs. Another constraint to use soluble FcγRI is its small molecular size (45 kDa), resulting in fast renal clearance [64]. It also makes it difficult to achieve high enough FcγRI therapeutic concentrations at the site of inflammation to be effective.

The combination of therapeutic antibodies with cytokines has great potential due to the potent pro-inflammatory effects and induction of FcγRI inside-out signaling. However, there are only a few clinical trials currently investigating anti-tumor mAb therapy with cytokines [119–121]. In these studies, there are often no control groups receiving monotherapy (either mAb or cytokine), for good ethical reasons. This does make it difficult to determine the effect of cytokine stimulation on mAb therapy in humans. Nonetheless, the results of these studies are very promising. Adding G-CSF to mAb therapy in relapsed leukemia patients produced good therapy responses for a short duration [119, 120]. Before stem cell transplantation, patients receive anti-thymocyte globulin (ATG), an infusion of horse- or rabbit-derived antibodies against human T cells and the precursor thymocytes that leads to depletion of T-cells by inducing CDC and ADCC. When ATG is combined with G-CSF infusion in these patients, neutrophil-mediated ATG cytotoxicity was significantly higher, thereby enhancing T-cell clearance [122]. Furthermore, the addition of G-CSF to ATG treatment in anemia patients resulted in reduced inflammation and days of hospitalization in the first three months [123], although it does not improve the long-term outcome and sustainability of remission [124].

Directly evaluating the influence of FcγRI inside-out signaling on therapeutic antibody therapy remains challenging, as it is difficult to determine the activation status of FcγRI, and the surface expression of FcγRI is often not measured in clinical trials. In addition, inside-out signaling is a rapid process which occurs minutes after cytokine stimulation, while clinical trials usually focus on long term effects of antibody therapy. Nonetheless, the *in vitro* and *in vivo* data on inside-out signaling is very promising in regard to enhancing FcγRI effector functions.

Concluding remarks & outlook

Therapeutic antibodies can exploit FcγR-mediated effector functions and targeting FcγRI is a promising strategy to enhance the efficacy of antibody-based therapy (Fig. 3). FcγRI plays an important role in mAb based therapy [5, 7, 12], and through antibody engineering, activating, or inhibitory immunomodulatory effector functions of FcγRI can be initiated [16, 54–56, 106]. Moreover, in various autoimmune

diseases, antigens and autoantibodies cross-link and form ICs that bind and activate FcγRI [67]. These inflammatory effector functions can cause extensive tissue damage in diseases like RA and SLE, but also chronic joint pain [52, 53]. In these autoimmune diseases, blocking IC-FcγRI interactions with mAbs that specifically block the ligand binding domain of FcγRI may be beneficial.

Using anti-FcγRI/ anti-tumor bsAbs is an attractive alternative to conventional mAbs for direct tumor killing as they exhibit dual specificity for the target and effector cells. They can be designed to recruit a range of cellular effectors, like T cells and NK cells [106, 107]. If these bsAbs bind an epitope outside of the ligand binding domain of FcγRI, there is no competition with human IgG, thus ADCC activity can be attained under normal serum conditions [16, 54]. Additionally, this design would make it possible for IgG to still mediate its function by binding with its Fc-tail to FcγRI [16]. Together, these bsAbs can result in optimal tumor killing.

In conclusion, the involvement of FcγRI in many inflammatory diseases makes FcγRI a promising target in treatment of autoimmune and malignant diseases. A better understanding of inside-out signaling in combination with antibody therapy will lead to better and safer therapies.

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