

Review

# Enhancing antibody-dependent cell-mediated cytotoxicity: a strategy for improving antibody-based immunotherapy

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## ABSTRACT

The targeting of surface antigens expressed on tumor cells by monoclonal antibodies (mAbs) has revolutionized cancer therapeutics. One mechanism of action of antibody-based immunotherapy is the activation of immune effector cells to mediate antibody-dependent cell-mediated cytotoxicity (ADCC). This review will summarize the process of ADCC, its important role in the efficacy of mAb therapy, how to measure it, and finally future strategies for antibody design that can take advantage of it to improve clinical performance.

**Statement of Significance:** Targeted mAb therapy represents a promising therapeutic strategy for many cancer types. ADCC is a crucial mechanism underlying targeted antibody-based immunotherapy approaches. Many patients have limited responses to mAb therapy and there is a great need for antibodies with enhanced clinical efficacy. Designing and engineering antibodies with enhanced ADCC eliciting properties will improve patient outcomes.

**KEYWORDS:** ADCC; monoclonal antibody; immunotherapy; FcγR; Fc glycosylation

## INTRODUCTION

Targeted therapy utilizing monoclonal antibodies (mAbs) has established immunotherapy as a powerful new tool in the fight against cancer. Antibodies directed towards tumor cell antigens can cause tumor cell death by both direct and indirect mechanisms. Direct mechanisms for therapeutic antibodies to induce cell death include blocking growth factor receptor signaling, direct transmembrane signaling, and acting as targeted vectors for toxic payloads such as radioisotopes [1]. The indirect mechanisms require engagement with components of the host immune system and are comprised of complement-mediated cytotoxicity, antibody-dependent cellular phagocytosis, and antibody-dependent cell-mediated cytotoxicity (ADCC), which is the subject of this review. The phenomenon of ADCC was first described in 1965 by Erna Möller who demonstrated that lymphoid effector cells only induced cytotoxic death

of tumor cells when in the presence of anti-serum from rabbits previously inoculated by those tumor cells [2]. It was eventually discovered that the factor in the anti-serum required for cell-mediated cytotoxicity was immunoglobulins [3] and the process was initially termed “antibody-dependent lymphocyte-mediated cytotoxicity” [4]. Subsequent research identified multiple cell types capable of facilitating cytotoxicity. Thus, ADCC was established as an immune mechanism involving three components: effector cells, antibodies, and target cells opsonized by the antibody. As mAb therapy has revolutionized cancer treatment, ADCC has become more relevant in a clinical context. Clinical trials have demonstrated that many mAbs function, in part, by eliciting ADCC. Now that an increasing number of therapeutic antibodies are receiving approval from the FDA and entering the clinic, a significant goal moving forward is to construct more effective mAbs. Future design strategies will have to incorporate an understanding

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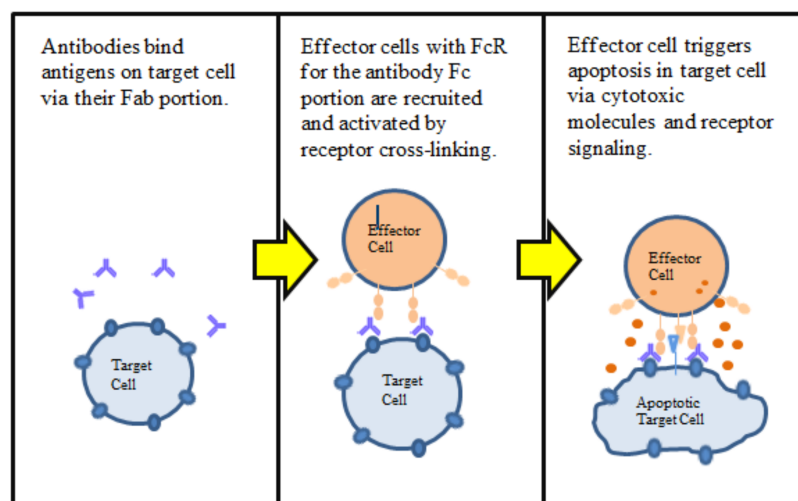


Figure 1. Diagram of ADCC.

of ADCC and how to best enhance its effects to improve cancer patient outcomes.

## ADCC MECHANISMS

ADCC is the process by which antibodies coat a target cell and recruit effector cells to induce target cell death via non-phagocytic mechanisms (Fig. 1). Antibodies can bind to their specific antigens on the target cell surface via their antigen-binding fragment (Fab) portions and interact with effector cells via their fragment crystallizable region (Fc) portions thereby acting as bridges that link the effector to a target. While several classes of human antibodies can mediate ADCC, including IgG, IgA, and IgE, IgG1 is the most used subclass for cancer therapeutic antibodies [5]. In order for an effector cell to carry out ADCC it must express Fc receptors (FcR) that will bind the antibody [6]. The known classes of FcR include  $Fc\gamma R$ , which bind IgG;  $Fc\alpha R$ , which bind IgA; and  $Fc\epsilon R$ , which bind IgE.  $Fc\gamma R$  are the most important for tumor cell clearance by myeloid cells and are comprised of activating  $Fc\gamma RI$  (CD64),  $Fc\gamma RIIA$  (CD32A),  $Fc\gamma RIIIA$  (CD16A), and inhibitory  $Fc\gamma RIIB$  (CD32B) receptors [7]. Once the  $Fc\gamma R$  binds antibody it triggers receptor cross-linking and downstream signal propagation. Activating  $Fc\gamma R$  signal via their immunoreceptor tyrosine-based activation motifs while inhibitory  $Fc\gamma R$  signal via their immunoreceptor tyrosine-based inhibitory motifs [8]. Many effector cells also express other receptor types such as the inhibitory killer inhibitory receptors (e.g. KIR) and activating NKG2D receptors on natural killer (NK) cells. The delicate balance amongst the activating and inhibitory pathway signaling ultimately determines effector cell response. Myeloid cells capable of acting as ADCC effectors are NK cells, monocytes, macrophages, neutrophils, eosinophils, and dendritic cells [9]. Once these effector cells have been activated they mediate target cell death through three key mechanisms: cytotoxic granule release, Fas signaling, and elaboration of reactive oxygen species. The main and best characterized mechanism utilized in ADCC is the release of perforins and

granzymes from effector cell granules. Upon activation, effector cells such as NK cells polarize and exocytose their granules in a calcium-dependent manner [10]. Perforin and Granzyme B work in concert to induce cell death. Perforins create pores in the cell membrane that facilitate granzyme B entry into the target cell, resulting in DNA fragmentation and apoptosis [11]. Additionally, research has suggested that activated effector cells will upregulate Fas ligand expression in order to cause apoptosis in the target via Fas signaling [12]. While multiple myeloid lineage cells are capable of ADCC, in the context of cancer immunotherapy NK cells appear to be the major effector cell type *in vivo*. The clinical efficacy of many targeted mAb therapies has been demonstrated to be NK cell dependent [13]. NK cells highly express activating  $Fc\gamma RIIIA$  and do not express the inhibitory  $Fc\gamma RIIB$ ; therefore modifying antibody interactions specifically through  $Fc\gamma RIIIA$  has become of interest for cancer immunotherapy.

## ROLE OF ADCC IN IMMUNOTHERAPY

Currently used mAbs are known to employ a variety of the indirect mechanisms mentioned above to exert cytotoxic effects on tumor cells. Although each of these mechanisms of action is believed to be important, there has been some debate about their role *in vivo*. Many antitumor mAbs were shown to mediate ADCC *in vitro*; however for a time the relevance to therapeutic efficacy was unknown. Seminal work from Clynes *et al.* demonstrated that ADCC was a substantive contributor to the *in vivo* activity of the mAbs trastuzumab and rituximab using mouse models [14]. Their results indicated that clinical efficacy depended upon the balance of engagement with activating  $Fc\gamma RIII$  and inhibitory  $Fc\gamma RIIB$ . This provided the initial rationale for designing mAbs that could preferentially bind the activating receptor. Additional mechanistic studies in mice confirmed that  $Fc\gamma R$  on innate immune system mononuclear cells were required for tumor clearance [15]. Subsequently, de Haij *et al.* demonstrated that ADCC was essential for mAb therapy in a novel mouse model where a mutant

Fc $\gamma$ R incapable of ADCC was unable to clear tumors [16]. One drawback to the majority of these studies was that the mAbs used to assess whether ADCC played a significant role in *in vivo* efficacy utilized additional mechanisms of action such as signaling perturbation. Therefore, these results proved ADCC was required but not necessarily whether ADCC by itself would be sufficient for tumor clearance. Recent work with mAbs to tumor-associated antigens that rely solely on ADCC as their mechanism of action confirmed that ADCC alone is able to mediate therapeutic benefit [17].

Since Fc $\gamma$ R is crucial for mAb efficacy in mouse models, its role in human cancer patients was explored using clinical trial data to correlate Fc $\gamma$ R polymorphisms with clinical outcomes. In humans Fc $\gamma$ RIIA is polymorphic at position 131 and alleles exist that code for either a histidine (H) or arginine (R). Fc $\gamma$ RIIA is polymorphic at position 158 with alleles for either a valine (V) or phenylalanine (F). Individuals with Fc $\gamma$ RIIA-131H/H and Fc $\gamma$ RIIA-158V/V genotypes have Fc $\gamma$ R with higher affinity for IgG1 and therefore enhanced ADCC [18,19]. This augmented ADCC was demonstrated in several studies to cause Fc $\gamma$ RIIA-158V/V lymphoma patients to have better clinical response to rituximab, a mAb against CD20 [20–22]. Interestingly, although the inhibitory Fc $\gamma$ RIIB is also polymorphic, no association between inhibitory receptor genotype and rituximab response was observed [23]. Both activating Fc $\gamma$ RIIA and Fc $\gamma$ RIIA genotypes associated with higher affinities for Fc were strong predictors for improved survival when colorectal patients were treated with cetuximab, a mAb targeting the epidermal growth factor receptor (EGFR), and metastatic breast cancer patients were treated with trastuzumab, a mAb targeting human epidermal growth factor receptor 2 (HER2) [24,25]. However, in nonmetastatic breast cancer patients there was no correlation between Fc $\gamma$ R genotypes and trastuzumab efficacy [26]. This suggests that the role of ADCC may be different depending on cancer stage. In more recent studies, the Fc $\gamma$ R polymorphisms in patients treated with cetuximab or trastuzumab were directly linked to the ability to carry out ADCC by *in vitro* studies using patient-derived immune cells [27,28]. In neuroblastoma patients treated with anti-GD2 antibodies, a similar association between the high-affinity Fc $\gamma$ RII/IIIA genotypes and survival was found, and appeared to be linked to an increase in ADCC activity [29]. These multiple analyses confirm that patients with higher-affinity Fc $\gamma$ R have better clinical outcomes when administered by mAb therapy, irrespective of cancer type and the mAb target antigen.

In addition to examining Fc $\gamma$ R polymorphisms, other studies have used patient samples from clinical trials to investigate the relative importance of ADCC to therapeutic success. For example, patients with HER2-positive breast cancer treated with trastuzumab, docetaxel, or another treatment had immunohistochemistry performed on their tumor samples to measure granzyme B expression as a surrogate marker of ADCC activity. Those who received trastuzumab were found to have better overall survival and had much higher levels of ADCC compared to the other cohorts [30]. Furthermore, patient-derived *in vitro* models demonstrated ADCC as a major therapeutic mechanism of

rituximab in non-Hodgkin lymphoma [31] and anti-CD38 antibodies in multiple myeloma [32,33]. There is now strong evidence that ADCC plays a critical role in facilitating mAb antitumor responses in patients. In fact, additional variables that would affect ADCC such as target antigen expression level and density, mAb isotype, and mAb dose all correlate with clinical response [34]. Now that ADCC is recognized as a determining factor for mAb therapy success, research and development of novel mAbs have shifted towards designing mAbs with improved capacity to mediate ADCC.

## MEASURING ADCC

Strong evidence for the critical role of ADCC in targeted mAb clinical efficacy has inspired efforts to engineer mAbs with enriched ADCC functionality. Surprisingly, techniques currently employed to quantify ADCC require continued development in order to properly assess and compare the properties of newly developed mAbs. The first assay for calculating cytotoxicity was published in 1968 and involved radiolabeling target cells with  $^{51}\text{Cr}$  [35].  $^{51}\text{Cr}$  release could be used as a surrogate for ADCC activity and this quickly became the gold standard assay. However, the use of radioactive substances poses regulatory and safety challenges and generally works best only when high amounts of cytotoxicity are expected. Modern fluorometric techniques have largely replaced radiolabeling with fluorescent markers that allow for more precise measurements of cellular cytotoxicity. Labeling of target cells with fluorescent dyes allowed for sensitive determination of cytotoxicity via flow cytometry [36]. Expanding upon these initial flow cytometric techniques, the VITAL assay was developed to measure cytotoxicity of multiple target populations simultaneously both *in vitro* and *in vivo* [37]. There are several newer techniques that rely on similar principles as the  $^{51}\text{Cr}$  release assay but replace radioisotopes with natural cell products. As cells are killed they release lactate dehydrogenase and other proteases that can be quantified by supplying fluorogenic substrates in order to more accurately assess cytotoxicity without the need to perform any labeling or manipulation of target cells [38,39]. A potential drawback of relying on reporter molecules is that they may spontaneously release even from live cells. Impedance-based analysis avoids this issue and has shown promise as a quantitative measure of cell-mediated cytotoxicity that can continuously measure ADCC over time [40]. More recently, scientists have recognized the need for a sensitive, reliable, and accurate cytotoxicity assay that is compliant with regulatory requirements. One such method involves combining flow cytometric and labeling-based techniques for high-throughput single-cell computer image analysis [41]. An alternative method genetically modifies target cells to express a reporter protein for a standardized assay [42]. Another variable to consider is the effector cell populations used in the assay. Currently, the most widely used cells for quantifying ADCC are peripheral blood mononuclear cells and purified NK cells from donors. Unfortunately, the Fc $\gamma$ R and KIR molecule genotype differences inherent in diverse donor-derived

cells will cause high inter-assay variability. In order to address these limitations an immortalized NK cell line that expresses the high-affinity CD16V variant has been developed that allows for more standardized ADCC assays [43]. Additional engineered effector cell lines have been created for single assay thaw and use format to further reduce variability [44]. Robust ADCC assays are not only needed for assessing mAb quality but also for predicting patient responses. Clinically available blood samples can be used without isolating NK cells in a rapid ADCC reporter assay that could be applied to cancer patients [45]. Establishing a new gold standard technique for accurately quantifying cell-mediated cytotoxicity for widespread use will be essential for proper comparison of newly designed mAbs that aim to improve therapeutic benefit by enhancement of ADCC.

### IMPROVING THERAPEUTIC BENEFIT BY ENHANCING ADCC

Now that ADCC is recognized as a crucial mechanism of action for targeted mAb cancer therapy there has been increasing interest for clinically testing mAbs engineered with properties that enhance ADCC. While there are many methods to augment ADCC, including supplementation of cytokines or manipulation of the effector cells, we focus here on the modification of therapeutic antibodies' structures [46,47]. The main strategy to enhance ADCC functionality has been to alter the Fc portion of the mAb to increase binding affinity to the activating FcγRIIIA via site-directed mutagenesis, changing Fc domain glycosylation, and/or removing Fc domain fucosylation. Creation of IgG1 variants with improved binding to activating FcγR by mutagenesis has been an effective strategy for increasing ADCC efficiency *in vitro* [48,49]. In addition to modification of Fc residues, asymmetrical engineering of the Fc portion to create heterodimers of different heavy chains yielded more stable antibodies with enhanced ADCC functionality [50]. The use of mAbs engineered by mutagenesis for optimal FcγR binding to improve therapeutic efficacy

has been further validated by preclinical animal models [51,52]. For several different antibodies, it has also been shown that alterations of the glycosylation patterns can be used to increase affinity for activating FcγR in order to boost ADCC activity [53–55]. In one study, optimization of FcγRIIIA binding only affected the ADCC properties of a subset of effector cells, particularly NK cells, and further optimization for FcγRIIIA was required to enhance ADCC mediated by other effector cells [56]. Of the oligosaccharides attached to the Fc domain fucose sugar units appear to play the largest role in determining binding to FcγRIIIA. Removal of fucose improved affinity by up to 50-fold and afucosylated mAbs mediated higher levels of ADCC both *in vitro* and *in vivo* [57,58]. These results led to the development of methods for producing mAbs that lacked fucose in their Fc region [59]. Afucosylated versions of commonly used therapeutic mAbs have superior efficacy *in vivo* [60,61] and have also entered into clinical trials [62,63]. As many of the currently available antibodies are highly fucosylated [64], this represents an important opportunity to improve the clinical efficacy of mAb therapy. Another important mAb therapeutic strategy has been to target inhibitory immune checkpoints on T cells. While mAbs against molecules such as PD-1 are either designed or engineered to eliminate their ADCC activity to prevent immune cell fratricide, novel antibodies targeting the PD-L1 expressed on tumor cells are being designed to both block the immune checkpoint pathway and have enhanced ADCC of the tumor cells [65].

Evaluating antibodies for clinical use based on their ability to facilitate ADCC is not a new concept [66] and mAbs to novel targets are being developed with ADCC in mind [67]. The introduction of targeted mAb therapy has revolutionized the treatment of cancer; however, there is still room for improvement. Detailed analysis of the mechanisms of action of mAbs has revealed that amplifying effector functions, specifically ADCC, is a promising approach to increase clinical benefit. The next generation of therapeutic antibodies must take advantage of engineering strategies to increase benefits for patients. Although many of the new mAbs have shown enhancement of efficacy

**Table 1.** ADCC-Enhancing Antibodies Approved or in Clinical Trials

Antibody	Target	Modification	Status*
Obinutuzumab	CD20	Reduced fucosylation	Completed Clinical Trials. FDA Approved for CLL in 2013.
Mogamulizumab	CCR4	Afucosylated	Completed Clinical Trials. Approved in Japan for T-Cell Lymphoma. FDA Approved for non-Hodgkins lymphoma in 2018.
Margetuximab	HER2	Fc Mutagenesis	Phase 3 Clinical Trial (NCT02492711)
MOR208	CD19	Fc Mutagenesis	Phase 3 Clinical Trial (NCT02763319)
Ublituximab	CD20	Reduced fucosylation	Phase 3 Clinical Trial (NCT02612311)
MEDI-551	CD19	Afucosylated	Phase 2 Clinical Trial (NCT01466153)
Gatipotuzumab	MUC1	Altered glycosylation	Phase 2 Clinical Trial (NCT01899599)
Tomuzotuximab	EGFR	Altered glycosylation	Phase 2 Clinical Trial (NCT02052960)
Ocaratuzumab	CD20	Fc Mutagenesis	Phase 2 Clinical Trial (NCT00003874)
RO5083945 (GA201)	EGFR	Altered glycosylation	Phase 2 Clinical Trial (NCT01326000)
TrasGEX	HER2	Altered glycosylation	Phase 1 Clinical Trial (NCT01409343)

\*A single representative of the most advanced clinical trial is included.



in preclinical models, studies in patients will ultimately reveal if this approach has general merit. A list of mAbs targeting tumor antigens engineered to augment ADCC that are currently enrolled in or have completed clinical trials is presented in Table 1. These trials will determine if ADCC-augmenting antibodies have acceptable safety profiles and meaningfully impact patient outcomes. The data implicating ADCC as a crucial player in targeted mAb cancer therapy is encouraging and suggests that advances in antibody engineering will ultimately lead to more powerful therapeutic antibodies.

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