Engineering antibodies to enhance their interactions with the immune system holds great promise for treating cancer. Here we review various factors involved in defining the ideal profile for such drugs.

Optimizing engagement of the immune system by anti-tumor antibodies: an engineer's perspective

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A unique property of monoclonal antibodies, and a principal reason for their success as cancer therapeutics, is their ability to engage the immune system. A growing set of data supporting the relevance of Fc-mediated effector functions to anti-tumor efficacy has motivated efforts to enhance the interactions between antibodies and Fc receptors expressed on immune cells. Although current approaches have considerable promise for improved clinical performance, the immunobiology of tumors, antibodies, and Fc receptors continues to evolve. In this review we discuss what is known and what is not known about the interactions between therapeutic antibodies and the immune system, with the goal being progress toward clear target profiles for effector engineering efforts.

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Introduction

Despite a small number of therapeutically successful anti-cancer antibodies with *bona fide* clinical efficacies, it is becoming very clear that monoclonal antibodies (mAbs) can be improved further for optimized interactions with the human immune system. One strategy is to begin with a naturally evolved set of features and perturb them to favorably impact efficacy. Along these lines, an approach that is currently at the forefront of mAb-improving technologies is optimization of antibodies for enhanced engagement with the Fc gamma receptors (FcγRs) expressed on immune effector cells (Box 1, Table 1). The basis of the approach is that engineering antibodies with optimal affinities for certain of those FcγRs will lead to greater effector activation and greater killing of antibody-coated tumor cells. As the products of these technologies begin to enter clinical trials, and as a growing set of new Fc modification technologies emerge behind them, we ask the question: what are the optimal FcγR affinities and selectivities? If one could design the perfect FcγR selectivity for an oncology antibody, what would that be? Perhaps more realistically, if a compromise must be made between FcγR selectivity profiles, which is the best one? We also ask what we might expect out of the antibodies of the future in terms of their *in vivo* effects on their target cells. The answers ultimately depend on knowledge of effector cells and their functions, and whether or not they are in the right state at the right place at the right time. Although with today's data one cannot make a definitive choice, there is certainly enough information to begin the conversation, which is our intent in writing this review.

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BOX 1

Technologies for optimizing immune engagement

Early work with antibody isoforms, variants, and glycoforms established a clear correlation between FcγR affinity and *in vitro* effector function, paving the way for a variety of Fc engineering efforts aimed at enhancing anti-tumor potency (Table 1). A straightforward approach to optimizing FcγR affinity is amino acid modification, and progress in this area has been made using alanine scanning and site-directed mutagenesis, computational structure-based design, and selection-based methods [59–63]. Overall a large set of Fc variants has been generated that provides a spectrum of FcγR binding profiles. The primary goal of these studies, however, has been improved FcγRIIIa affinity, driven by the more clear relevance of this receptor from polymorphism data (see main text) and the dominance of NK cells among other PBMC components in ADCC assays. Toward this goal, variants have been generated that provide up to 100-fold greater affinity for FcγRIIIa, resulting in enhanced ADCC as high as 100-fold.

Success at selectively improving the affinity of FcγRIIIa relative to the inhibitory receptor FcγRIIb has been more tempered. As of yet there is no overwhelming evidence for an *in vitro* benefit from modifications that selectively enhance FcγRIIIa affinity relative to FcγRIIb. Whether this is due to inadequacy of the current set of selective variants, immaturity of assays for differentiation, or lack of an actual biological effect of FcγRIIb on FcγRIIIa is not known. The evidence for the opposed biochemistry of FcγRIIa and FcγRIIb is somewhat more established, suggesting that selectivity between these two receptors may be more easily discerned *in vitro* or *in vivo*. Unfortunately, because of the near identical extracellular domains of these receptors (93%), engineering of Fc mutations that distinguish between them is more challenging. Clark and colleagues have dissected IgG isotype sequences to engineer variants that reduce binding to FcγRIIb [64]; however, these same variants had even greater reduction of affinity for FcγRIIa. In our own work, we have screened over 2500 amino acid variants of the Fc domain and have discovered a small handful that selectively increase affinity for FcγRIIa while reducing or not increasing affinity to FcγRIIb (Richards *et al.*, manuscript in preparation). Such variants may represent the next generation for Fc engineering; according to the current understanding of these receptors, selective engagement of FcγRIIa over FcγRIIb may positively impact phagocytosis and potentially tip the balance toward APC activation, antigen presentation, and adaptive T cell immunity.

Another method for enhancing FcγR affinity is glycoform engineering, motivated by observations that antibodies with reduced fucose content from Lec13 and YB2/0 cells lines demonstrated enhanced ADCC *in vitro*. Glycoengineering of more commercially applicable CHO lines followed, including generation of cells with inducible expression of the enzyme β(1,4)-*N*-acetylglucosaminyltransferase III (GnTIII) [65], and genetic knockout of the enzyme α-1,6-fucosyltransferase (FUT8) [66]. The successes of these efforts at improving FcγRIIIa affinity and ADCC have motivated similar attempts in non-mammalian expression systems. In the process of engineering away nonhuman and thus potentially immunogenic glycoforms in yeast, plants, and moss, several groups have taken the extra step of removing fucosylation pathways [67–69]. Although other glycoform modifications have not been found to enhance ADCC, recent work in mice suggests that sialylation of the Fc carbohydrate is a biological mechanism for regulation of FcγR affinity and cytotoxicity [70], with intriguing potential for application to autoimmune and inflammatory diseases.

There has been some debate about the relevance of particular structural aspects of the Fc glycoform to enhanced effector function ADCC. The maturing view is that though certain sugar structures can negatively impact antibody Fc properties [71], lack of fucose is the most important if not sole factor for enhancement [72]. This is supported by a recent study showing that the mechanism of enhancement is via alleviation of a steric interaction between the Fc fucose and carbohydrate on the receptor [41]. Whether this result implies that differences in FcγRIIIa glycosylation [73,74] may impact potential *in vivo* benefit remains to be seen. The glycosylated receptor mechanism predicts that defucosylation should only enhance affinity to human FcγRIIIa/b and potentially mouse FcγRIV because of lack of an asparagine at the analogous position in the other receptors. This is consistent with observations in some studies [41,56]; however, other data show increased binding of defucosylated mAbs to human FcγRIIb, mouse FcγRIIb, and mouse FcγRIII [3,68,75]. Whether these discrepancies are due to assay differences or differences in glycoform production is not clear. Overall, the current view is that defucosylation enhances affinity only to FcγRIIIa among the human FcγRs, highlighting a key distinction between amino acid and glycoform engineering (Table 1).

The existing data come from a variety of sources. First, there is detailed knowledge of the measurable anti-tumor functions of various effector populations. Second, there are mouse models that deconvolute the influence of different FcγRs as well as effector cells. Next, there are a growing number of human polymorphism studies that provide valuable, but incomplete, glimpses into the relevance of certain FcγRs to the clinical efficacy of particular antibodies and indications. Finally, there are data showing the relative abilities of different effector populations to infiltrate a tumor. In this review we consider these disparate sources of data to provide a framework for thinking about when, where, and why to apply a given Fc receptor selectivity.

Fc receptors link antibodies to the cellular immune system

The principal mechanism by which IgG antibodies engage the cellular immune system is via interaction of the Fc domain with FcγRs [1,2]. The human FcγR family contains six known members in three subgroups, including FcγRI (CD64), FcγRIIa,b,c (CD32a,b,c), and FcγRIIIa,b (CD16a,b). There are a number of subtle differences between the receptors, summarized in Table 2, that determine the biological complexity of the system. FcγRI binds with high affinity (10^{-9} M) to monomeric IgG and is not thought to distinguish between unbound IgG and immune complexes (IC). By contrast, FcγRII and FcγRIII bind with low affinity (10^{-5}–10^{-7}) to monomeric IgG, and the dynamic range provided by binding of these receptors to IC is thought to be an important trigger for immune activation. Central to FcγR biology is the balance between activating and inhibitory signals. Four of the receptors are activating on the basis of their possession of a cytoplasmic immunoreceptor tyrosine based activation motif (ITAM), which is either encoded directly (FcγRIIa,c) or gained by association with a common ITAM γ-chain (FcγRI and FcγRIIIa). By contrast, FcγRIIb possesses an inhibitory motif (ITIM) in its cytoplasmic domain, and signaling through this receptor negatively regulates effector functions. FcγRIIIb is unique in that it is linked to the membrane with a glycosyl phosphatidyl inositol (GPI) anchor and therefore does not signal. Receptor expression differences on the various immune cells, discussed in detail below

TABLE 1

Technologies for FcγR optimization

Group	Engineering approach	Reference[a]	Most advanced clinical candidate[b]	FcγR partnerships and acquisitions[c]
AME (Lilly)	Amino acid engineering	Allan *et al.* [59]	Anti-CD20, Phase I	AME acquired by Lilly in 2004 for ~$400M
Biolex	Glycoengineering in plants	Cox *et al.* [67]	N.D.	N.D.
Bioren (Pfizer)	Amino acid engineering	Crea *et al.* [92]	N.D.	Bioren acquired by Pfizer in 2005, amount undisclosed
Biowa (Kyowa Hakko)	Glycoengineering in CHO	Yamane-Ohnuki *et al.* [66]	Anti-GD3, Phase II	Biogen Idec, Genentech, Igeneon, Medarex, MedImmune, UCB
Diversa & Medarex	Amino acid engineering	Hansen *et al.* [60]	N.D.	N.D.
Genentech	Amino acid engineering	Shields *et al.* [62]	N.D.	N.D.
Glycart (Roche)	Glycoengineering in CHO	Umaña *et al.* [65]	N.D.	Glycart acquired by Roche in 2005 for ~$180M
GlycoFi (Merck)	Glycoengineering in yeast	Li *et al.* [68]	N.D.	GlycoFi acquired by Merck in 2006 for ~$400M
Greenovation	Glycoengineering in moss	Nechansky *et al.* [69]	N.D.	N.D.
Macrogenics	Amino acid engineering	Stavenhagen *et al.* [63]	N.D.	N.D.
Xencor	Amino acid engineering	Lazar *et al.* [61]	Anti-CD30, Phase I	Boehringer Ingelheim, Centocor, Genentech, MedImmune, Roche

[a] Patent applications are provided only when a journal publication is not available.
[b] N.D., not disclosed.
[c] Deals and acquisitions are as listed on company websites in March 2007. Only partnerships disclosed to be for Fc engineering are listed. Acquistion deals are not necessarily related to Fc engineering. N.D., none disclosed.

TABLE 2

Human FcγR properties

Receptor	Gene	Expression[a]	Cytoplasmic	ECD % ID	Alleles[b]	Relative affinities[c]		
						hIgG1	hIgG2	mIgG3
FcγRI	CD64	MΦ DC neutrophil	ITAM, association		N.A.			
FcγRIIa	CD32a	MΦ DC neutrophil	ITAM, genetic	93% to FcγRIIb	H131 R131	≈	10X 1X	1X >1X
FcγRIIb	CD32b	MΦ DC neutrophil B cell	ITIM, genetic	93% to FcγRIIa, 98% to FcγRIIc	I232 T232	≈	≈	≈
FcγRIIc[d]	CD32c	MΦ neutrophil NK	ITAM, genetic	98% to FcγRIIb	N.A.			
FcγRIIIa	CD16a	MΦ DC NK	ITAM, association	98% to FcγRIIIb	V158 F158	5-7X 1X	N.B.	
FcγRIIIb	CD16b	Neutrophil	None, GPI-linked	98% to FcγRIIIa	NA1 NA2	>1X 1X[e]	N.B.	

[a] MΦ, macrophages; NK, NK cells; DC, dendritic cells. FcγR expression is dependent on activation state, typically modulated by cytokines. FcγRs are also expressed on other cell types that are not discussed in this review.
[b] N.A., not applicable; no functionally different alleles reported.
[c] Relative affinities for V/F158 FcγRIIIa/hIgG1 determined by calorimetry [42] and Biacore [[41], Xencor unpublished results]. Relative affinities for H/R131 FcγRIIa/hIgG2 determined by Biacore [Xencor unpublished results]. To our knowledge H/R131 FcγRIIa/mIgG3 affinities have not been confirmed, and binding of mIgG3 to FcγRIII is not known. The symbol'≈' indicates that the IgG binds both alleles with approximately the same affinity. N.B. indicates that no detectable binding has been reported.
[d] FcγRIIc expression on the indicated cell types varies per individual. FcγRIIc expression on DCs is not known. Identity between FcγRIIc and FcγRIIb may vary slightly with FcγRIIc isoform.
[e] FcγRIIIb NA1 and NA2 alleles have been shown to bind IgG1 with equal affinity [93], but the NA1 allele mediates greater phagocytosis with IgG1-opsonized cells [94].

for each cell type, are fundamental to their diverse ability to control cellular immune response.

The other component of mAb-mediated effector function is complement activity. Although the focus of this review is on FcγRs and cellular effector functions, a brief discussion is provided on the possible role of complement in anti-tumor activity and engineering efforts to optimize it (Box 2).

The degree to which natural IgG antibodies interact with FcγRs bears on our attempts to understand the mechanisms of therapeutic mAbs. Despite decades of studying natural antibody response to disease, it remains unclear how differences between the isotypes, at the detailed level of Fc receptor specificity, dictate their use in immune reaction. The simplistic view that there are two apparently high effector function IgGs (human IgG1 and IgG3; murine IgG2a/c and IgG2b) and two apparently low effector function IgGs (human IgG2 and IgG4; murine IgG1 and IgG3) ignores potentially useful information. Are there distinct cell types, effector functions, and/or cytokine signatures that are tapped by human IgG1 versus IgG3, or by IgG2 versus IgG4? Recent work in mice showing that the ratio of affinities for activating various inhibitory FcγRs correlates with *in vivo* activity [3] is progress in the right direction. Although extension of this concept to humans is complicated by the balance of the near identical activating and inhibitory FcγRII receptors (discussed below), a more sophisticated understanding of natural and variant IgG effector properties is needed to deduce how the immune system utilizes Fc receptors to control disease.

BOX 2

What about engineering mAbs for optimized complement activity?

The best-characterized antibody-dependent (classical) complement mechanism of target destruction is noncellular, namely complement-dependent cytotoxicity (CDC) carried out by the membrane attack complex (MAC). There are also two cellular mechanisms, both mediated by interaction between opsonic iC3b on target cells and CR3 expressed on the effector cells, most notably macrophages and NK cells. CR3-dependent cellular cytotoxicity (CR3-DCC) is a direct outcome of the CR3/iC3b interaction, and because it is activated by cell wall β-glucan it is not thought to be relevant for cancer therapy. However, another mechanism involving CR3/iC3b enhancement of FcγR-mediated effector functions does not require microorganism danger signals, and may be a relevant anti-tumor mechanism. Interestingly, this process is enhanced by opsonic complement protein C5a, which not only is chemotactic for effector cells, but also selectively increases expression on macrophages of activating FcγRs relative to the inhibitory receptor FcγRIIb [76,77]. In this way C5a and CR3/iC3b serve as a potential means of crosstalk and amplification between complement and FcγR effector pathways.

There is a lack of firm support for a relationship between complement and mAb anti-cancer efficacy. The dependence of anti-CD20 activity on complement observed in some mouse models [78,79] is inconsistent with results in others [24,80]. Likewise, although some groups have documented correlations between expression of complement regulatory proteins and lower clinical response to Rituxan [81,82], others have observed neither a correlation nor differences in complement-mediated cytotoxicity *in vitro* using tumor cells from the different response groups [83]. Nonetheless there have been successful efforts to optimize mAb/complement interactions and complement activity in order to enhance mAb anti-tumor potency [84,85]. It is important to note, however, that studies measuring C1q affinity and CDC activity *in vitro*, as well as complement dependence in mouse models, are blind to whether the key component for cell killing in a clinical setting may be MAC-dependent CDC, CR3-DCC, and/or CR3-enhancement of ADCC. Because of the gaps in our understanding of these mechanisms as they relate to tumor therapy, it remains to be seen whether engineered improvements in CDC *in vitro* will enable more potent antibodies in the clinic.

A diverse set of immune cells carry out a variety of FcγR-mediated effector functions

A wide variety of immune effector cells express antibody FcγRs and thereby have the ability to mediate the therapeutic effects of anti-tumor antibodies. They can accomplish this via an array of antibody-dependent effector functions, including cytolysis, phagocytosis, release of soluble factors such as cytokines and chemokines, and other more complex activities (Figure 1). The usual suspects for antibody-dependent anti-tumor effects include, in order of reputation, natural killer (NK) cells, monocytes/macrophages, neutrophils, and dendritic cells (DCs).

NK cells are the best appreciated among anti-tumor effectors, probably because their activity is so readily measured in the most commonly used ADCC assays, in which the spilling out of natural (e.g. lactate dehydrogenase) or artificial (e.g. chromium) target cell ingredients is measured to monitor the extent of lysis. When peripheral blood mononuclear cells (PBMCs) are used as effectors, lysis in these assays is mediated almost completely by the NK cell population. NK cells have the unique distinction that they typically only express the activating receptor FcγRIIIa and are not subject to regulation by the inhibitory receptor FcγRIIb (certain individuals express the activating receptor FcγRIIc and very rarely FcγRIIb, depending on allelic polymorphism [4]). The major FcγR-induced effector activities of NK cells are cytolysis of target cells through lytic granule release or apoptosis via secretion of TNF family ligands (e.g. TNF, FasL) [5]. They are also potent producers of other cytokines such as IFNγ. NK cells are regulated through a series of activating receptors, such as NKG2D, and inhibitory receptors of the killer Ig-like receptor (KIR) family. KIRs suppress killing when they interact with autologous (self) class I major histocompatibility complex (MHC) molecules on normal cells. Target cells lacking a matching MHC are, however, attacked by NK cells, via binding to activating NK receptors, establishing the so-called 'missing-self' paradigm of NK cell function. Engagement of the activating receptor FcγRIIIa (CD16a) by antibody-coated (opsonized) target cells can partially override the KIR inhibitory signal, resulting in killing of the coated cells. Interestingly, additional killing can be observed when anti-KIR or anti-MHC blocking antibodies are combined with anti-tumor antibodies (Lou Weiner, personal communication).

Cells of the myeloid lineage, including monocytes/macrophages, neutrophils, and DCs, have an overlapping array of FcγR expression profiles [6–9]. All express FcγRIIa and at least one splice variant of the inhibitory receptor FcγRIIb. Monocytes/macrophages and DCs also express FcγRIIIa and FcγRI depending on their source and activation state. Neutrophils express FcγRIIIb rather than FcγRIIIa, and FcγRI when activated by G-CSF. Although some data suggest that the GPI-linked FcγRIIIb can signal, possibly in collaboration with FcγRIIa [10], other results

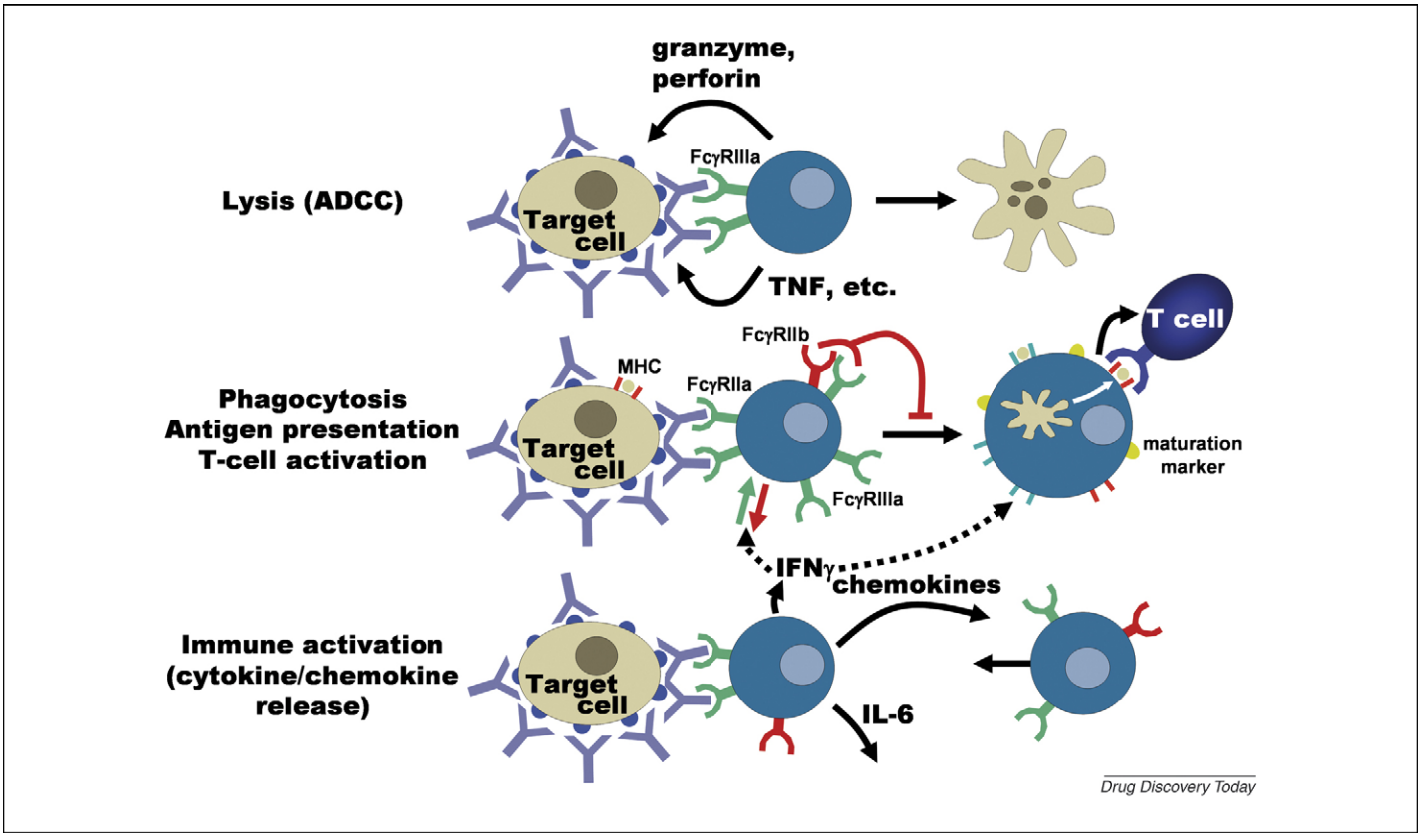

FIGURE 1

FcγR-mediated effector functions. Activation of FcγRs by antibody-coated target cells can elicit a variety of cellular effector functions. NK cells, which express the activating FcγRIIIa, can cause direct killing through release of cytotoxic granules and/or secretion of apoptotic TNF family members. Phagocytic cells s uch as neutrophils, monocyte/macrophage, and dendritic cells (DC) can engulf target cells when FcγRIIa and FcγRIIIa are triggered, but are subject to regulation by the inhibitory receptor FcγRIIb. Phagocytosis by antigen presenting cells such as macrophage and DC can also lead to MHC presentation of tumor antigen and activation of tumor-specific T cells (see Box 3). FcγR activation can also lead to release of cytokines and chemokines that could further amplify these other effector functions. For example, IFNγ can modulate FcγR expression in favor of activating FcγR and increase MHC expression on target and antigen-presenting cells. Chemokines can recruit additional effector cells to the site of activation.

indicate that it does not contribute to neutrophil effector function [11]. Macrophages and neutrophils are classic phagocytes, and can phagocytose opsonized target cells through engagement of FcγRs. They can also induce apoptosis of target cells through the release of reactive nitrogen (RNI) and oxygen (ROI) intermediates, or lyse them through the release of cytolytic granules. Which of these anti-tumor activities are most relevant for *in vivo* efficacy is difficult to discern.

A more intriguing aspect of macrophages and DCs with respect to anti-tumor therapy involves their roles as professional antigen presenting cells (APCs). FcγR-mediated phagocytosis, besides leading to the destruction of a target cell, can facilitate a potentially more robust anti-tumor effect known as cross-priming, in which these cells process and present tumor-derived antigens on their surface class I MHC, thus acquiring the ability to activate T cells. Cross-priming can activate cytotoxic T lymphocytes (CTLs) that recognize MHC/tumor antigen complexes, ultimately leading to attack on the tumor cells. We consider this kind of *in vivo* vaccination effect, catalyzed by an anti-tumor antibody, to be a Holy Grail of antibody therapy (Box 3), because it theoretically could lead to long-lasting adaptive anti-tumor immunity and long-term remission. Indeed, it is such effects that are sometimes invoked to explain the long-term responses observed in lymphoma patients after therapy with the anti-CD20 antibody Rituxan® (rituximab) [12].

We should emphasize that all of these cell types exist in a broad spectrum of states that are heavily influenced by their environments, making it difficult to classify them into distinct subsets or precisely predict their anti-tumor behaviors. This uncertainty warrants caution in broadly extrapolating *in vitro* effects into *in vivo* expectations. What is clear, however, is that these effector cells have a well-documented variety of FcγR-mediated effects, ranging from direct killing of antibody-coated target cells via lysis, apoptosis, and phagocytosis, to indirect effects such as cytokine/chemokine release and promotion of adaptive immune responses.

From analysis of cellular FcγR expression and functional activities, it is difficult to predict *a priori* which FcγRs are most relevant to *in vivo* efficacy. Accurately extending such analysis to clinical relevance for anti-tumor antibodies requires thorough consideration of the large set of *in vivo* data, as discussed below. Nonetheless, as FcγRIIIa is the only FcγR expressed on NK cells and is also expressed on macrophages and DCs, it is a strong contender for clinical importance. Alternatively, FcγRIIa which is not expressed on NK cells, is important for the function of neutrophils, monocytes, macrophages, and DCs. The inhibitory receptor FcγRIIb is expressed on the same cell subsets as FcγRIIa, which, together with

BOX 3

The Holy Grail? FcγR engagement of the adaptive immune system

The common goal of myriad cancer therapies is to amplify the involvement of the adaptive immune system, leading to the generation and/or activation of tumor-killing cytotoxic T lymphocytes (CTL). Although the notion of tumor immunogenicity is an old one, recent work has begun to shed light on the mechanism by which T cells infiltrate and eliminate tumors [86], and the properties of infiltrated T cells that correlate with favorable prognosis [87]. These activated anti-tumor T cells recognize, via the T cell receptor (TCR), complexes of tumor-associated antigens (TAA) with class I MHC molecules displayed on the tumor cell surface. Triggering of the TCR then induces lysis of the tumor cell. Induction of adaptive immunity against tumor cells begins with professional APC such as macrophages, B cells, and DCs that process TAA and present them to T cells as complexes with MHC molecules. Depending on the origin of the TAA, the MHC can be class I or class II, which can react with the TCR of CD8+ or CD4+ T cells, respectively. T cells recognizing these complexes on APCs can then be activated as amplifiers of the response (CD4+ helper T cells) or as direct effectors (CD8+ CTLs) that can attack the tumor cells.

Typically, exogenous antigens are presented on class II MHC, and internal antigens are presented on class I MHC. Thus, DC phagocytosis of a tumor cell would normally lead to presentation of the TAA on class II MHC, leading only to activation of CD4+ helper T cells. However, a relatively recent observation is that anti-tumor antibodies can break this rule – through undeciphered mechanisms – and induce a phenomenon called cross-presentation. That is, if a DC phagocytoses a tumor cell through engagement of its FcγRs, TAA can also be presented on class I MHC. This is crucial, as it is with class I MHC/TAA complexes that DCs can directly activate the potent tumor-killing CTLs, a process referred to as cross-priming.

Several recent *in vitro* studies demonstrate that opsonized tumor cells can be used as an effective vehicle to induce tumor-specific T cell responses [88–90]. Co-culture of DCs, antibody, and tumor cells resulted in more potent activation of tumor-specific CTLs. Furthermore, blocking antibodies to MHC class I molecules [88,90] or FcγRIII and FcγRII [89] eliminated the enhanced cross-presentation, confirming a direct role of FcγRs in the process. Interestingly, opsonization of tumor cells alone does not enhance uptake and DC maturation. Incubation with a cocktail of inflammatory cytokines (IL-1β, IL-6, PGE2, and TNFα) was necessary to induce full maturation of DCs, a prerequisite for T cell activation. Although cross-presentation involves the presentation of various TAAs to CTLs, it has also been shown that tumor-specific CD4+ immunity is induced as well [90].

DCs express both activating FcγRIIa and inhibitory FcγRIIb molecules on the surface. Boruchov *et al.* have elegantly shown that both the number of cells expressing these receptors and the density of expression can be modulated with various agents including cytokines [6]. The ultimate outcome of FcγR-mediated phagocytosis and cross-presentation appears to depend on the relative engagement of activating versus inhibitory FcγRs. In human *in vitro* studies, blockade of FcγRIIb promotes DC maturation, T cell activation, and production of IL-12 without the addition of inflammatory cytokines [6,16]. IFNγ modulation of FcγR expression in favor of FcγRIIa also promotes IgG-induced maturation. In mice, blocking FcγRIIb resulted in enhanced tumor immunity [91] using immune complex as the antigen.

Although these studies were aimed initially at tumor vaccine strategies, the results have enormous implications for mAb therapy.

The picture is emerging that the relative engagement of immune complexes with FcγRIIa versus FcγRIIb determines an on/off switch for DC maturation, cross-presentation, and T cell activation. The notion of tipping this balance clinically using cytokines or Fc engineering (Box 1) suggests a way forward for optimizing antibody therapy not only for innate effector functions, but also for enhancing adaptive immunity.

their close homology (93% identity of their extracellular domains), implicates these receptors as a co-evolutionary activating/inhibitory pair. Various studies have demonstrated the importance of FcγRIIIa and FcγRIIa for the activities described above. An ongoing question is to what extent FcγRIIb downmodulates the activities of each of the activating FcγRs, the resolution of which had been hampered by the lack of a selective anti-FcγRIIb versus FcγRIIa antibody reagent. Earlier data, based mostly on indirect observations, suggested significant FcγRIIb inhibition of FcγRIIa-mediated phagocytosis by monocytic cells [13,14]. Notably, an FcγRIIb effect on FcγRIIIa-mediated phagocytosis was absent [13]. These observations, coupled with similar observations in murine model systems (reviewed below), have led to propagation of the paradigm that a high activating/inhibitory (*A*/*I*) FcγR ratio, in IgG affinity and/or receptor expression, is important for maximal antibody activity [3,8]. Building on this concept, van Mirre *et al.* demonstrated an association between higher FcγRIIa/FcγRIIb expression levels and human neutrophil activation by immune complexes [15]. Interestingly, *A*/*I* expression dependence was not apparent for neutrophil phagocytosis of antibody-coated bacteria. In our own observations, we have measured macrophage-mediated phagocytosis with antibody Fc variants possessing a range of *A*/*I* affinity ratios and have thus far failed to observe an inhibitory influence of human FcγRIIb (Richards *et al.*, manuscript in preparation). More recently, selective FcγRIIb-blocking antibodies have been reported [16,17]. So far, they have only been applied to the study of DC effects [6,16] and have revealed a strong influence of FcγRIIb on FcγRIIa-mediated DC maturation and cross-priming (Box 3). We expect that these useful reagents will soon be used to investigate the relevance of FcγRIIb in monocytes, macrophages, and neutrophils, and to further define the role of FcγRIIb in human immunobiology.

Cytokines, FcγRs, and effector cell crosstalk

For all of these cell types, expression of the various FcγRs can be modulated by cytokines. The most generalizable effects, with some exceptions, involve IFNγ, IL-4, and IL-10. IFNγ, the classic Th1-type cytokine, increases expression of activating FcγRs and decreases expression of the inhibitory FcγRIIb [6,8]. IL-4, the classic Th2-type cytokine, decreases expression of activating FcγRs and increases that of FcγRIIb [8]. IL-10 is more pleiotropic and increases expression of all FcγRs [6,14]. Beyond these general patterns, a range of other cytokines and soluble factors has been shown to modulate FcγR expression and exhibit intriguing synergies.

The role of the FcγR/cytokine relationship in anti-tumor therapy becomes more interesting when one considers that not only do cytokines modulate FcγR expression, but also FcγR activation can induce production of cytokines. As mentioned earlier, NK cells produce IFNγ when FcγRIIIa is activated. Immune complexes (IC) induce monocytes/macrophages to produce inflammatory cyto-

kines such as TNFα, IL-6, and IL-1, an effect that is considered to play a role in the pathological IC-mediated inflammation in autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). Thus, there is considerable potential for crosstalk and amplification effects when one or more effector cells are activated by antibody engagement of their FcγRs. Although direct FcγR-mediated killing of target cells by lysis, apoptosis, or phagocytosis is intuitively considered to be the primary anti-cancer mechanism in antibody therapy, FcγR-mediated cytokine and chemokine release may collectively be of equal or higher importance, particularly because of the potential for effector cell crosstalk and stimulation of other arms of the immune system. The simplest effect that can be expected is that FcγR-induced chemokine release will lead to recruitment of additional effector cells to the tumor. Secreted cytokines such as IFNγ can upregulate class I MHC on tumor cells, enhancing their immunogenicity, as well as on effector cells, increasing their potential for cross-priming. Most intriguingly, cytokines can also favorably alter the FcγR state of surrounding effector cells, further amplifying the potential for the anti-tumor activities discussed above (Figure 1). Because of these and other desirable anti-tumor properties, considerable effort has been applied toward administering certain cytokines systemically or locally as anti-tumor therapies [18]. While systemic administration often leads to prohibitive toxicities, localized cytokine administration can lead to robust anti-tumor effects. It is intriguing to speculate that engineered enhancements in Fc/FcγR affinities (Box 1, Table 1) could provide tailored and localized cytokine release in the tumor microenvironment.

In vivo studies—what they do and do not tell us

Although there are many caveats to extrapolating effects from murine models to humans, *in vivo* models are invaluable for elucidating the biology of FcγRs and generating sound hypotheses for testing in humans. Studies with FcγR knockout mice or blocking antibodies, effector cell depletion, and antibody isotype comparisons have greatly expanded our knowledge of the role of mouse FcγRs and effector cells as mediators of antibody efficacy.

Murine FcγR biology includes three activating receptors (FcγRI, FcγRIII, and FcγRIV) and one inhibitory receptor (FcγRIIb). Murine FcγRI is expressed on monocytes, macrophages, and DCs. Murine FcγRIII, as in humans, is expressed on monocytes, NK cells, DCs, and neutrophils; it is the only FcγR expressed on murine NK cells. The more recently discovered FcγRIV (CD16-2) [19,20] is expressed on monocytes, macrophages, DCs, and neutrophils. Finally, FcγRIIb is expressed on a broad range of hematopoetic cells including DCs, macrophages, and neutrophils. There are some similarities in the properties and functions of mouse and human FcγRs, including the presence of activating and inhibitory receptors, signaling biochemistry, and types of immune cells that utilize them to mediate effector functions. However, there are also important differences that must be considered when extrapolating results of murine *in vivo* studies. These include differences in homology, isotype affinities, and disparate expression patterns of each mouse FcγR and its closest human homolog (Table 3). For example, though FcγRIV's closest human sequence homolog is FcγRIIIa, its expression pattern and functional profile are more similar to that of human FcγRIIa.

TABLE 3

Human and Mouse FcγRs

Cell type	FcγR expression	
	Human[a]	Mouse[b]
MΦ	FcγRI	FcγRI
	FcγRIIa	FcγRIV
	FcγRIIb	FcγRIIb
	FcγRIIIa	FcγRIIII
DC	FcγRI	FcγRI
	FcγRIIa	FcγRIV
	FcγRIIb	FcγRIIb
	FcγRIIIa	FcγRIII
Neutrophil[c]	FcγRI	
	FcγRIIa	FcγRIV
	FcγRIIb	FcγRIIb
	FcγRIIIb	FcγRIII
NK	FcγRIIIa	FcγRIII

[a] FcγRIIc is expressed variably on human effector cells and is not included for simplicity.
[b] Mouse and human receptors are aligned as suggested functional analogs.
[c] FcγRI is expressed on activated but not resting neutrophils in humans. To our knowledge inducible expression of FcγRI on activated mouse neutrophils has not been shown.

Genetic knockout mice were crucial in demonstrating convincingly that FcγRs are relevant to the *in vivo* activity of anti-tumor mAbs. Early studies showed that the common γ-chain, utilized by all murine activating FcγRs, is essential for antibody-mediated immune responses [21], anti-tumor efficacy [22], and tumor vaccination efficacy [22]. In seminal work, Clynes, Ravetch and colleagues demonstrated with $\gamma^{-/-}$ mice and several antigen/antibody systems, including the anti-Her2 antibody Herceptin® (trastuzumab) and the anti-CD20 antibody Rituxan® (rituximab), that activating FcγRs are crucial for optimal antibody efficacy against tumors [22,23]. Consistent with these observations Tedder and colleagues have shown that depletion of normal B cells from bone marrow, blood, spleen, and lymph node by a panel of murine anti-CD20 antibodies is dependent on the common γ-chain [24,25].

In a tour-de-force assessment of the relative contributions of murine IgGs and FcγRs, Tedder and colleagues examined the B cell depleting capabilities of murine anti-CD20 isotypes in the context of various perturbations, including genetic knockouts of activating FcγRI and FcγRIII or the addition of blocking antibodies to FcγRIV [25]. There are three major isotypes of murine antibodies, each with unique FcγR profiles: IgG1, IgG2 (a, b and c subtypes), and IgG3. The Tedder studies showed that most if not all of the effector function of the highest potency isotypes IgG2a/c and IgG2b was attributable to FcγRIV, which is expressed on monocytes and macrophages but not NK cells [25]. FcγRIV-blocking antibodies almost completely abrogated IgG2a/c-mediated and IgG2b-mediated B cell depletion in wild-type (WT) mice. This result reconciled observations in this and the earlier study [24] that while B cell depletion in $\gamma^{-/-}$ mice was minimal, B cells were still deleted in the FcγRI$^{-/-}$, FcγRIII$^{-/-}$, and FcγRI$^{-/-}$/FcγRIII$^{-/-}$ context. The dependence of *in vivo* activity on FcγRIV and expression of this receptor on phagocytic populations are consistent with the demonstration that anti-CD20 B cell depletion is minimal in mice treated to remove macrophages [24]. The dominant role of macrophages and FcγRIV is further consistent with the observed isotype dependence in these studies: IgG2a/c and IgG2b have preferential affinity for FcγRIV.

Although the B cell depletion capacity of the most potent isotypes was definitively tied to FcγRIV, this result should not necessarily lead to the generalization that antibody-mediated tumor clearance in mice is carried out exclusively by this receptor. On the contrary, the study also revealed that when the murine IgG1 isotype was used its efficacy crucially depended on FcγRIII. The different cell types and FcγR-dependent effector mechanisms tapped by the different IgG isotypes is a critical consideration for interpreting results from mouse models. Herlyn *et al.* [26] have reported that only IgG2a had high activity against tumor xenografts *in vivo*, solely mediated by macrophages without the involvement of T or NK cells. Of note, the authors observed that IgG1 antibodies exhibited *in vitro* cytotoxicity similar to IgG2a antibodies but were inefficacious *in vivo*.

The attenuating effect of the inhibitory receptor FcγRIIb on antibody immunotherapy has been observed in mouse models under certain conditions. Employing syngenic and xenograft models, Clynes *et al.* [23] compared the therapeutic efficacies of antibodies in FcγRIIb$^{-/-}$ versus WT mice. A murine IgG2a anti-melanoma antibody prevented metastasis at lower concentrations when FcγRIIb was not present to inhibit activating FcγRs. Consistent with this result, in murine models of lymphoma and breast carcinoma, Rituxan and Herceptin were active at reducing tumors in FcγRIIb$^{-/-}$ mice at significantly lower concentrations than required in WT mice. It is worth noting, however, that the improvements to activity were not observed at higher antibody doses typical for therapeutic administration. Increased potency of antibodies in FcγRIIb$^{-/-}$ mice was also observed in the antibody-induced B cell depletion studies [25]. The most significant increases occurred in lymph node B cell populations, where the potency of an IgG2c antibody in FcγRIIb$^{-/-}$ mice was increased 10-fold relative to its potency in WT mice. Since FcγRIIb is expressed on myeloid lineage cells, this result further confirms their important role in antibody-driven anti-tumor responses. The lymph node B cell depletion by the FcγRIII-dependent isotype IgG1 was also enhanced in the FcγRIIb$^{-/-}$ mice, suggesting that at least some of the efficacy of this isotype is mediated by macrophages expressing FcγRIII (rather than NK cells, which do not express FcγRIIb).

Although the FcγR-mediated activity of murine IgGs has been well studied, much work has also been done with human isotypes, particularly human IgG1 as it is the most commonly used scaffold for therapeutic antibodies. IgG1 is also generally the most effective of the human isotypes in mouse models, though IgG4 is often comparable in activity [27,28], oddly so given its low effector function with human cells. In contrast to the monocyte/macrophage-dependence of the most active murine antibodies, the story with human IgG1 is more complex and implicates a diverse cellular population of effectors. Zeng *et al.* demonstrated that efficacy of an anti-ganglioside GD2 human IgG1 against neuroblastoma [29] is fully abrogated in the absence of NK cells. Hernandez-Ilizaliturri *et al.* [30] reported that neutrophils and NK cells, but not macrophages, contributed to prolonged survival in the murine B cell lymphoma model treated with the human IgG1 antibody Rituxan. A recent study that tested a human IgG1 anti-CD70 mAb demonstrated that depletion of neutrophils, NK cells, or macrophages all had a negative impact on efficacy against a lymphoma xenograft [31]. And of further note, Eisenbeis *et al.* generated data strongly implicating NK cells for Rituxan-mediated efficacy in a mouse lymphoma model [32]. This study is of particular interest, since it used a hu-PBL-SCID model wherein human lymphocytes are engrafted into the mice to serve as effector cells, thus matching human FcγRs with human antibodies as is generally desired.

An unfortunate practicality is that most xenograft models must be performed in immunocompromised mouse systems such as nude and SCID, which lack the adaptive T and B cells of the immune system. Luckily, a small number of studies have provided a glimpse of the potential of antibody therapies in the context of a fully functioning immune system. Such studies have demonstrated the importance of the cooperation of phagocytic cells (possibly macrophages) and tumor-specific CD8+ cells for antibody-mediated tumor regression. Dyall *et al.* [33] created a system to probe effector cell dependence of an anti-CD4 antibody by working with a murine CD4+ lymphoma transfected with chicken ovalbumin (OVA) to monitor CTL epitopes. Only phagocytic (CD11b+) cells and CTLs (OVA-specific CD8+ cells) were obligate for antibody-mediated ablation of established tumors. Macrophages were able to infiltrate these tumors independent of the anti-CD4 antibody. However, only this tumor-specific antibody, signaling through FcγRs, could facilitate the expansion of OVA-specific CD8+ cells that subsequently infiltrated and cleared the tumors. In the absence of tumor-specific CTLs, antibody therapy was ineffective at tumor clearance. Another example of T cell dependence in antibody efficacy was documented by Reilly and colleagues [34]. Using mice that were engineered to spontaneously develop mammary cancer (via expression of rat Her2/neu), this group demonstrated that the survival benefit of treatment with Her2-specific antibodies is severely attenuated when either CD8+ or CD4+ T cells were removed. These two studies nicely demonstrate the bridging of the innate and adaptive immune systems by antibody/FcγR interactions, presumably through priming of tumor-specific CTLs by DCs or macrophages (Box 3). They also highlight the incomplete view one gets when working with immunocompromised models that are more typically used for preclinical testing of antibodies.

In summary, the mechanism of action of antibodies *in vivo* is strongly dependent on their isotype. In mouse models where isotype dependence has been systematically studied, murine IgG2a/c and IgG2b are the most potent, and their activities are mediated predominantly by monocyte populations expressing FcγRIV and subject to downmodulation by engagement of FcγRIIb. Other studies, particularly with human IgG1 antibodies, highlight a potentially important role for NK cells and neutrophils. These differences are presumably based largely on the relative binding properties of the various isotypes to murine FcγRs, but may also have some dependence on properties of the target and tumor. In immunocompetent models, T cells have also been shown to be important for complete efficacy of the administered antibodies. Although tempting, extrapolating the roles of murine effector cells directly to the design of human therapeutic antibodies should be done with a dose of healthy skepticism, particularly due to incomplete parallels between murine and human effector cells, antibody isotypes, and FcγRs.

Human clinical data—links to therapeutic relevance

A variety of information relating to the role of Fc receptors in antibody-mediated cancer therapy can be derived from human clinical studies. The two most compelling of these are associations

of functionally relevant FcγR polymorphisms with clinical responses to administered antibodies and the data collected on the infiltration and/or recruitment of various effector populations into tumor tissues.

Polymorphisms

Before application to antibody therapies, FcγR polymorphism associations had been intensely studied as predictors of disease incidence or susceptibility, particularly for infectious [35,36] and autoimmune diseases [37]. The basic concept is that possession of the higher function (tighter IgG binding) alleles of activating FcγRs should provide a predisposition for fighting bacterial infections (the relationships for autoimmune disease are more complex). Studies have shown, for example, that possession of the R131 polymorphic form of FcγRIIa is a risk factor for pneumonia, periodontitis, and fulminant meningococcal septic shock, consistent with *in vitro* studies demonstrating inferior phagocytosis of IgG2-opsonized bacteria by macrophages that are homozygous for this lower affinity form of FcγRIIa [38].

More recently, and central to the present discussion, several investigators have examined the relationship between FcγR polymorphisms and clinical responses to antibody therapies (Table 4). Two independent studies [39,40] demonstrated that Rituxan-treated follicular lymphoma patients who were homozygous for the higher affinity form of FcγRIIIa (V/V158) had significantly prolonged periods of progression-free survival relative to patients heterozygous or homozygous for the lower affinity F158 form. Because Rituxan is of the IgG1 isotype, it has a fivefold affinity preference for V158 FcγRIIIa relative to F158 [41,42]. Consistent with these observations, *in vitro* studies demonstrate higher average cytotoxic potency and activation of V/V158 relative to F/F158 NK cells [43]. The results clearly confirm the importance of FcγRIIIa for antibody-mediated anti-tumor efficacy, at least for CD20 and probably for the hematological setting in general, and still stand as the most compelling pieces of clinical data justifying the significant investment of many companies in FcγRIIIa-enhancing antibody technologies (Box 1, Table 1). Interestingly, or perhaps confusingly, Weng and Levy also documented a survival relationship with the two forms of FcγRIIa (H131 versus R131), despite absence to date of any evidence that there is a functional difference for IgG1 isotype antibodies. Indeed, we have used surface plasmon resonance measurements (Biacore) to directly com-

TABLE 4

FcγR polymorphism and clinical outcome

Study	Treatment	IgG	Indication[a]	End Points & patients[b]	Response & significance[c]		
					V/F158 FcγRIIIa	H/R131 FcγRIIa	I/T232 FcγRIIb
Cartron *et al.* [39]	Anti-CD20	IgG1	Follicular NHL	12 month OR, $n = 49$	VV 90% VF, FF 51% **$P = 0.03$**	N.I.	N.I.
Weng and Levy [40]	Anti-CD20	IgG1	Follicular NHL	12 month OR, $n = 87$	VV 75% VF, FF 26% **$P = 0.002$**	HH 55% HR, RR 26% **$P = 0.027$**	N.I.
Farag *et al.* [95]	Anti-CD20	IgG1	CLL	2 month PR, $n = 30$	VV 33% VF 42% FF 50% $P > 0.2$	HH 78% HR 31% RR 65% $P = 0.7$	N.I.
Weng *et al.* [96]	Idiotype vaccine post-chemo		Follicular NHL	PFS, $n = 136$	VV 8.21 years VF, FF 3.38 years **$P = 0.09$**	HH 4.83 years HR, RR 4.68 years $P = 0.947$	N.I.
Weng and Levy [48]	Anti-CD20	IgG1	Follicular NHL	TTP, $n = 92$	VV 534 VF, FF 170 **$P = 0.023$**	HH 434 HR, RR 140 **$P = 0.005$**	II 203 days II, IT 276 days $P = 0.739$
Kim *et al.* [97]	Anti-CD20[d] + chemo	IgG1	DLBCL	ORR, $n = 113$	VV 98% VF 90% FF 50% **$P < 0.001$**	HH 95% HR 92% RR 75% $P = 0.137$	N.I.
Cheung *et al.* [45]	Anti-GD2 +GM-CSF post-chemo	mIgG3	Neuro blastoma	PFS & Hazard Ratio, $n = 136$	PFS VV 5.4 years VF 11.3 years FF 8.0 years Hazard VV 1.63 VF, FF 1 $P = 0.3$	PFS HH 6.8 years HR 10.2 years RR 47.0 years Hazard RR 0.57 HH, HR 1 **$P = 0.049$**	N.I.
Musolino *et al.* [44]	Anti-Her2 + taxane	IgG1	Metastatic breast cancer	RR & PFS, $n = 40$	**Significant**, data not yet disclosed	**Significant trend**, data not yet disclosed	Not yet disclosed

[a] NHL, non-Hodgkin's lymphoma; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B cell lymphoma.
[b] OR, objective response; PR, partial response; PFS, progression-free survival; TTP, time to progression; ORR, overall response rate; RR, response rate.
[c] Bold indicates significant correlations ($P < 0.05$). N.I., not Investigated.
[d] Notably, no correlation with V/F158 FcγRIIIa polymorphism was noted in the chemotherapy only arm (no anti-CD20 antibody).

pare the affinities of IgG1 for H131 and R131 FcγRIIa and have found them to be identical. This suggests that the clinical correlation is due to some unknown relationship of FcγRIIa, or more interestingly that clinical outcome is partly due to a Rituxan-elicited immune response that consists of IgG2 antibodies, which do bind differentially to the H/R131 FcγRIIa alleles (Table 2). Since these original groundbreaking studies, a number of additional polymorphism studies have been published (Table 4). Several have again revealed the importance of FcγRIIIa in antibody-treated hematological diseases. Assigning the enhanced anti-tumor effects of FcγRIIIa (V/V158) to an effector cell type is challenging, given that FcγRIIIa can be expressed on lymphoid and myeloid cells, including NK cells, macrophages, and DCs. The current gestalt, however, is that the effects are dominated by NK cells.

Although clinical investigators are currently looking for FcγRIIIa associations in antibody therapy of solid tumor diseases, few results have been published to date. A recent AACR abstract reports that the FcγRIIIa V/V158 genotype was significantly correlated with the response rate and progression-free survival of 20 Herceptin-treated metastatic breast cancer patients [44]. Any further data that emerge will be quite informative, particularly in light of some skepticism that exists on the role of FcγR in antibody efficacy against solid tumors (see discussion of infiltration below). Besides the early and unexplained FcγRIIa associations observed by Weng and Levy [40], the only published FcγRIIa association with antibody therapy is in the solid tumor neuroblastoma. Cheung *et al.* observed with anti-GD2/GM-CSF cotherapy of neuroblastoma that the FcγRIIa (R/R131) genotype correlated with progression-free survival [45]. The study clearly demonstrates that under the right circumstances, FcγRIIa-mediated anti-tumor effects can have considerable impact. Furthermore, since FcγRIIa is expressed on phagocytic myeloid cells such as neutrophils, macrophages, and DCs, the results emphasize the importance of at least one of these cell types for antibody efficacy against solid tumors. Given that the authors only observe the association when GM-CSF is provided as cotherapy, the strongest hypothesis is that the enhanced anti-tumor activity in R/R patients comes from the GM-CSF-stimulated neutrophil or monocyte/macrophage populations.

We should emphasize that this rare window into the relevance of FcγRIIa for anti-tumor efficacy appeared with the use of outdated technology—a murine IgG3 antibody. Murine IgG1 and IgG3 antibodies have long been known to prefer the so-called 'high responder' R131 allele of FcγRIIa (this can be disorienting given that human IgG2 prefers instead the H131 form). Unfortunately, opportunities to explore the anti-tumor role of FcγRIIa more systematically are hindered by the lack of a functionally relevant preference of human IgG1 for either H/R form. A major blind spot thus exists for the human antibody isotype most commonly used in cancer therapy. Some hope remains in the study of the second most therapeutically popular human isotype IgG2, though it is frequently used in settings where effector function is unwanted. Although IgG2 does not exhibit any NK cell mediated ADCC activity, it can mediate phagocytosis, particularly when interacting with the H131 form of FcγRIIa [38]. One example of a clinically applied IgG2 antibody is Vectibix® (panitumumab), an anti-EGFR antibody for treatment of colon and other cancers, and we enthusiastically advocate polymorphism studies with this agent.

Given the potential importance of the inhibitory receptor, studies on the impact of FcγRIIb polymorphisms could be very informative. There has been some characterization of promoter and transmembrane-domain (I/T232) polymorphisms for FcγRIIb, particularly as they affect susceptibility to the autoimmune disease SLE. The functional relevance of this polymorphism is beginning to be established [46,47]. However, where studied with antibody therapy, no association between the I/T232 alleles and clinical efficacy has been observed [48].

Effector cell infiltration

While *in vitro* data demonstrating interactions between antibodies, target cells, and effector cells can be quite intriguing, one should also consider whether or not, in the *in vivo* setting, all of those entities are present together in the right place in the right state at the right time. The answer may depend dramatically on the type of tumor under consideration.

Much of the interest in antibodies and effector function comes from studying ADCC reactions with target cells. We, and others before us, have observed that NK cells mediate essentially all of the ADCC measured with standard assays using PBMCs as effector cells. In the majority of cases, robust ADCC can be observed *in vitro* with antibodies targeted to cell lines representative of a wide variety of hematological and solid tumor cells. However, this situation is arguably artificial. For hematological diseases, one can make the argument that NK cells will be readily available and prepared to mediate these effects *in vivo*. But for solid tumors, the dogma exists that NK cells are poor tumor infiltrators [49], and one wonders how misleading *in vitro* ADCC might be. When measured, typically very small numbers of NK cells are found in direct contact with tumor cells, but they can be seen in the tumor stroma. A glimmer of hope for NK cells comes from a recent study demonstrating that tumor infiltration of NK cells increases after treatment with Herceptin [50]. The mechanism leading to increased localization is unclear, but we speculate that the NK cells are recruited after engagement of other effector populations and an ensuing chemokine release, consistent with well-established responsiveness of NK cells to CXC chemokines [51]. Further corroboration of the importance of NK cells could come from definitive demonstration of an FcγRIIIa polymorphism association with Herceptin efficacy, though the expression of FcγRIIIa on other effector populations remains a confounding factor.

The most famous of tumor-infiltrating cells are macrophages [52], usually referred to as TAMs (tumor-associated macrophages). Their frequent presence raises the possibility that they are key mediators of antibody efficacy against solid tumors, consistent with many of the observations in mouse models that monocyte populations are crucial for antibody-mediated tumor reduction. A potential problem exists, however: TAMs have recently earned notoriety for being part of the problem [53], existing in an immunosuppressive, tumor-supporting, M2 state (akin to the Th2 state of helper T cells) with reduced FcγR expression [52]. TAMs are thought to exist in a symbiotic relationship with tumors and can even produce proangiogenic factors to further promote tumor survival. DCs are other FcγR-positive effector populations with a healthy reputation for infiltrating tumors. Like macrophages, tumor-infiltrating DCs (TIDCs) exist in a variety of states, and there is evidence that a common tumor escape strategy is to

downmodulate activation and migratory DC pathways [54], both essential for the initiation of anti-tumor immune response. These common issues raise the obvious question about the antibody reactivity of TAMs or TIDCs, whether M2 polarization and DC suppression can be reversed with antibody therapies directed against tumor antigens and whether reversal could potentially be dependent on indirect effects such as Th1 cytokine release from other effector cells. One also wonders if this will be an issue with newly recruited macrophages or DCs. A related question on the state or phenotype of TAMs and TIDCs is their levels of FcγR expression, in both the absolute and relative sense. Because cytokines can modulate the balance of activating versus inhibitory FcγRs on macrophages and DCs, it is possible that antibody therapy, via FcγR-mediated cytokine release, can contribute to tipping the balance in favor of an anti-tumor immune response. Interestingly, there is at least one published example demonstrating re-direction of M2 macrophages and inactive DCs using specific stimuli [55].

Target FcγR profiles and tailored effector responses

As discussed in detail in Box 1, a variety of technologies have emerged for modifying the affinity of human antibodies to the various FcγRs. With so much effort and promise in this area, it is healthy to ask which receptors and which selectivities will probably provide the most clinical return on our engineering investments. The high affinity of FcγRI for monomeric IgG is thought to preclude a capacity to distinguish between unbound antibody and immune complexes. Together with the lack of impact observed in FcγRI$^{-/-}$ mice, the data suggest that this receptor is not a key player in antibody anti-cancer activity. The absence of cytoplasmic signaling by FcγRIIIb and the variable expression and poor understanding of FcγRIIc have relegated these receptors to the same 'irrelevant' category. Of course, we must be cautious that the lack of functional orthologs of the latter receptors in mice, as well as absence of polymorphism signals from clinical studies for these three receptors makes us partially blind to their clinical relevance. Nonetheless, the current data support the greatest roles for FcγRIIa, FcγRIIb, and FcγRIIIa, and therefore these receptors in our view should receive the emphasis for engineering.

Fc engineering technologies can be roughly grouped as technologies that perturb the glycosylation state of the antibodies (defucosylation, in particular) and those that modify the amino acid sequence. Defucoyslation appears to elicit a specific enhancement in affinity for FcγRIIIa [41,56], while amino acid modifications create a more diverse range of effects. At first glance, the ideal FcγR selectivity profile of an engineered antibody would be high affinity for all activating FcγRs and low affinity for the inhibitory FcγRIIb. Beyond the fact that there are technical challenges to this approach, we should also consider whether the answer depends on the situation. Given the wide variety of immune effector mechanisms, their regulation, and their capacity for tumor infiltration, this certainly seems plausible if not likely. At the highest level, one might consider treating hematological versus solid tumors with a different FcγR selectivity. With the current clinical polymorphism data indicating an important role for FcγRIIIa [39,40], we speculate that pure FcγRIIIa-mediated effects are a good bet for hematological diseases. For solid tumors, however, there are a number of other considerations that might impact the choice of ideal antibody. First, there is the issue of effector cell infiltration of solid tumors. Since the more regularly infiltrating effector cells include those of the FcγRIIa/IIb-expressing myeloid lineage (macrophages, DCs, neutrophils), an antibody that selectively engages FcγRIIa might be most efficacious. However, macrophages and DCs can also express FcγRIIIa, complicating this decision, and despite the lack of strong support for an NK cell role in solid tumor therapy, one is generally hesitant to give up FcγRIIIa effects. Another factor that might influence the choice is the MHC class I expression levels on the tumors, which can vary extensively among different tumor types. Indeed, different tumors have varying propensities for downregulating class I [57], with breast and prostate cancers having the highest rates of MHC downregulation [58]. Although this is a tumor strategy for evasion of adaptive immunity, it does potentially expose them to higher NK cell killing because the MHC/KIR inhibitory interaction is reduced. A final factor in choosing FcγRIIIa selectivity versus FcγRIIa is the potential for autoimmune toxicity. Given published data on the role of FcγRIIa in promoting DC cross-priming and adaptive immunity, these effects, while useful for tumor killing, might be less desirable in situations where the target antigen is widely or highly expressed in normal tissues.

Concluding remarks

Years of thoughtful studies by scientists and clinicians have built a solid framework for thinking about how to engineer antibodies for optimal engagement of the immune system and greater tumor eradication. Observations from disparate systems tell us that FcγRs mediate a variety of effector functions that are important for clinical efficacy of therapeutic antibodies. They are also beginning to tell us more about the ways that antibodies can be further tuned to make them work even better. However, a number of gaps still exist in our knowledge, and we expect these will be systematically filled as the current set of hypotheses is thoroughly explored. Our own wish list includes more polymorphism studies, greater definition of the role of FcγRIIb in human FcγR biology, and further clarity on the relative roles of various effector populations. Finally, we should see data emerging over the next few years revealing the impact that the first wave of Fc technologies has made on clinical efficacy, confirming that we are indeed on the right track.

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