Fcγ receptors and immunomodulatory antibodies in cancer

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Abstract

The discovery of both cytotoxic T lymphocyte-associated antigen 4 (CTLA4) and programmed cell death protein 1 (PD1) as negative regulators of anti-tumor immunity led to the development of a number of immunomodulatory antibodies as cancer treatments. Preclinical studies have demonstrated that the efficacy of immunoglobulin G (IgG)-based therapies depends not only on their ability to block or engage their targets but also on the antibody's constant region (Fc) and its interactions with Fcγ receptors (FcγRs). Fc–FcγR interactions are essential for the activity of tumor-targeting antibodies, such as rituximab, trastuzumab, and cetuximab, where the killing of tumor cells occurs at least in part due to these mechanisms. However, our understanding of these interactions in the context of immunomodulatory antibodies designed to boost anti-tumor immunity remains less explored. In this Review, we discuss our current understanding of the contribution of FcγRs to the *in vivo* activity of immunomodulatory antibodies and the challenges of translating results from preclinical models into the clinic. In addition, we review the impact of genetic variability of human FcγRs on the activity of therapeutic antibodies and how antibody engineering is being utilized to develop the next generation of cancer immunotherapies.

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[H1] Introduction

Antibodies are members of the immunoglobulin superfamily and are structurally composed of two domains: the fragment antigen-binding (F(ab)) domain, which is responsible for antigen recognition, and the fragment crystallizable (Fc) domain, which engages with Fc receptors (FcRs). The F(ab) comprises the light chain and heavy chains that fold to form the complementarity-determining region (CDR), which contains six hypervariable loops that determine antigen recognition. The light chain in mammals originates from the lambda (λ) or kappa (κ) genes (most clinical antibodies contain a κ light chain; see Table 1). The Fc region is made of either the α, δ, ε, γ or μ heavy chain, found in immunoglobulin A (IgA), IgD, IgE, IgG, and IgM antibodies, respectively which defines the antibody isotype. The F(ab) and the Fc domains are bound together by a short sequence of amino acids that provides flexibility and is known as the hinge region [1]. There are three types of FcRs: type I, which includes the canonical Fcγ receptors (FcγRs); type II, which contains C-type lectin receptors such as CD209 and CD23 [2]; and the intracellular receptors, neonatal Fc receptor (FcRn) and TRIM-21 responsible for maintaining antibody half-life and degradation respectively [3, 4].

Antibodies of the IgG subclass are the most broadly used in the clinic due to their potent effector function and ease of production. Although IgA [5], IgE [6], and IgM [7], are also being investigated as therapeutic agents, for the remainder of this Review we will focus on IgG. IgGs are categorized into subclasses with differences in structure and affinities to FcγRs. In humans (h) and mice (m), there are four subclasses: hIgG1, hIgG2, hIgG3, hIgG4, and mIgG1, mIgG2a/c, mIgG2b, and mIgG3. The activity and binding to FcγRs of the different subclasses are driven primarily by differences in the length of the hinge region and the number of disulfide bonds present [8]. In the clinic, hIgG1, hIgG2, and hIgG4 have proven to be highly successful in the treatment of human cancers and are a critical part of many treatment regimens [9]. Despite the ability of hIgG3 to elicit strong antibody-mediated effector functions, its clinical development has been limited by concerns of immunogenicity (due to its polymorphic nature), stability in vivo and manufacturing concerns compared to other hIgG subclasses [10].

The role of FcγRs in the efficacy of antibodies targeting tumor antigens so called 'tumor-targeting antibodies', has been broadly recognized across different cancer types [11]. However, in the case of immunomodulatory antibodies that target immune checkpoints or aim to deplete immune cell populations, the impact of FcγRs on their activity remains less well understood. In this Review, we will examine the evidence for how FcγRs modulate the activity of immunomodulatory antibodies. We will assess the impact of IgG subclass, genetic polymorphisms, and the translational gap between pre-clinical and clinical testing of the

proposed mechanism of action of these antibodies and how the field can advance to improve the efficacy of these therapeutic agents.

[H1] FcγRs and their mechanisms of action

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[H2] FcγRs modulate the effector functions of IgG.

FcγRs are a family of receptors that bind monomeric and immune-complexed (IC) IgG and can elicit activating or inhibitory functions (Figure 1). Engagement of these receptors drives antibody effector functions, including antibody-dependent cellular cytotoxicity (ADCC) and/or

55   antibody-dependent cellular phagocytosis (ADCP) [1], antigen presentation, and the release of chemokines and cytokines [12, 13]. In humans and mice, there are three families of receptors. These include FcγRI (also known as CD64), FcγRII (also known as CD32) and FcγRIII (also known as CD16) that bind IC IgG. Mice express these three receptors, as well as the unique high-affinity FcγRIV (also known as CD16-2) [14]. FcγRI, FcγRIII, and FcγRIV

60   are considered to be activating receptors whilst FcγRII is inhibitory (Supplementary Figure 1). The human FcγR system is more complex than that of the mouse, as exemplified by the presence of receptor gene families for hFcγRI (*FCGR1A, FCGR1B* and *FCGR1C*), hFcγRII (*FCGR2A*, *FCGR2B* and *FCGR2C*) and hFcγRIII (*FCGR3A* and *FCGR3B*) as well as several allelic variants. *FCGR1B* and *FCGR1C* do not encode functional proteins, while *FCGR2C* is a

65   pseudogene that is expressed in a subset of the population [15]. Typically, humans express FcγRI, FcγRIIa (also known as CD32a), FcγRIIb (also known as CD32b), FcγRIIIa (also known as CD16a) and FcγRIIIb (also known as CD16b). All are considered activating, besides the glycosylphosphatidylinositol (GPI) anchored FcγRIIIb that lacks a signaling domain [16], and the inhibitory FcγRIIb that modulates activating FcγRs [17].

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Human and mouse FcγRI, FcγRIII, and FcγRIV signal via an immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor protein, known as the Fc receptor gamma (FcRγ) chain [14, 18-20]. Human FcγRIIa and FcγRIIc are the only FcγRs that contain an endogenous ITAM in their cytoplasmic tail and do not require an adaptor protein to signal [21]. Interestingly,

75   FcγRIIIa in human natural killer (NK) cells also associates with the CD3 ζ chain, driving more potent signaling as this adaptor protein has three sets of ITAM domains compared to only one set present in the FcRγ chain [22]. Although FcγRIIIb lacks direct signaling capacity, this receptor has been shown to interact with FcγRIIa in cis to potentiate intracellular signaling that enhances neutrophil-mediated ADCP [23]. However, the contribution of FcγRIIIb to antibody

80   effector functions remains controversial as in vitro studies have shown that it can also be detrimental for neutrophil mediated ADCC [24]. FcγRIIb inhibits antibody effector functions mediated by activating FcγRs by binding to immune complexes and signaling through the

endogenous intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM) [25]. FcγRIIb has been shown to restrict the ability of myeloid cells to kill tumor cells following anti-CD20 (rituximab, hIgG1) and anti-human epidermal growth factor receptor 2 (HER2) (trastuzumab, hIgG1) treatment in human xenograft models of B cell lymphoma and breast cancer [26]. However, recent evidence suggests that human FcγRIIb does not require its ITIM to inhibit the depletion of malignant B cells and regulatory T (T_reg) cells, following treatment with CD20, CD25 (also known as IL-2Rα), and OX40 depleting antibodies [27]. This study proposes that the primary mechanism of inhibition is elicited through FcγRIIb competing with activating FcγRs on the same cell for the Fc of therapeutic antibodies rather than through ITIM signaling. FcγRIIb has also been shown to elicit other ITIM-independent mechanisms that modulate the activity of some therapeutic antibodies, such as antibody bipolar bridging and hyper-crosslinking of agonistic antibodies. Antibody bipolar bridging is a phenomenon that has been shown to impact B cell targeting antibodies such as those against CD20 (e.g., rituximab) and CD57 (e.g., alemtuzumab). In these cases, FcγRIIb engages the antibody on the surface of the B cell in a *cis* formation, promoting rapid internalization and degradation, and ultimately reducing the killing capacity of the antibody [28]. Although formal evidence is limited to those antibodies targeting B cells, this mechanism could happen to any cell expressing FcγRIIb. Hyper-crosslinking of some agonistic antibodies targeting the tumour necrosis factor (TNF) receptor superfamily (TNFRSF) also requires FcγRIIb engagement on an accessory cell in *trans* to hyper-crosslink a particular receptor to deliver an agonistic signal. It has been described as one of the main mechanisms of action for anti-CD28 super-agonists in humans [29] and for multiple agonistic antibodies in mouse models, including those against CD40 [30, 31], OX40 [32], 4-1BB [33], and CD27 [34].

[H2] FcγR affinities and immune contexture.

FcγRs are classified as high and low affinity, depending on their ability to bind monomeric or IC IgG (Figure 1 and Supplementary Figure 1). FcγRs with affinities above 10^7 M^{-1} can bind monomeric and IC IgG and are considered high affinity, while FcγRs with affinities below 10^7 M^{-1} can only bind to IC IgG and are considered low-affinity [35]. Humans have one high-affinity FcγR, hFcγRI, with the remainder considered low affinity. Mice have two high-affinity FcγRs, mFcγRI and mFcγRIV (although mFcγRIV has approximately an order of magnitude lower affinity for mIgG2a than mFcγRI [36]), and two low-affinity FcγRs, mFcγRII, and mFcγRIII. *In vivo*, high-affinity FcγRs are thought to be saturated with monomeric IgG being unable to exert effector functions. However, despite binding monomeric IgG, these high-affinity receptors constantly dissociate, leading to a dynamic steady state of binding and release [37, 38]. In contrast, low-affinity FcγRs remain free of ligands in physiological conditions and can bind circulating IC IgG to mediate signaling. The affinity and binding of the different IgG for FcγRs

have been measured *in vitro* using immobilized FcγRs and quantified using surface plasmon resonance (SPR) for monomeric IgG and fluorescence-based methods for IC IgG [35]. In the case of human IC IgG, hIgG1, hIgG3, and hIgG4 bind to all FcγRs while hIgG2 binds to all FcγRs except for FcγRI and FcγRIIIb [35, 39] (Figure 1). In the case of mouse IC IgG, mIgG1 only binds to mFcγRII and mFcγRIII, and mIgG2a/c and mIgG2b bind to all FcγRs [39], whilst mIgG3 only binds to mFcγRI [40]. While these studies provide a strong pillar to aid the understanding of IgG–FcγR interactions, *in vivo* the avidity of these receptors is affected by multiple variables such as the antibody: antigen ratio, the size of the immune complex, and the location of the receptor [41]. For the remainder of this Review, we will focus on hIgG1, hIgG2, and hIgG4, as well as mIgG1, mIgG2a/c, and mIgG2b, as these subclasses are the most commonly used for the treatment of human and mouse cancers, respectively.

The different affinities of the IgG subclasses for FcγRs and the cellular expression level of each FcγR have been used to define the likelihood of eliciting 'weak' or 'strong' antibody effector functions. This is known as the activating-to-inhibitory (A/I) ratio [42]. Based on this concept, a mathematical model has been developed that has the potential to predict the activity of a therapeutic antibody based on IgG subclass affinity, binding valency, and receptor abundance [43]. Typically, therapeutic antibodies with a hIgG1 backbone are used to engage activating FcγRs and eliminate target cells. In contrast, hIgG2 and hIgG4 antibodies are used to block a receptor–ligand interaction without engaging activating FcγRs, due to their relatively low affinity for FcγRs as measured by SPR [35]. However, the ability of hIgG2 and hIgG4 to still bind activating FcγRs, especially when complexed, could drive effector functions in unexpected scenarios [44, 45]. As FcγRIIa and FcγRIIIa are primarily expressed on NK cells, granulocytes, and macrophages, they are considered the primary effector cell of IgG in humans [46, 47]. Human NK cells are highly relevant in this context due to their intrinsically cytotoxic properties, high expression of FcγRIIIa, and lack of FcγRIIb, meaning they can easily elicit ADCC [48].

In mice, mIgG2a is used when antibody effector functions are required (similar to hIgG1), while mIgG1 (similar to hIgG4) is used when blocking the biological activity of the target. Therapeutic antibodies of the mIgG2a subclass require engagement with the high-affinity mouse FcγRs, mFcγRI, and mFcγRIV on myeloid cells to deplete their target. These include TA99 antibodies targeting melanoma metastasis in the lung and liver [49-51] and CD20 antibodies targeting subcutaneous lymphomas [52]. Similar rules apply to immunomodulatory antibodies targeting intratumoral Treg cells (anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA4) [53] and anti-CD25 [54]) and intratumoral programmed cell death protein 1 ligand 1 (PDL1)+ cells [55]. mFcγRIII only partially contributes to the activity of tumor-targeting and immunomodulatory

antibodies [51, 52]. This could be explained by the low affinity of mFcγRIII for mIgG2a and its inability to efficiently associate with the CD3 ζ chain in mouse NK cells, which dampens its function [56].

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These data suggest two critical differences between humans and mice: 1) The relevance of the myeloid compartment in mice compared to the NK and myeloid cells in humans, and 2) The relevance of the high-affinity FcγRs in mice compared to the relevance of the low-affinity FcγRs in humans. In mice, the high-affinity mFcγRI and mFcγRIV on myeloid cells are the

165   primary drivers of antibody-effector functions. mFcγRIV, in particular, is unique to this species [14], structurally homologous to hFcγRIIIa [57], but considered of high affinity due to its ability to bind monomeric IgG [58]. Therefore, the potential importance that mFcγRIV has in the context of tumor-targeting antibodies represents a problem when trying to extrapolate data or conclusions from mice into humans as it does not have a functional counterpart. We believe

170   that at least part of the complexity of translating observations made in mice into the clinic may be the result of these two substantial differences, highlighting the need for better mouse models (See Box 1).

[H2] FcγR expression in blood, tissues, and tumors.

175   FcγRs have been extensively characterized on peripherally circulating immune cells within human blood. hFcγRI is expressed on monocytes [59], dendritic cells [59], and can be upregulated on activated neutrophils [45]. hFcγRIIa is expressed on all myeloid cells and platelets [60, 61], and hFcγRIIb is expressed on B cells [62], monocytes [62], and subsets of dendritic cells [62, 63]. hFcγRIIIa is expressed on NK cells [64] and monocytes [65], while

180   hFcγRIIIb expression is restricted to neutrophils and basophils [66]. A recent study has quantified the number of molecules of each FcγR in blood leukocytes of humans and mice [67] (Figure 1 and Supplementary Figure 1). Classical monocytes (CD14$^+$CD16$^-$) express the highest levels of hFcγRI as well as hFcγRIIa, while non-classical monocytes (CD14$^-$CD16$^+$) express hFcγRIIa, and FcγRIIIa, with low levels of hFcγRIIb on both monocyte subsets [67].

185   In mice, mFcγRI has a similar expression pattern in blood, being found primarily in classical (Ly6C high) and non-classical (Ly6C low) monocytes [67]. However, differences in hFcγRIIa and mFcγRIII expression (most homologous between mouse and human) are evident; in mice, mFcγRIII expression is higher on classical monocytes, with lower levels on non-classical monocytes, neutrophils, and NK cells. In humans, high expression is found on monocytes,

190   neutrophils, and dendritic cells. In addition, the expression of mFcγRIV is restricted to non-classical monocytes and granulocytes, showing similarities with its ortholog hFcγRIIIa, with the exception that mFcγRIV is not expressed on mouse NK cells [67]. The expression of hFcγRIIIa is similar between non-classical monocytes and NK cells, while neutrophils uniquely

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express hFcγRIIIb, with no functional equivalent FcγR in mice [67]. hFcγRIIb is expressed mainly on B cells and at low levels on monocytes, while mFcγRII is expressed at high levels on B cells and monocytes [67].

In tissues, the expression of mouse and human FcγRs has been described for some tissue-resident macrophage (TRM) populations in the bone marrow, spleen, liver and lung [68] (Figure 1); however published studies remain limited. Our understanding of the expression of human FcγRs within tumors is also limited, and the available data suggests high levels of heterogeneity between immune cell populations. For example, human melanoma samples show high expression of FcγRI, FcγRIIa, FcγRIIb, and FcγRIIIa on tumor-infiltrating granulocytes, monocytes, and macrophages when assessed by flow cytometry [45]. Of note, tumor-infiltrating NK cells express lower levels of hFcγRIIIa than those in the blood, suggesting specific downregulation within the tumor, which could be an additional mechanism of immune evasion [45]. These specific studies can inform on how the tumor microenvironment (TME) modulates FcγR expression in a particular tumor, but they are difficult to compare. However, publicly available datasets provide an opportunity to comprehensively characterize FcγR expressing cells across multiple tumor types.

To further our understanding of the expression of FcγRs in human tumors, we used publicly available single-cell RNA sequencing data to assess the expression of the different FcγRs in key immune cell populations. We analyzed data from non-small-cell lung carcinoma (NSCLC), renal cell carcinoma (RCC), and colorectal carcinoma (CRC) datasets (Figure 2). Across all tumors, *FCGR1A* and *FCGR2A* were restricted to myeloid cells, while *FCGR3A* was expressed in monocytes, macrophages, dendritic cells, and NK cells. In NSCLC and RCC, NK cells expressed lower levels of *FCGR3A* compared to the normal tissue and the blood, as observed in melanoma [45]. *FCGR2B* expression was upregulated in macrophages and monocytes across the three tumor types. The upregulation of hFcγRIIb within tumors has recently been described in macrophages that reside in the adipose tissue of breast cancer [69] and in mesothelioma-infiltrating monocytes [70]. The FcγR expression on type I conventional dendritic cells (cDC1) across the three tumor types analyzed is consistently low compared to normal tissue, again suggesting modulation by the TME. Furthermore, most immune cells within CRC express low levels of all FcγR mRNA compared to NSCLC and RCC, with *FCGR3A* displaying the most striking downregulation. Although this data still requires validation at the protein level, it highlights the diversity of FcγR expression across different tumor types.

[H1] *FCGR* genetic polymorphisms

Human FcγR genes (FCGR) are located in the long arm of chromosome 1 (1q), with *FCGR1A* in position 21.2, while the low-affinity FCGRs are clustered at position 23.3 [11]. The proximity among the low-affinity FCGRs causes copy number variations that occur in distinct but recognizable copy number variable regions (CNRs) [71]. This means that different individuals will have deletions or duplications of the loci containing the low to medium-affinity FCGRs, potentially impacting protein expression with relevance to the activity of antibody therapeutics. In addition to the CNRs, single nucleotide polymorphisms (SNPs) have been described for all FCGRs, and for some, the change in their affinity for IgG subclasses has been correlated with the response to therapeutic antibodies. In this section, we discuss the impact of these variations on the response to antibody-based therapies.

[H2] Copy number variable regions

Four CNRs have been described, CNR1 includes *FCGR2C* and *FCGR3B*, CNR2 includes *FCGR2A* and *FCGR2C*, CNR3 includes *FCGR3A* and *FCGR2C* and CNR4 includes *FCGR2C*, *FCGR3B* and *FCGR2B*. However, only CNR1, CNR2, and CNR3 have immune associations that could impact the response to antibody-based therapies [72]. Deletions of CNR4 are rare and only heterozygous deletions have been reported, leading to loss of *FCGR2B* on B cells but without associations with disease or response to therapies [72].

Amplifications and homozygous or heterozygous deletions in CNR1 are the most prevalent followed by CNR2 and CNR3 as found in a cohort of healthy individuals that included Europeans, Chinese and African individuals [72]. Changes in CNR1 lead to changes in the copy numbers of *FCGR3B* and expression of *FCGR2B* on NK cells in the case of deletions [72]. Neutrophils isolated from healthy donors with low copy numbers of *FCGR3B* (CNR1 deletion) showed higher levels of ADCC in the presence of trastuzumab or cetuximab and tumor cells expressing the target antigen, compared to those with higher copy numbers (CNR1 amplification) [24]. FcγRIIb on NK cells (CNR1 deletion) decreases their ability to kill tumor cells in the presence of rituximab, suggesting that patients carrying this feature would be poor responders to tumor-targeting antibodies [73]. Deletions in CNR2 cause the generation of a chimeric gene between *FCGR2A* and *FCGR2C* that decreases the levels of FcγRIIa in neutrophils and monocytes while amplifications lead to the expression of this chimeric gene on NK cells [72]. Amplifications and deletions on CNR3 are expected to primarily affect the expression of *FCGR3A* but due to its overlap with CNR2, no distinction has been made in the literature when reporting differences in the copy numbers. However, *FCGR3A* copy numbers are associated with its expression and correlate with the capacity of NK cells for ADCC, which

is increased when more copies of the gene are present [74]. It would be expected that patients with cancer carrying fewer copies of *FCGR3B* may be better responders to tumor-targeting antibodies, while those with loss of *FCGR3A* would have reduced responses. However, this hypothesis requires testing using data from appropriate clinical trials.

[H2] Single nucleotide polymorphisms

The nomenclature of the SNPs in *FCGR1*, *FCGR2* and *FCGR3* regarding their amino acid position has led to inconsistencies across different studies when naming each SNP. In this work, we have considered the amino acid position based on the full protein proposed by the Human Genome Variation Society [11, 75].

Five SNPs have been described for the high-affinity *FCGR1A*; three, located at the extracellular (V39I), transmembrane (I301M), and intracellular (I338T) domains, and two that result in stop codons (R92* and Q224*) [76]. The frequency of these SNPs is less than 0.5% in the overall population as estimated from the 1000 Genomes Project Phase 3 [76], and to date, they have not been linked to human pathologies. FcγRI SNPs do not affect binding to monomeric IgG but have been found to reduce binding to IC hIgG1 (V39I) and affect the receptor signaling capacity (V39I, I301M and I338T) [76]. Both hIgG1 and hIgG4 have the potential to bind FcγRI and elicit antibody effector functions, therefore it is possible that therapeutic antibodies of these subclasses would display reduced activity in the small fraction of patients bearing these SNPs. However, these associations have not been formally evaluated.

SNPs in *FCGR2* and *FCGR3* have also been associated with susceptibility to autoimmune diseases and responses to antibodies targeting CD20, HER2, EGFR, and CTLA4 [11]. *FCGR2A* has several known SNPs, with FcγRIIa-R166H (previously known as FcγRIIa-H131R) being the most widely studied as it changes the affinity of IgG for the receptor [77]. FcγRIIa-R166H has an allelic frequency of approximately 44-67% carrying *FCGR2A*[166H] and 33-56% carrying *FCGR2A*[166R] across the European, Chinese, and African populations [78]. Homozygous expression of FcγRIIa-166H results in increased binding to hIgG1 and hIgG2 without changes in its affinity for hIgG3 and hIgG4 [35]. Functionally, the FcγRIIa-166H SNP correlates with increased phagocytosis and degranulation of neutrophils [77, 79]. *FCGR2B* has one well-described SNP, FcγRIIb-I232T that is thought to affect its localization in lipid rafts of the cell membrane, resulting in an enhanced macrophage inflammatory response to immune complexes and opsonized cells [80, 81]. However, recent evidence has questioned the localization of FcγRIIb in lipid rafts, suggesting a structural role for the FcγRIIb-I232T variant in reducing the inhibitory function of the receptor [82]. Although not formally tested, this

polymorphism could enhance the activity of therapeutic antibodies in the context of cancer
due to the reduced functionality of the inhibitory FcγR.

In the case of *FCGR3A*, two SNPs have been described; FcγRIIIa-V176F (previously known
as FcγRIIIa-V158F) [83] and the triallelic FcγRIIIa-66L/R/H (also known as FcγRIIIa-48L/R/H)
[84]. FcγRIIIa-V176F has been identified to have an allelic frequency of approximately 65%
carrying FcγRIIIa-176F and 35% carrying FcγRIIIa-176V across European, Chinese, and
African populations [78]. Homozygous expression of the FcγRIIIa-176V SNP affects the IgG
binding domain, increasing the affinity of the FcγR to all hIgG subclasses [35]. Homozygous
FcγRIIIa-66H carriers have an increased susceptibility to viral infections due to a decreased
association with the co-receptor CD2 [85, 86], while NK cells from patients carrying
homozygous FcγRIIIa-176V have increased killing activity against cells opsonized with a sub-
saturating concentration of rituximab [87]. The homozygous FcγRIIIa-176V has also been
associated with a better response to rituximab in a small cohort of 49 patients with non-
Hodgkin lymphoma [88]. However, a recent study looking at more than two thousand patients
with non-Hodgkin lymphoma and treated with rituximab found no association between *FCGR2*
or *FCGR/3* SNPs and progression-free survival, shedding doubt on the physiological
relevance of these SNPs [89]. In the case of trastuzumab and cetuximab, in a small cohort of
54 patients with breast cancer and 69 patients with colorectal cancer, those homozygous for
FcγRIIIa-176V responded better to treatment, thus supporting the physiological relevance of
this SNP [90, 91]. *FCGR3B* has three common polymorphic variants known as NA1, NA2, and
SH that are the result of 6 SNPs present in its third exon [92]. No differences have been
observed in the binding of NA1, NA2, and SH to IgG1 and IgG3 [35], and no correlations with
the response to therapeutic antibodies have been reported.

While the impact of some of these SNPs in IgG binding *in vitro* is clear, their *in vivo* impact
and physiological relevance remain, at least in part, controversial. It is worth considering that
most studies linking the presence of FCGR SNPs to the clinical activity of tumor-targeting
antibodies focus on the presence of the SNP but fail to consider additional key factors such
as the expression (at the protein level) and cellular distribution of the FcγR variants. Based on
the complexity of the FcγR family, we believe that a more comprehensive approach
incorporating SNP status in addition to FcγR expression and cell distribution inside and outside
the tumor is required to improve our understanding of their physiological and therapeutic
relevance.

[H1] FcγRs and immunomodulatory antibodies

The heterogeneous expression of the FcγRs within tumors, their target density as well as antibody–FcγR interactions driven by the specific IgG subclass (and epitope interactions) add considerable complexity to the design, function, and understanding of therapeutic immunomodulatory antibodies in cancer immunotherapy. With an increasing number of immunomodulatory targets and pharmaceutical companies developing antibodies against such targets, it is common to see multiple antibodies with different IgG subclasses targeting the same molecule or pathways, complicating clinical data interpretation. These factors are further complicated by our limited understanding of the biology of some of these targets, and whether depletion, blockade, or agonism should be the desired outcome. Therefore, the overall expression of all FcγRs, and the A/I ratio, is a critical determinant of what the immunomodulatory antibody will achieve. In this section, we will discuss the most relevant findings in the field of 'blocking' and 'agonistic' immunomodulatory antibodies and how understanding their interaction with FcγRs within the TME could help accelerate and optimize their design for maximal therapeutic gain.

[H2] Antibody engineering

Antibody engineering is a powerful tool for the modulation of FcγR binding, influencing the activity, half-life, and manufacturing of therapeutic antibodies. Point mutations in the Fc [93] and modifications to the glycosylation site at N297 have been used extensively to modify the binding of IgG to complement, FcRn and FcγRs in order to influence their mechanism of action [94].

One of the most common mutations is the S228P substitution used in therapeutic hIgG4 antibodies. Under physiological conditions, hIgG4 can spontaneously swap half molecules with other hIgG4 molecules to become bispecific, contributing to the anti-inflammatory effects of this subclass [95]. The S228P mutation prevents spontaneous Fab-arm exchange and stabilizes the antibody *in* vivo to maintain a bivalent target binding [96]. As alluded to above, another common strategy is to glycoengineer the glycans attached to N297 within the CH2 domain of the Fc [97]. For example, the removal of fucose (known as afucosylation) from the glycan profile at N297 of hIgG1 can increase binding to FcγRIIIa for enhanced effector functions [98]. Conversely, replacing the asparagine in position 297 with alanine (N297A) or glutamine (N297Q), amongst other amino acid changes can eliminate the glycan profile, reducing interactions with FcγRs [99]. The CD20 antibody, obinutuzumab was the first afucosylated hIgG1 that entered the clinic and demonstrated some evidence of superior efficacy compared to the wild-type hIgG1, rituximab [100]. In the field of immune checkpoint

inhibitors, afucosylated CTLA4 [101, 102] and CD25 [103, 104] antibodies are currently in early-phase clinical trials as T_{reg} cell-depleting agents. Recently, avelumab, an afucosylated hIgG1 PDL1 antibody, showed better tumor control in mouse models expressing human FcγRs, suggesting that this modified version could soon enter the clinic [105]. Atezolizumab (anti-PDL1) contains the N297A mutation to reduce FcγR binding further compared to the commonly used hIgG4 antibodies. However, this mutation does not completely abrogate FcγR binding [106], and has been shown to increase the propensity of antibody aggregation during manufacturing [107], which may lead to the generation of anti-drug antibodies (ADAs) in patients, reducing clinical efficacy [108].

Beyond these, many mutations and their combinations have been evaluated in the generation of clinical-stage antibodies [94]. Durvalumab, another FDA-approved PDL1 antibody, contains three point mutations to significantly reduce FcγR binding (Table 1). Tislelizumab, a novel hIgG4 anti-programmed cell death protein 1 (PD1), contains, in addition to S228P, five mutations aimed at reducing FcγR binding [109] (Table 1). Beyond mutations in the Fc and changes in the glycosylation of therapeutic antibodies, novel strategies are being investigated. One example is mutations in the hinge region of hIgG2 antibodies that increase their capacity to deliver agonistic signals [110]. Whether these and others will be taken to the clinic successfully remains to be seen as several barriers, such as effective manufacturing and full elucidation of mechanisms of action, remain to be solved.

[H2] Targeting inhibitory immunomodulatory receptors

[H3] CTLA4. CTLA4 outcompetes CD28 for its binding to CD80 and CD86 on antigen-presenting cells, acting as a negative regulator of T cell activation [111]. Antibodies blocking CTLA-4 on activated effector T (T_{eff}) cells allow CD28 to interact with CD80 and CD86 triggering anti-tumor immunity [112] (Figure 3a). CTLA4 was originally described to be expressed by activated T_{eff} cells [111]. Today, it is accepted that CTLA4 is expressed preferentially and at the highest levels within mouse and human tumors by T_{reg} cells rather than on CD4$^+$ and CD8$^+$ T_{eff} cells [45]. In mice, there is compelling evidence that CTLA4 antibodies that engage activating FcγRs, preferentially deplete T_{reg} cells within the TME [53, 113, 114] through interactions with FcγRIV on tumor-associated macrophages (TAMs) [53]. T_{reg} cell depletion was shown to be the main driver of the increase in the T_{eff} to T_{reg} cell ratio in mouse melanoma models and was critical for tumor rejection and long-term survival [53, 113, 115, 116]. More recently, a study evaluating (at the single cell level) the impact of depleting and blocking CTLA-4 antibodies on the adaptive and innate immune compartment within tumors confirmed that only the depleting mIgG2a subclass was able to promote T_{reg} cell

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depletion and tumor control [117]. Most importantly, this single-cell RNA sequencing analysis of tumor-infiltrating immune cells revealed that in addition to promoting T_{reg} cell depletion, the depleting mIgG2a subclass could promote direct activation of the myeloid compartment in tumors through the engagement of activating FcγRs [117]. Strikingly, the combination of T_{reg} cell depletion and FcγR signaling appeared to reprogram the tumor-infiltrating myeloid compartment, reducing its immunosuppressive nature. Whether the CTLA4 antibodies in the clinic can contribute to myeloid reprogramming and activation remains to be demonstrated. Still, the data from mouse models support the value of FcγR engagement in innate cell reprogramming within the TME and the need for further research to develop novel therapeutics aimed at promoting this feature.

The FDA has approved two CTLA4 antibodies, ipilimumab (hIgG1) and tremelimumab (hIgG2) (Table 1) [118, 119] developed and selected based on their ability to block CTLA4 binding to CD80 and CD86. Despite being developed as blocking agents, these antibody subclasses are likely to interact with the FcγRs within the TME, thus impacting their *in vivo* activity (Figure 3a). Both hIgG1 and hIgG2 can engage activating FcγRs (although hIgG2 with a lower affinity than hIgG1 (Figure 1), reducing its depleting capacity), potentially driving antibody effector functions and leading to the depletion of CTLA4-expressing cells [45]. The presence of heterozygous and homozygous FcγRIIIa-176V in patients with melanoma, whose tumors were infiltrated with immune cells has been correlated with a better response to treatment with ipilimumab [45]. Supporting these findings, *in vitro* studies demonstrated the ability of ipilimumab to bind to FcγRIIIa on non-classical monocytes and promote T_{reg} cell depletion [120]. In the same study, higher infiltration of tumors by these monocytes correlated with better response to ipilimumab in patients with melanoma [120]. A reduction in frequency of T_{reg} cells has been documented in patients with bladder cancer [121] and in the lymph nodes of patients with early-stage melanoma [122] following ipilimumab and tremelimumab treatment, respectively. In a recent clinical trial, ipilimumab treatment prior to nivolumab (anti-PD1) in patients with melanoma correlated with better survival, which was suggested to be driven by the depletion of intratumoral T_{reg} cells [123]. In contrast, a clinical trial that evaluated the impact of ipilimumab or tremelimumab on T_{reg} cell number and fraction in patients with melanoma, prostate cancer, and bladder cancer concluded that T_{reg} cells are not depleted following treatment with either of these antibodies [124]. However, factors such as the timing of sample collection and the small cohort of patients evaluated have been proposed to impact the conclusions of this work [125, 126]. Nevertheless, there is an ongoing controversy in the field and a need to better understand the individual impact of clinical CTLA4 antibodies on T_{eff} cells, T_{reg} cells, and the rest of the TME. Understanding this may be key to further improvements or the development of novel, more potent CTLA4 antibodies.

Despite the ongoing debate about the mechanism of action of CTLA4 antibodies in humans, multiple new agents are being developed to promote effective intra-tumoral T_{reg} cell depletion. As mentioned above, these include afucosylated versions of CTLA4 antibodies currently being evaluated in clinical trials [102]. In addition, antibodies targeting other receptors preferentially expressed on T_{reg} cells and not expressed (or expressed at very low levels) on tumor-infiltrating T_{eff} cells are also in clinical evaluation, including C-C chemokine receptor 8 (CCR8) antibodies [127-132], and a novel CD25 antibody that targets CD25 on T_{reg} cells without interfering with interleukin 2 (IL-2) signaling on T_{eff} cells [103, 104]. While there is a growing pipeline of new antibodies targeting T_{reg} cells in cancer, it remains unclear whether T_{reg} cell depletion can be effectively achieved within human tumors. Engaging ADCC and/or ADCP in solid tumors is likely to require, in addition to a selective target (such as CTLA4, CCR8, and CD25), an innate immune effector compartment able to mediate effector function. Limited tumor infiltration by NK cells, low levels of activating FcγRs (and/or high expression of the inhibitory FcγRIIb), and expression of other inhibitory receptors on myeloid cells preventing phagocytosis (i.e. signal-regulatory protein α (SIRPα) [133], sialic acid-binding Ig-like lectin 10 (Siglec-10) [134], PD1 [135], leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1) [136]) are potential barriers to the efficacy of these new therapeutics in patients and need to be evaluated in new trials incorporating T_{reg} cell depleting agents.

[H3] PD1. PD1 is an inhibitory receptor upregulated upon T cell receptor (TCR) engagement and expressed at high levels on the surface of tumor-reactive T_{eff} cells [137]. Engagement of PD1 by its ligand PDL1, expressed on tumor and antigen-presenting cells (APCs), results in the inhibition of T cell activation [138] (Figure 3b). In mice, using PD1 antibodies with a depleting mIgG2a subclass led to poor tumor control compared to PD1 antibodies using mIgG1 or Fc-null mIgG1 [55, 139]. This effect correlated with the loss of CD8+ T_{eff} cells within the tumor that was dependent on mFcγRI, highlighting the relevance of using an IgG subclass that does not engage activating FcγRs for maximal anti-tumor activity *in vivo* [55]. Interestingly, in the study from Dahan et al. [55], both rat IgG2a and mouse IgG1 anti-PD1 (with low activating FcγR binding capacity) were negatively modulated by mFcγRII when compared to Fc-null mIgG1 anti-PD1. This effect could be attributed to unintended PD1 agonism, or removal of the antibody from PD1-expressing cells by FcγR+ myeloid cells [55, 139, 140]. In humanized FcγR mouse models, the optimal IgG subclass is less clear. In an OT-1 immunization model, an hIgG4 Fc-null anti-PD1 significantly enhanced the ovalbumin-specific T cell response compared to wild-type hIgG4 [139]. However, in a tumor setting, both hIgG4 and hIgG1 Fc-null anti-PD1 showed similar anti-tumor activity [55].

The FDA has approved several antibodies targeting PD1, which block its interaction with PDL1 (Table 1), using the hIgG4 subclass engineered with the S228P mutation (Figure 3b). Of interest, tislelizumab differs from the other FDA-approved PD1 antibodies as it has been engineered with a set of mutations in its constant region to decrease FcγR binding (Table 1), potentially preventing deleterious effects of Fc–FcγR interactions (Figure 3b). Several studies have suggested a negative impact of FcγR binding to anti-PD1 hIgG4, including capturing of ant-PD1 antibodies by FcγR-expressing TAMs [140] and depletion of the PD1^{hi} tumor reactive T cells that have the capacity to kill tumor cells [139]. In vitro studies using the F(ab)$_2$ of tislelizumab with a hIgG4 backbone demonstrated depletion of activated PD1$^+$ T cells, thus further supporting the development of the Fc-null hIgG4 [109]. The *in vivo* interaction between FcγRs and PD1 antibodies leading to the depletion of PD1^{hi} T cells could help explain clinical observations such as hyperprogression following anti-PD1 treatment (see Box 2). Upcoming clinical data will help expand our understanding of the mechanisms of action of PD1 antibodies and the impact of FcγR expression on their activity. Elucidating the correct subclass (and mutations) for optimal efficacy based on mouse studies remains to be fully determined. Nevertheless, based on these considerations and in agreement with previous work [44, 55], we believe that maximal activity of anti-PD1 antibodies will most likely be achieved by 'pure' PD1-blocking agents lacking binding to FcγRs.

[H3] PDL1. PDL1 is the main ligand of PD1, and the PDL1–PD1 axis is a major controller of central and peripheral immune tolerance [141, 142]. Within the TME, PDL1 can be expressed by tumor cells, the myeloid compartment, and activated T$_{eff}$ cells [143]. Whether the activity of PDL1 antibodies is dependent on the expression of PDL1 on the tumor cells or on immune cells remains a matter of active research [144, 145]. When comparing PDL1 antibodies with the same F(ab)$_2$ but with a mIgG1 or a mIgG2a backbone, only the depleting mIgG2a subclass delivered significant tumor control compared to untreated mice [55]. Tumor control correlated with a reduction in the frequency of PDL1$^+$ monocytes, likely due to direct depletion, suggesting that depleting subclasses may be superior in vivo over non-depleting PDL1 antibodies [55]. Importantly, the dependence of PDL1 antibodies on FcγR engagement in mice varies depending on the models and the mouse strain used [146]. More recently, in a humanized mouse model, glycoengineering of PDL1 antibodies to increase their binding to FcγRIIIa increases anti-tumor activity and this could be further enhanced in combination with an anti-FcγRIIb blocking antibody [105]. These data highlight the complexity of FcγR interactions with immunomodulatory antibodies targeting PDL1 and underscore the need to extend these types of studies to human samples to improve the design and use of these agents.

There are currently three FDA-approved PDL1 antibodies, atezolizumab, durvalumab, and avelumab, with differing FcγR-binding properties. Unlike atezolizumab and durvalumab, which have an Fc-null hIgG1 subclass, avelumab is a wild-type hIgG1 with intact FcγR binding [143, 147, 148] (Figure 3c). Avelumab has been shown to mediate ADCC of allogeneic cancer cell lines following engagement of hFcγRIIIa, like other tumor-targeting hIgG1 antibodies [148-150]. It might be predicted that eliminating PDL1+ cells would lead to better tumor control due to expression on tumor cells and potentially suppressive myeloid cells [143] (Figure 3c). However, as T_{eff} cells and APCs also express PDL1 in the TME [143], it is possible that avelumab would deplete these populations as well *in vivo*, leading to sub-optimal anti-tumor activity. More recently, survival benefit from avelumab was associated with the presence of two or more high-affinity alleles of the SNPs in *FCGR2A* and *FCGR3A* in advanced urothelial cancer [151]. The same study found that patients with tumors enriched in NK cells and macrophages had higher overall survival, suggesting that these cells were key for antibody effector functions following avelumab treatment [151]. Future studies evaluating the activity of avelumab versus Fc-null PDL1 antibodies will provide some clues as to the clinical value of PDL1 antibodies with a depleting subclass. Most likely, the final *in vivo* activity of anti-PDL1 agents will depend on the distribution and level of expression of PDL1 in different immune cells and across different TMEs.

[H3] LAG3 and TIGIT. Lymphocyte activation gene 3 (LAG3) and T cell immunoreceptor with Ig and ITIM domains (TIGIT) are co-inhibitory receptors present on T_{eff} cells, associated with T cell exhaustion and reduced tumor-killing activity [152], but are also expressed on tumor-infiltrating T_{reg} cells [153, 154]. Initial studies in mouse tumor models showed that LAG3 (rat IgG1, non-depleting) and TIGIT (mIgG2a, depleting) antibodies lacked efficacy as single agents but synergized with PD1 or PDL1 antibodies [155, 156]. In mouse models, TIGIT antibodies using a depleting subclass (mIgG2a), have shown that T_{reg} cell depletion occurs in some cases [157], while not in others [158]. In addition, FcγR engagement and myeloid cell activation following anti-TIGIT mIgG2a have been proposed as an additional mechanism of action [159].

The FDA has recently approved relatlimab, a LAG3 antibody (hIgG4^{S228P}), in combination with anti-PD1 (nivolumab) for the treatment of advanced melanoma [160, 161]. Like most PD1 antibodies, relatlimab was developed to block the inhibitory activity of LAG3 without eliciting antibody effector functions, potentially explaining the choice of a hIgG4. However, no studies have systematically evaluated the impact of FcγR engagement for this antibody.

Tiragolumab, an anti-TIGIT hIgG1, has recently received breakthrough status from the FDA for treating NSCLC in combination with the PDL1 antibody atezolizumab [162, 163]. Although tiragolumab was initially developed as an immune checkpoint inhibitor, its hIgG1 backbone raises questions regarding its potential depleting activity against TIGIT$^+$ T$_{eff}$ cells and T$_{reg}$ cells within the TME. Results presented in a recent poster presentation described CD4$^+$ and CD8$^+$ T$_{eff}$ cell and NK cell activation, T$_{reg}$ cell depletion, and concomitant myeloid cell activation in tiragolumab-treated patients [164]. In keeping, another TIGIT blocking antibody currently in phase I/II clinical trials (EOS-448, hIgG1) [165], was also able to promote T$_{reg}$ cell depletion *in vitro* [158]. Consistent with what was originally described for CTLA4 antibodies [45, 53], preferential depletion of T$_{reg}$ cells over T$_{eff}$ cells correlated with a higher number of TIGIT molecules on the surface of T$_{reg}$ cells [158].

These results suggest that antibodies against TIGIT may work within a 'sweet spot' microenvironment where, in addition to their blocking activity, high target antigen density drives depletion (such as seen for T$_{reg}$ cells), while low target antigen density preferentially promotes target blockade (such as on T$_{eff}$ cells). However, a key point to consider is that the heterogeneity of TIGIT expression across cell types and tumor indications will likely impact upon and confound the interpretation of the *in vivo* activity of agents targeting this receptor with a depleting hIgG1 subclass. Whether, in addition to their blocking activity, an optimal (hIgG1) or a sub-optimal (hIgG4) depleting subclass is the most suitable format for TIGIT and LAG3 antibodies remain unknown.

[H2] Targeting activating immunomodulatory receptors.

The immuno-oncology field has actively pursued the development of antibodies that directly deliver activating signals into effector immune cells. These 'agonistic' antibodies target co-activating receptors of the B7 immunoglobulin superfamily (B7 IgSF) such as CD28 and inducible T cell co-stimulator (ICOS) or TNFRSF members such as OX40, 4-1BB, GITR, CD40 and CD27. Unfortunately, strong positive clinical outcomes remain elusive, highlighting a considerable gap in our understanding of the mechanism of action of agonistic antibodies *in vivo*.

Unlike immune checkpoint-blocking antibodies that aim to bind to their target, out-competing the natural ligand, the rules for the design of agonistic antibodies are still being written. The target epitope, affinity [166], and the ability to promote receptor supercluster formation (crosslinking) all contribute to the activity of agonistic antibodies [167]. Data from mouse models and humans underscores a critical role for FcγRIIb (FcγRII in mice) as a mediator of target crosslinking, acting as a scaffold to cluster the antibody–receptor complex and deliver

a positive signal as demonstrated for CD40 [30, 31], OX40 [32], 4-1BB [33], and CD28 [29, 168]. The data suggest that to deliver an agonistic signal into T cells, an antibody subclass like hIgG2 or hIgG4 with low binding affinity to activating FcγRs and a higher affinity for FcγRIIb may be the better choice. Interestingly, hIgG2 has been shown to deliver target-mediated agonism independently of any FcγR [169]. Agonistic activity is driven by the naturally occurring structural rearrangement of the disulfide bonds of the hIgG2 hinge region under physiological conditions from a flexible structure (hIgG2-A) into a more rigid structure (hIgG2-B) [110]. The arrangement of the hIgG2-B hinge results in an F(ab) that can adopt fewer structural conformations, promoting increased receptor crosslinking independent of the FcγR binding [170]. A recent study has provided some further insight into optimizing agonistic antibodies. The agonistic potential of both CD40 and 4-1BB hIgG2-B antibodies can be improved by decreasing the affinity of the F(ab) for the target, increasing receptor clustering, and enhancing downstream signaling through a combination of antibody rigidity and fast target off-rate [166]. Therefore, it could be hypothesized that an optimal agonistic antibody will have a relatively low affinity for its target [166], and a hIgG2-B subclass, including mutations to abrogate FcγR binding to deliver an agonistic signal into target cells, independent of FcγR engagement.

Beyond the intrinsic complexities of delivering an agonistic signal into T_{eff} cells, an additional challenge in cancer is the high levels of expression of B7 IgSFs and TNFRSFs on tumor infiltrating T_{reg} cells, which may promote activation of these cells in response to agonistic antibodies. However, this remains speculative, and there is still no clear understanding of how delivering agonistic signals into T_{reg} cells may affect their function. Indeed, studies evaluating the activity of agonistic anti-GITR and anti-OX40 suggest these agents promote T_{reg} cell inactivation *in vivo* [171, 172]. Below, we briefly summarize some of the receptors targeted with agonistic antibodies and how the antibody subclass may influence their agonistic potential.

[H3] CD28. CD28 is expressed in nearly all human peripheral CD4+ T_{eff} cells and around half of the CD8+ T cells [173]. CD28 is critical for T cell activation following initial TCR ligation by peptide–major histocompatibility complex (MHC), promoting proliferation and survival [174]. In mice, CD28 super-agonistic antibodies promoted the expansion of T_{reg} cells without affecting T_{eff} cells, with potential benefits for autoimmunity [175, 176], and treatment of B-cell chronic lymphocytic leukemia [177]. Theralizumab (also known as TGN-1412) was the first CD28 antibody tested in the clinic, designed with a wild-type hIgG4 subclass (lacking the S228P mutation) to act as a super-agonistic antibody targeting human CD28 independently of TCR engagement [178]. Importantly, the administration of theralizumab to healthy volunteers in phase I clinical trials resulted in a cytokine release syndrome that led to severe inflammatory

reactions and chronic organ failure [179]. Further *in vitro* studies suggested that hFcγRIIb hyper-crosslinking of theralizumab enhanced CD28 clustering and increased cytokine production by CD4$^+$ T$_{eff}$ cells, driving at least part of its toxicity, a characteristic not observed in pre-clinical studies [168]. Additionally, this antibody had a wild-type IgG4 without the S228P
595   mutation, suggesting it could have undergone Fab-arm exchange *in vivo*, potentially adding to its toxic effects [180]. Theralizumab was later bought by a new company that initiated clinical trials for its use in autoimmunity [181], and solid tumors [182]. More recently, CD28 agonism has been revisited as a target for the treatment of cancer. Bi-specific antibodies that target CD28 and a tumor-associated antigen aim to restrict CD28 activation to tumor-associated T$_{eff}$
600   cells. Pre-clinical studies have shown promising results without evidence of toxicity [183, 184], and this molecule has progressed into clinical trials [185].

[H3] ICOS. Structurally and functionally related to CD28, ICOS is a costimulatory receptor whose expression is induced on T cells following their activation [186] (Figure 4a). In tumors, ICOS is expressed primarily on T$_{reg}$ cells, with lower levels on T$_{eff}$ cells [45]. ICOS–ICOS ligand
605   (ICOSL) interactions are critical for the anti-tumor activity of CTLA4 antibodies, as shown in ICOS$^{-/-}$ mice, where the therapeutic effect of anti-CTLA4 is ablated [187]. Several ICOS antibodies with either a hIgG1 or hIgG4 backbone have been evaluated in the clinic (Table 1 and Figure 4a). The rationale for targeting ICOS arises primarily from clinical observations correlating ICOS expression on T$_{eff}$ cells with positive clinical outcomes in patients treated with
610   anti-CTLA4 [121] and pre-clinical work demonstrating that delivery of ICOS signals via overexpression of ICOSL in tumor cell-based vaccines synergize with CTLA4 antibodies to promote tumor control [188]. Two schools of thought have driven the development of human ICOS antibodies; one favored the development of anti-ICOS with a depleting hIgG1 (vopratelimab) to promote depletion of ICOS^{hi} T$_{reg}$ cells and activation (through receptor
615   crosslinking) of T$_{eff}$ cells expressing lower levels of ICOS on their surface [189, 190]. The second was more conservative and favored the development of anti-ICOS with a hIgG4 to agonize ICOS and reduce the chances of depleting activated ICOS^{hi} T$_{eff}$ cells (feladilimab) [191, 192] (Figure 4a). However, neither of these antibodies, has delivered significant clinical impact and it is not fully understood whether these molecules block the natural interaction of
620   ICOS and ICOSL, which, as mentioned above, is critical for anti-tumor immunity [187]. ICOS remains a target of interest, and the initial data in combining anti-CTLA4 with ICOSL expressing vaccines supports that view. A deeper understanding of the agonistic activity of the immunomodulatory antibodies targeting ICOS *in vivo* is needed, as well as a systematic characterization of the role and impact of FcγR and target density in their function.

625 **[H3] CD40.** Part of the TNFRSF, CD40 is a type I membrane protein expressed on APCs that interacts with the trimeric CD40 ligand (CD40L; also known as CD154), primarily found on activated CD4^{+} T cells [193]. CD40 activation enhances the immune response through dendritic cell licensing [194], as well as augmenting antigen cross-presentation [195] and enhancing germinal center **[G]** formation [196] (Figure 4b). Pre-clinical studies have proved

630 promising in the use of CD40 antibodies for anti-tumor immunity when using antibody subclasses that favor binding to the inhibitory FcγR. This is because the receptor can scaffold and hyper-crosslink anti-CD40 IgG when bound to its target, mimicking the binding of the trimeric CD40L and delivering activating signals to the recipient cell [30, 31]. Activating FcγRs can also scaffold antibodies but are more likely to result in depletion of the CD40^{+} cells [31].

635 To further enhance the efficacy of these antibodies, a number of different strategies have been explored. Antibody engineering has been used to increase the specific affinity for FcγRIIb, such as by using a set of mutations known as V11 on the hIgG1 subclass [197] (Figure 4b). Alternatively, FcγR independent agonism has been investigated using the hIgG2 subclass, which can promote CD40 agonism without binding to FcγRIIb [110]. Amongst the many CD40

640 antibodies that have reached clinical trials [198] (Table 1), hIgG1 with enhanced FcγRIIb binding [199-203], and hIgG2 antibodies [204-214], which do not need FcγRIIb [215, 216], are perhaps the most likely to elicit optimal therapeutic potential. However, despite their efforts, most clinical trials using CD40 antibodies have shown limited efficacy and liver toxicities as well as cytokine release syndrome (CRS, higher levels of IL-6 and TNF-alpha in plasma)

645 associated with the treatment [198, 217]. Intratumoral delivery of CD40 antibodies [218], and bispecific antibodies **[G]** targeting CD40 and a tumor-associated antigen [219], or a dendritic cell marker [220], have been developed to overcome these limitations. These recent developments could help bring the next generation of CD40 antibodies to the clinic and provide a blueprint for the progress of other agonistic antibodies for patients.

650 **[H3] OX40.** Human CD4^{+} and CD8^{+} T_{eff} cells transiently express OX40 following TCR stimulation [221] (Figure 5a). In human tumors, OX40 is most highly expressed on T_{reg} cells, with high levels also on CD4^{+} T_{eff} cells and little expression on CD8^{+} T_{eff} cells [45]. Several studies in mice have validated the use of agonistic antibodies targeting OX40 to promote anti-tumor immunity [222, 223]. Interestingly, both depleting (mIgG2a) and non-depleting (mIgG1)

655 antibody subclasses have shown a significant survival benefit, suggesting that depletion of OX40^{+} T_{reg} cells and receptor agonism are important for their mechanism of action [32]. Adding to this, the combination of OX40 and 4-1BB antibodies showed synergistic effects in mouse models driven by CD8^{+} and CD4^{+} T_{eff} cells that oligoclonally expand within the TME [224]. Human OX40 antibodies have been tested in the clinic using a hIgG1 or hIgG2 subclass

660 (Figure 5, Table 1). Ivuxolimab (also known as PF-04518600), the only hIgG2, was tested as

a monotherapy in a phase I trial and was found to promote the clonal expansion of CD8$^+$ T$_{eff}$ cells in the blood, with a partial response in 6% of patients with locally advanced or metastatic cancer [225, 226]. In the same study, ivuxolimab was combined with a 4-1BB antibody (utomilumab), showing some efficacy in a small number of patients with advanced solid cancer [227]. hIgG1 OX40 antibodies have also been tested in early-phase clinical trials (Table 1). Cudarolimab (also known as IBI101) [228, 229], and tavolimab were associated with an expansion of CD4$^+$ and CD8$^+$ T$_{eff}$ cells and in the case of the latter, with T$_{reg}$ cell depletion within the TME [230], despite lack of clinical activity. These studies have demonstrated that OX40 agonism can modulate T cell function within human tumors but highlights that further insight is needed to understand the factors limiting tumor control.

[H3] 4-1BB. 4-1BB is expressed on activated and memory CD4$^+$ and CD8$^+$ T$_{eff}$ cells, and its ligation via 4-1BB ligand (4-1BBL) drives activation and proliferation of these cells [231, 232] (Figure 5b). In mice, 4-1BB antibodies can activate CD8$^+$ T cells and NK cells, promoting tumor rejection [233, 234]. In addition, 4-1BB is a downstream target of forkhead box protein P3 (FOXP3) with high expression on resting T$_{reg}$ cells and further upregulation on activated T$_{reg}$ cells [235]. In mice, only 4-1BB antibodies with a depleting mIgG2a subclass drive T$_{reg}$ cell depletion with concomitant tumor reduction [33]. Interestingly, an anti-4-1BB mIgG2a designed to additionally hyper-crosslink the receptor through hinge engineering led to T$_{reg}$ cell depletion and T$_{eff}$ cell activation resulting in better tumor control than the wild-type mIgG2a [33]. Two main 4-1BB antibodies have been evaluated in the clinic (Table 1), urelumab (hIgG4) [236-244] and utomilumab (hIgG2) [245-251] (Figure 5b). Similar to ICOS and OX40, these antibodies have shown limited responses in clinical trials. Urelumab and utomilumab have shown increased expression of activation markers on circulating NK, CD4$^+$, and CD8$^+$ T$_{eff}$ cells as well as dendritic cells [252, 253]. However, urelumab displayed a poor safety profile due to its considerable liver toxicity [254], while utomilumab had a more acceptable safety profile [255]. Urelumab toxicity has been attributed to supraphysiological signaling via 4-1BB driven by hIgG4 binding to FcγRIIb on liver sinusoidal endothelial cells and/or Kupffer cells [256, 257]. In addition, agonistic signaling via 4-1BB on liver myeloid cells is thought to induce IL-27 release, potentially contributing to the toxicity of urelumab [258]. The more favorable safety profile of utomilumab [259, 260], may relate partly to the fact that utomilumab is less agonistic than urelumab [261], and its hIgG2 subclass does not bind to FcγRIIb (Figure 1). A potential human 4-1BB antibody development strategy could be to decrease the F(ab) affinity of utomilumab or urelumab whilst engineering a hIgG2-B subclass to drive 4-1BB agonism independent of FcγRs [166].

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[H1] Concluding remarks.

Antibodies targeting tumors and immunomodulatory receptors have proven relevant in the fight against cancer. In the last decade, there has been an increased acceptance of the role that FcγRs play in the mechanism of action of these therapeutics. Despite the progress, there are still gaps in our understanding and a lack of cohesiveness in therapeutic approaches, as evidenced by antibodies targeting the same receptors using different IgG subclasses. A systematic evaluation of FcγR biology, polymorphisms, FcγR-expressing innate immune cells, and a critical understanding of the target are needed to optimize clinical success. We expect that an improved preclinical evaluation of therapeutic antibodies will come from studies using a mixture of mouse models expressing human FcγRs, together with explants derived from human tumors and immune-proficient patient-derived xenografts (immune-PDX; see Box 1) [262]. This is the case with PD1 [55, 263], PDL1 [55, 262], and CD25 antibodies [103], for which a better understanding of their mechanism of action has come from using cutting-edge humanized mouse models as well as human tissues. In the clinical context, a greater understanding of the rules of FcγR engagement in humans is required and trials are needed to collect and analyze tumors using multiple techniques, such as immunohistochemistry, whole exon sequencing, and whole transcriptome sequencing. A good example is the JAVELIN Bladder 100 trial that not only examined the presence of polymorphisms across FcγRs but also the frequencies of NK cells and macrophages in the context of anti-PDL1 treatment (avelumab, hIgG1) of bladder cancer [151]. This study combined multiple datasets with the use of cutting-edge bioinformatic tools that correlated the efficacy of avelumab with CD8$^+$ T cell infiltration, as well as FcγR-expressing NK and myeloid cells amongst other parameters. We believe that these factors will become paramount as we try to understand the complexities of new antibody-like modalities, such as bi-specifics and antibody–drug conjugates, to ensure successful clinical translation of their therapeutic potential.

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Glossary

- **IgG subclass** – Defined by the differences in the length of the hinge and number of disulfide bonds, the subclass affects the binding affinity to different FcγRs and influences the potency of IgG effector functions

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- **Valency –** The number of binding sites present on the antibody to enable binding to the antigen. IgG typically has a valency of two (bivalent).

- **Surface plasmon resonance (SPR) –** an optical technique used to measure molecular interactions. The refractive index of polarised light changes upon the binding of the analyte to the ligand, producing a sensorgram which allows the

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interpretation of the binding kinetics of two molecules.

- **Bispecific antibodies –** antibodies with two distinct binding sites directed against two different antigens.

- **Tumor targeting antibodies** – Therapeutic IgG antibodies designed to bind specifically to a tumor cell and elicit destruction of the cell

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- **Immunomodulatory antibodies** – Therapeutic IgG antibodies designed to bind to immune cells, to either block an inhibitory signal or agonize an activating receptor and enhance the immune response

- **Antibody–drug conjugates –** antibodies chemically tagged to a cytotoxic drug that are designed to bind to a specific antigen and release the drug to the target cell.

1480
- **Anti-drug antibodies (ADAs) –** the immunogenicity of a drug can provoke the immune system into generating an adaptive immune response against said drug. This can result in antibodies that can neutralise the drug's mechanism of action.

- **Anaphylaxis –** a rare but severe allergic reaction that is caused by the systemic release of inflammatory mediators and cytokines resulting in non-specific immune

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activation.

- **Antibody dependent cellular cytotoxicity** - (ADCC). Engagement of activating FcγRs can result in the release of cytotoxic granules to kill the target cell. Predominantly mediated by NK cells, granulocytes and myeloid cells

- **Antibody dependent cellular phagocytosis** - (ADCP). Engagement of activating

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FcγRs can result in the phagocytosis of opsonized target cells, resulting in their destruction. Predominantly mediated by myeloid cells and granulocytes

- **Germinal center –** a structure present in lymphoid organs where activated B cells diversify their immunoglobulin genes by somatic hypermutation to generate high affinity antibodies.

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- **Kupffer cells –** resident liver macrophage cells that maintain liver function and act as first line defence of the innate immune system.

- **Copy number variable regions** - (CNRs). When a cluster of two or more genes have deletions or duplications of the gene loci altering protein expression
- **Copy number variations –** when a specific segment of DNA is repeated within the genome of an individual and shows variation in the number of repeats across a population.
- **Single nucleotide polymorphisms** - (SNPs). When a single nucleotide change within a protein sequence (present in >1% population) affects the protein function and potentially patient phenotype
- **Pseudogene –** non-functional segments of DNA that resemble functional genes containing coding deficiencies such as frameshift mutations or premature stop codons.
- **Patient derived explants** - (PDE). An *ex vivo* system designed to maintain fragments of human tumors (and the accompanying microenvironment) for experimentation and therapeutic testing
- **Immune proficient patient-derived xenografts – (**Immune-PDX**).** – An in vivo system utilising immunocompromised mice to engraft fragments of a human tumor (and the accompanying microenvironment) for experimentation and therapeutic testing.
- **Assembloids** - Organoid-like culture that contains the tumor and associated microenvironment, maintained in a 3D structure *ex vivo*

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Author contributions

1560 F.G-C., A.S., C.C., I.M., D.Q. and K.L. researched data for the article. F.G-C., A.S., C.C., I.M., K.S.P., K.L. and S.A.Q. contributed substantially to discussion of the content. F.G-C., A.S., and S.A.Q. wrote the article. F.G-C., A.S., K.S.P. and S.A.Q. reviewed and/or edited the manuscript before submission.

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Competing interests

The authors declare no competing interests.

Table of Contents Summary

A number of immunomodulatory antibodies for cancer treatment have been developed following the discovery of negative regulators of anti-tumour immunity, such as PD1 and CTLA4. The efficacy of these antibodies is determined not only by their ability to block or engage their target but also by their interactions with Fcγ receptors. This Review outlines our current knowledge of these interactions and discusses how we can use this knowledge to generate more effective cancer immunotherapies in the future.

[H1] Data availability and methodology

The scRNA-seq analysis in Figure 2 used publicly available data from the following sources: GSE121638 [264], GSE131907 [265], GSE127465 [266]. GSE178341 [267], GSE148071 [268], SRZ190804 [269]. (https://trace.ncbi.nlm.nih.gov/Traces/index.html?view=analysis&acc=SRZ190804), (https://github.com/czbiohub/scell_lung_adenocarcinoma), (http://blueprint.lambrechtslab.org), Braun et. al. [270], Bi et. al. [271] (https://singlecell.broadinstitute.org/single_cell/study/SCP1288/tumor-and-immune-reprogramming-during-immunotherapy-in-advanced-renal-cell-carcinoma#study-summary), Chan et al (https://data.humantumoratlas.org/) [272], and Cheng et. al.[273] (data accessed via a request to corresponding author).

For scRNA-seq analysis, raw counts matrices from each study were merged and analyzed by the Seurat package (v4.0.6). Low quality cells were filtered out for cell barcodes with <200, or >6000 unique molecular identifiers (UMIs), <300 or >5000000 expressed genes and percent of mitochondrial gene expression >20%. The remaining cells were normalized using the SCTransform function from the Seurat package. Dimensional reduction by CCA (canonical correlation analysis) was performed to find anchors with 3000 genes which would be further used by the IntegrateData function to correct for batch effects. Principal components analysis (PCA) was conducted on the integration-transformed expression matrix and the top 30 PCs were used for graph-based cluster and subsequent dimensionality reduction using Uniform Manifold Approximation and Projection (UMAP). Clusters were calculated by the FindClusters function with a resolution between 0-1, and a final resolution of 0.4 was selected by elbow plot.

Figure legends.

1605 **Figure 1: Human Fcγ receptors, their expression pattern, and affinity for therapeutic IgG1, IgG2 and IgG4.** Fc γ receptors elicit the action of immunoglobulin (IgG) antibodies. They are broadly considered as activating, signaling via an associated immunoreceptor tyrosine-based activation motif (ITAM) (FcγRI, FcγRIIa, FcγRIIc, and FcγRIIIa), inhibitory, signaling via an associated immunoreceptor tyrosine-based inhibitory motif (ITIM) (FcγRIIb)

1610 or have no direct signaling capacity (FcγRIIIb). FcγRI is high affinity, binding monomeric and immune-complexed (IC) IgG; the remaining FcγRs are considered low affinity, binding only IC IgG. Their affinity for IgG was measured using surface plasmon resonance (K_A) and ability to bind IC determined by flow cytometry. These receptors are broadly expressed across innate immune cells in both the periphery and tissues. Human IgG3 is not a common subclass used

1615 in the clinic and therefore, is not shown in this figure. For the Affinity for therapeutic IgG, symbols '+/-', indicate the level of binding to IC, '-' = no binding and '+' = binding (+++ > ++ > +). For the Effector cell expression, Symbols '+/-', indicate the level of expression on the different immune cell subsets, '+/-' = heterogenous expression and '+' = consistent expression (+++++ > ++++ > +++ > ++ > +). Ab, antibody; ADCC, antibody-dependent cellular cytotoxicity;

1620 ADCP, antibody-dependent cellular phagocytosis; BM, bone marrow; class, classical; CTLA4, cytotoxic T lymphocyte-associated antigen 4; HER2, human epidermal growth factor receptor 2; MO, monocytes; ND = not determined; NK, natural killer; non-class, non-classical; SNP, single nucleotide polymorphism; TRM, tissue-resident macrophages. References for the affinity or association constant K_A [35]; immune complex binding for human FcγRs [39]; allelic

1625 frequency of FcγRIIa and FcγRIIIa [78]; FcγR expression in the periphery [67] and FcγR expression in tissues [68].

Figure 2. Fcγ receptor mRNA expression in key immune subsets from patients with non-small-cell lung cancer, renal cell carcinoma and colorectal cancer. The expression of Fcγ receptor (FCGR) mRNA across key immune subsets from patients with non-small-cell lung cancer, renal cell carcinoma, and colorectal cancer was extracted from publicly available single-cell RNA sequencing (scRNA-seq) datasets. The relative expression of FCGR mRNA was assessed in matched blood (B), normal tissue (N), and tumor (T) from three different tumor types to compare the changes in the expression of FcγRs across key immune subsets. Expression data was normalized within samples to correct for gene expression variability and across tumor types during the data harmonization and batch effect correction. cDC, conventional dendritic cells.

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Figure 3: The mechanism of action of monoclonal antibodies directed against T cell immune checkpoints in cancer. A) Cytotoxic T lymphocyte-associated antigen 4 (CTLA4) is upregulated by activated T cells and can outcompete CD28 for its ligands CD80 and CD86. CTLA4 antibodies block CTLA4, allowing CD28–CD80 or CD86 interactions to drive T cell activation. Immunoglobulin G1 (IgG1) antibodies block CTLA4 at low expression levels on CD8$^+$ effector T (T$_{eff}$) cells, while high expression on regulatory T (T$_{reg}$) cells may result in their depletion via interactions with activating Fcγ receptors (FcγRs). IgG2 antibodies have weaker interactions with activating FcγRs, so are less likely to result in target cell depletion and are more akin to a 'blocking' antibody. B) Programmed cell death protein 1 (PD1) interacts with PD1 ligand 1 (PDL1) to elicit inhibitory signaling and dampen the immune response of activated T cells. PD1 antibodies block PD1 from interacting with PDL1 to maintain the activation of T cells within the tumor microenvironment. Wildtype IgG4 are designed to block PD1, but may interact with activating FcγRs to deplete PD1$^+$ cells or the inhibitory FcγRIIb to cause internalization of the antibody. IgG4 Fc null antibodies can be considered 'true' blockers. C) Antibodies have also been designed against PDL1 to block their interactions with PD1. IgG1 antibodies block PDL1 on cells that express low levels of the receptor and have the additional function of interacting with activating FcγRs and depleting PDL1$^+$ high cells. IgG1 Fc-null antibodies can be considered 'true' blockers. ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; ITAM, Immunoreceptor tyrosine-based activation motif; MDSC, myeloid-derived suppressor cell; mAb, monoclonal antibody; NK, natural killer.

Figure 4: The mechanism of action of monoclonal antibodies directed against T cell co-stimulatory receptors ICOS and CD40 in cancer. A) Inducible T cell co-stimulator (ICOS) is a co-stimulatory receptor upregulated by activated T cells, which interacts with ICOS ligand (ICOSL) found on APCs to drive strong T cell activation. Antibodies directed against ICOS can mimic ICOSL binding and independently stimulate T cells. Immunoglobulin G1 (IgG1) antibodies can interact with activating Fcγ receptors (FcγRs) to deplete ICOS^{hi}-expressing cells such as regulatory T (T$_{reg}$) cells and activated CD8$^+$ effector T (T$_{eff}$) cells. IgG4 can interact with Fcγ receptor IIb (FcγRIIb) to elicit ICOS agonism in the target cells through cross-linking of the receptor. B) CD40 is a co-stimulatory receptor found on antigen presenting cells (APCs) such as dendritic cells (DC) and is agonized by CD40 ligand (CD40L) predominantly found on CD4$^+$ T cells. Antibodies directed against CD40 can mimic the action of CD40L to elicit activating signaling in the target cell. IgG1 antibodies in this context can crosslink CD40 through FcγRIIb binding. A set of mutations called V11 have been introduced to IgG1 to enhance FcγRIIb binding, and therefore increase FcγRIIb-mediated cross linking. IgG1 may also deplete CD40$^+$ immune cells and mediate liver toxicity and cytokine release syndrome

(CRS) through the agonism of CD40+ myeloid cells. IgG2 antibodies have weak interactions with FcγRs, so they may elicit weak depletion or agonism. If the IgG2-B isoform is present, CD40 agonism can be produced independently of FcγRs. ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; ITAM, Immunoreceptor tyrosine-based activation motif; mAb, monoclonal antibody; NK, natural killer

Figure 5: The mechanism of action of monoclonal antibodies directed against T cell co-stimulatory receptors OX40 and 4-1BB in cancer. A) OX40 is a co-stimulatory receptor upregulated by activated T cells, which interacts with OX40 ligand (OX40L) found on APCs to drive strong T cell activation. Antibodies directed against OX40 can mimic OX40L binding and independently stimulate T cells. Immunoglobulin G1 (IgG1) antibodies can interact with activating Fcγ receptors (FcγRs) to deplete OX40^{hi}-expressing cells such as regulatory T (T$_{reg}$) cells. They may also interact with the inhibitory Fcγ receptor IIb (FcγRIIb) to elicit agonism of OX40^{low}-expressing cells such as CD4+ T effector (T$_{effs}$) cells. IgG2 have weak interactions with FcγRs, so may produce weak depletion or agonism. If the IgG2-B isoform is present, OX40 agonism can be elicited independently of FcγRs. B) 4-1BB is a co-stimulatory receptor upregulated by activated T cells, which interacts with 4-1BB ligand (4-1BBL) found on APCs to drive strong T cell activation. Antibodies directed against 4-1BB have been found to mimic 4-1BBL and independently stimulate T cells. IgG4 antibodies agonize 4-1BB on cells such as CD8+ and NK cells through cross-linking mediated by FcγRIIb. IgG4 antibodies may also stimulate liver toxicity through agonism of 4-1BB+ myeloid cells in the liver resulting in the release of interleukin 27 (IL-27). IgG2 antibodies have weak interactions with FcγRs, so they may elicit weak depletion or agonism. If the IgG2-B isoform is present, 4-1BB agonism can be produced independently of FcγRs. mAb, monoclonal antibody; NK, natural killer

Table 1. Monospecific antibodies approved, under review or in late-stage clinical trials. Data was obtained from the Antibody Society (antibodysociety.org, assessed: June 2023) and corroborated from the websites of the relevant pharmaceutical and biotechnology companies. Antibodies shown were selected based on their subclass and Fc-modifications to highlight those discussed in the text.

[b1] BOX 1. Mice and beyond – pre-clinical models to better predict clinical outcomes

'Next generation' mouse models have been developed by the Ravetch [274], group and Regeneron Pharmaceuticals [275], to recapitulate the full human Fcγ receptor (FcγR) expression in one model, moving beyond single human FcγR (hFcγR) transgenic mice. The

first mouse model was developed by the Ravetch group by crossing single hFcγR transgenic mice and breeding the progeny with mouse FcγR (mFcγR) knockout mice (FcRα null) [274]. These mice have the low-affinity single nucleotide polymorphisms (SNPs) of *FCGR3A*[176F] and

1695 *FCGR2A*[166R], *FCGR2B*[232I,] and *FCGR3B* (SNP not stated) and the high-affinity *FCGR1A*. This model has been instrumental in understanding the mechanism of action of multiple immunomodulatory antibodies [45, 55, 105, 197], and has been further improved through the addition of the human neonatal Fc receptor (hFcRn) and the hIgG1 heavy chain to better recapitulate the human setting [276, 277]. However, these mice display high copy number

1700 variations and an anomalous FcγR expression pattern on some cells compared to humans [17, 278], limiting conclusions drawn from this model. The model developed by Regeneron removed the low-affinity mFcγR locus and replaced it with the entire low-affinity hFcγR locus in FcRα^{-/-} mice [275]. This mouse has the high-affinity SNPs of *FCGR3A*[176V] and *FCGR2A*[166H], *FCGR2B*[232I], *FCGR3B*[NA2], and *FCGR2C*[STOP] and has been used to better understand hFcγR

1705 mediated anaphylaxis [275, 279]. The original model described expressed mFcγRI [275] but this has since been replaced with hFcγRI and further improved with the addition of hFcRn, and the hIgG1 heavy chain and kappa light chain [279, 280]. Despite the improved genetic insertions of the hFcγR loci, this model shows some anomalies in expression such as the overexpression of hFcγRIIb on blood monocytes and low levels of hFcγRIIIa on natural killer

1710 (NK) cells compared to humans [275]. Alternatives to mouse models including immune proficient patient-derived xenografts (Immune-PDX) [262], and patient-derived explants (PDE) [263], are being developed and have been used to test PD1 [263], PDL1 [262], and CD47 antibodies [281]. These models offer better translatability but are limited by patient variability and maintenance of tissue viability ex vivo. The use of stem cell derived-innate immune cells

1715 [282] and assembloids that enable cell types to be combined [283], could help further dissect the contribution of hFcγRs to antibody therapeutics by the in vitro reconstruction of tissues, providing an alternative research platform in the future.

[bH2] BOX 2. Non-responders to immunomodulatory antibodies and hyperprogressive

1720 **disease**

Hyperprogressive disease (HPD), whereby patients experience a substantial increase in their disease burden following treatment with anti-programmed cell death protein 1 (PD1), anti-PD1 ligand 1 (PDL1), or anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA4) therapies, is a clinical phenomenon described in case reports or retrospective studies [284]. Increased

1725 numbers of Fcγ receptor (FcγR)-expressing M2-like macrophages [285], proliferating regulatory T (T$_{reg}$) cells [286], PD1 expression on the tumor cells [287], and the genomic landscape [288] are some of the mechanisms proposed for HPD. In vitro studies have shown

that human PD1 antibodies can mediate crosslinking between PD1$^+$ effector T (T$_{eff}$) cells and FcγRI$^+$ macrophages, resulting in macrophage-mediated phagocytosis of PD1$^+$ T$_{eff}$ cells [109].

1730 Supporting these findings, in patient-derived xenograft models, tumor growth was enhanced by treatment with PD1antibodies but not by anti-PD1 F(ab)$_2$ fragments highlighting the importance of the Fc in driving this phenomenon [285]. Whether hIgG4-mediated phagocytosis of T$_{eff}$ cells occurs in patients receiving PD1 antibodies (or other hIgG4-based antibodies) remains to be fully elucidated. As hIgG4 binds to hFcγRI, hFcγRIIa and to hFcγRIIIa, under

1735 the right conditions it could mediate effector functions, eliciting CD8$^+$ T$_{eff}$ depletion by myeloid cells and/or NK cells expressing these receptors. Associations between the presence of FcγR-expressing cells and single nucleotide polymorphisms (SNPs) in FcγRIIIa or FcγRIIa that increase their affinity for hIgG4 will help us gain a better understanding of this phenomenon.

Target	Antibody	Isotype (human)	Fc modification	Role of Fc modification	Late stage clinical trial/Approval granted	Initial indication	Company
CTLA4	Ipilimumab	IgG1 kappa	None		FDA approved	Metastatic melanoma	Bristol-Myers Squibb
	Tremelimumab	IgG2 kappa	None		FDA approved	Antineoplastic, Liver cancer	AstraZeneca
PD1	Tizlelizumab	IgG4 kappa	S228P/E233P/F234V/L235A/D265A/R409K	Hinge stabilization/Abrogate FcγR binding	Phase III Clinical Trial	Esophageal squamous cell carcinoma	Novartis
	Pembrolizumab	IgG4 kappa	S228P	Hinge stabilization	FDA approved	Melanoma, Non-small Cell Lung Cancer, Renal cell carcinoma	Merck
	Nivolumab	IgG4 kappa	S228P	Hinge stabilization	FDA approved	Melanoma, Non-small cell lung cancer, Hodgkin's lymphoma	BMS
PDL1	Durvalumab	IgG1 kappa	L238F/L239E/P335S	Abrogate FcγR binding	FDA approved	Bladder cancer	AstraZeneca
	Avelumab	IgG1 lambda	None		FDA approved/EMA approved/Approved in China	Merkel cell carcinoma	Pfizer
	Atezolizumab	IgG1 kappa	N297A	Abrogate FcγR binding	FDA approved	Bladder cancer	Roche
LAG3	Relatlimab	IgG4 kappa	S228P,K447> del	Hinge stabilization	FDA and EMA approved	Melanoma	Bristol-Myers Squibb
TIGIT	Tiragolumab (MTIG7192A/RG-6058)	IgG1 kappa	Not disclosed		Phase III Clinical Trial	Non-small cell lung cancer, Esophageal Cancer	Genentech
ICOS	Feladilimab (GSK3359609)	IgG4 kappa	S228P	Hinge stabilization	Phase III, discontinued	Solid tumours, Multiple myeloma, Non-small cell lung cancer, Head and neck cancer	GlaxoSmithKline
	Vopratelimab (JTX-2011)	IgG1 kappa	Not disclosed		Phase II, discontinued	Non-small Cell Lung Cancer Solid tumours; Urogenital cancer	Jounce Therapeutics, Inc
CD40	2141-V11	IgG1, kappa	G237D/H268D/P271G/A330R	Increase FcγRIIb binding	Phase I/II	Solid tumors: skin cancer, glioblastoma	Rockefeller University
	Sotigalimab	IgG1, kappa	S267E	Increase FcγRIIb binding, decrease FcγRIIa binding	Phase I	Solid tumors: Colorectal adenocarcinoma, Pancreatic ductal adenocarcinoma	Bristol-Myers Squibb
	Selicrelumab (CP-870,893)	IgG2, kappa	None		Phase I/II	Solid tumors	Pfizer
	CDX-1140	IgG2, kappa	None		Phase I/II	Solid tumors	Celldex Therapeutics
OX40	Ivuxolimab (PF-04518600)	IgG2 kappa	Not disclosed		Phase II, failed	Breast cancer, Renal cell carcinoma, Solid tumours, Acute myeloid leukaemia, Squamous cell cancer, Follicular lymphoma	Pfizer
	Tavolimab (MEDI-0562)	IgG1 kappa	Not disclosed		Phase II, completed	Ovarian cancer, solid tumors	MedImmune/AstraZeneca

	Cudarolimab (IBI 101)	IgG1 kappa	Not disclosed		Phase I	Advanced solid tumors	Innovent Biologics
41BB	Urelumab (BMS-663513)	IgG4 kappa	S228P	Hinge stabilization	Phase II, discontinued/recruiting	Non-Hodgkin's lymphoma, Solid tumours, Glioblastoma, Multiple myeloma	Bristol-Myers Squibb
	Utomilumab	IgG2 lambda	Not disclosed		Phase III (Terminated/Not yet Recruiting)	Breast cancer, Oropharyngeal cancer, Solid tumours, B-cell lymphoma, Diffuse large B cell lymphoma, Follicular lymphoma, Ovarian cancer	Pfizer

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