# Isotype selection for antibody-based cancer therapy

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**Abstract**

The clinical application of monoclonal antibodies (mAbs) revolutionised the field of cancer therapy as it enabled the successful treatment of previously untreatable types of cancer. Different mechanisms play a role in the anti-tumour effect of mAbs and both target engagement with the Fab arm as well as Fc-mediated effector functions contribute to the efficacy of treatment. Because Ig isotypes differ in their ability to bind to FcRs on immune cells as well as in their ability to activate complement, they differ in the immune responses they activate. Therefore, the choice of antibody isotype for therapeutic mAbs is dictated by its intended mechanism of action. Considering that clinical efficacy of many mAbs is currently achieved only in subsets of patients, optimal isotype selection and Fc optimisation during antibody development may represent an important step towards improved patient outcome. Here, we discuss the current knowledge of the therapeutic effector functions of different isotypes and Fc-engineering strategies to improve mAbs application.

**Keywords: mAbs, isotype, Fc tail, effector functions, cancer**

## Introduction

Monoclonal antibodies (mAbs) have become an increasingly important class of drugs with a global market comprised of a total of 93 mAbs with marketing approval[1] and cancer being their most prevalent target disease[2]. Significant breakthroughs made in the areas of hybridoma technology and recombinant antibody production enabled the development of highly specific mAbs. However, for most mAbs not only the antigen-binding Fab arm determines their therapeutic efficacy, but also the antibody isotype. More specifically, the Fc tail largely dictates the downstream effector functions of an antibody through its interaction with FcRs and complement and thus the subsequent activation of the immune system **(fig.1, table 1** ). Therefore, the final outcome of the binding of an antibody to its target is critically dependent on the chosen isotype. Moreover, Fc- or glyco-engineering of the chosen isotype can be used to further optimise its effector functions and half-life. In this review we focus on optimal isotype selection for three mAb types receiving much clinical attention, which are according to their mechanism of action: (a) tumour antigen-targeting, (b) immune checkpoint inhibiting and (c) TNFR family targeting agonistic mAbs.

## Tumour antigen-targeting mAbs

### Mechanism of action of tumour antigen-targeting mAbs

The first generation of mAbs approved for clinical application - and still the most common group of mAbs in cancer therapy – consisted of mAbs directly targeting tumour antigens. These tumour antigens are, to a greater or lesser extent, important for the growth, survival and invasiveness of the tumour. The interference with tumour cell signalling pathways affects cell proliferation and leads to tumour cell death (e.g. anti-HER2, anti-EGFR)[3,4]. However, several observations in humans and mice suggest that Fc-mediated activation of immune cells is an important additional mechanism of action of many of these mAbs[4,5] (**fig.2)**

. Tumour cell bound antibodies can bind with their Fc tail to activating FcRs present on effector cells such as natural killer (NK) cells, macrophages or neutrophils, which then mediate tumour cell lysis[5]. This can occur via release of cytotoxic mediators (antibody-dependent cell-mediated cytotoxicity - ADCC) or via phagocytosis of tumour cells (antibody-dependent cell-mediated phagocytosis - ADCP). In addition, with their Fc tail, antibodies can activate the complement cascade through binding of C1q which can result in tumour cell lysis via several different mechanisms[6]. These include the formation of membrane attack complex (MAC) that directly induces the lysis of target cells (CDC) or the attraction of immune cells through the chemo-attractive activity of the complement components C3a, C4a and C5. Furthermore, the opsonisation by C3b and C4b marks the target cells for complement-dependent cell-mediated cytotoxicity (CDCC) by NK cells, macrophages/monocytes and granulocytes, or for complement-dependent cell-mediated phagocytosis (CDCP) by myeloid cells[6]. Antibody-mediated cell death also leads to the release of tumour antigens and formation of immune complexes (IC) which facilitates the initiation of anti-tumour T cell responses, sustaining the tumour control and rejection. During this process, binding to FcγRs and activation of complement have been shown to play a critical role in the uptake of IC and cross-presentation of IC-derived tumour antigens by dendritic cells (DCs) *in vivo* [7–9].

In conclusion, in addition to blocking important signalling pathways in tumour cells with their Fab arm, tumour-targeting antibodies furthermore deliver their effect through Fc-mediated ADCC, ADCP and CDC. Therefore, an antibody isotype with the highest capacity to induce these effects should show improved clinical efficacy. We will discuss different strategies to improve IgG Fc-effector functions, as well as the potential use of alternative isotypes such as IgE and IgA (**fig.4a** ).

**Optimising IgG effector function**

IgG Fc-effector functions are mediated via complement and FcγRs which are either activating (FcγRI, FcγRIIa/IIc, FcγRIIIa, FcγRIIIb[10]) or inhibitory (FcγRIIb)[11]. Since most effector cells co-express both activating and inhibitory FcγRs, the outcome of IgG binding is a result of the relative binding affinity, receptor availability and signalling capacity. The relative receptor affinity is defined as the activating-to-inhibitory (A/I) ratio[12](**fig.3** ) and differs across IgG subclasses. Thus, depending on their A/I ratio, IgG subclasses can be defined as highly activating with strong Fc-effector function (IgG1, IgG3) or poorly activating with limited Fc-effector function (IgG2, IgG4)[13]. Although less relevant for effector functions, an additional IgG receptor is the neonatal Fc receptor (FcRn) which mediates IgG transport through the placenta as well as IgG cellular recycling, providing IgG with a relatively long serum half-life and thus favourable pharmacokinetic properties[14]. FcRn also binds albumin with similar effects[14].

Currently, most of the clinically approved tumour-targeting mAbs are of IgG1 isotype which was shown to be superior to other isotypes and subclasses in inducing ADCC by mononuclear cells as well as CDC *in vitro* [15]. IgG1 achieves most of its Fc-effector functions via FcγRIIIa present on macrophages and NK cells (ADCC, ADCP), as well as via complement activation[16]. Furthermore, IgG1 shows favourable biopharmaceutical characteristics with regard to production and purification. However, independent of their specificity all tumour-targeting IgG used hitherto in the clinic displayed a therapeutic effect only in a subset of patient. Therefore, over the years different strategies have been explored to further optimise tumour-targeting mAbs, many of which focused on improving Fc-mediated functions.

*Improving activating-to-inhibitory (A/I) ratio*

One of the common approaches to improve the IgG Fc-effector functions is to optimise A/I ratio by increasing the affinity for the activating FcγRs on one hand and decreasing the binding to the inhibitory FcγRIIb on the other. Improved A/I ratio was successfully achieved by glycoengineering. The most relevant modification is afucosylation of N297 glycan which significantly increased the affinity for FcγRIIIa improving the ADCC effect *in vitro* [17], which was mirrored by improved *in vivo* anti-tumour responses in mouse models[18]. Two afucosylated mAbs already received marketing approval (mogamulizumab[19], obinutuzumab[20]) and several others are currently in clinical trials[21]. Another commonly used strategy to improve A/I ratio is the introduction of point mutations in the Fc tail[16]. The most promising mAb in this group is margetux-

imab, an anti-HER2 antibody featuring five point-mutations in its Fc tail resulting in improved binding to FcγRIIIa and FcγRIIa, as well as a decreased FcγRIIb binding[22]. This translated into improved ADCC *in vitro* [22], enhanced anti-tumour activity *in vivo* [22] and higher response rate and progression-free survival in HER2-positive metastatic breast cancer when compared to its non-Fc-engineered analogue trastuzumab[23]. Therefore, glyco- and Fc- engineered IgG1 mAbs with optimised A/I ratio are superior to non-engineered IgG1, most likely due to enhanced ADCC.

*Optimising CDC*

CDC has been recognised as an important mechanism of action for some therapeutic mAbs such as anti-CD20[24–26]. Thus, strategies to optimise Fc-mediated complement activation are currently being developed.

Intrinsically, due to its naturally occurring pentameric and hexameric forms, IgM demonstrates the highest capacity for complement activation. However, IgM has not received much attention in therapeutic mAbs development and only a few tumour-targeting IgM mAbs have been evaluated in clinical trials[27]; most prominently with PAT-SM6 receiving orphan drug designation by EMA and FDA for multiple myeloma[28,29].

Among IgG subclasses, IgG1 and IgG3 are good complement activators. Although IgG3 seems to be more potent, manufacturing issues, instability and shorter *in vivo* half-life make it a less attractive candidate for drug development. A way to combine the advantages of both IgG1 (favourable manufacturing characteristics) and IgG3 (enhanced CDC) was achieved through the construction of IgG1/IgG3 chimeric antibodies[30]. The optimal construct, called 113F, combined the CH1 and the hinge of IgG1 with the CH2 of IgG3 and a CH3 which was partly of IgG3 and partly of IgG1 origin. The nonfucosylated version of this chimeric antibody showed enhanced CDC and ADCC comparable to nonfucosylated IgG1, in addition to preserved protein A binding, important for the purification process. The improved efficacy of this chimeric construct was confirmed in cynomolgus monkeys where an anti-CD20 113F antibody construct showed greater B-cell depletion if compared to IgG1 (both antibodies were nonfucosylated for improved ADCC). This study indicates that the combination of optimised complement activation and A/I ratio represents a promising strategy for the improvement of tumour-depleting antibodies.

Other strategies for enhanced complement activation include the introduction of point mutations to improve IgG1 binding to C1q[16], a key component for the initiation of the complement cascade. Importantly, mutations that potentiate CDC can be combined with ADCP and ADCC-enhancing mutations in a single IgG1[31], thus broadening the effector function of these antibodies. Finally, the mutations that favour IgG hexamer formation also significantly enhance C1q fixation and thus CDC[32,33]. However, currently it remains to be seen whether these Fc mutations that enhance CDC in *in vitro* and in*ex vivo* studies translate into improved clinical efficacy.

**Use of alternative Ig isotypes:**

**IgE**

Several epidemiological studies have suggested a protective effect of some allergies and IgE antibodies against specific types of tumours[34,35], providing a rationale for exploring the potential use of mAbs of the IgE isotype as anti-tumour agents. IgE can mediate its Fc-effector function via two activating receptors - the high-affinity FcεRI and the low-affinity FcεRII. While predominantly expressed by mast cells (MC) and basophils, FcεRI expression can also be found on eosinophils, DCs and myeloid cells, although 10-100 fold lower than on fully matured and activated MC[36]. Compared to the IgG class, IgE offers several advantages that can be of interest for cancer therapy. For instance, it shows two orders of magnitude higher affinity for its receptor FcεRI than IgG for its high affinity receptor FcγRI[37]. Because of such a high FceRI affinity, IgE is locally retained on the cells expressing FceRI and has excellent bioavailability in tissues, which is of great importance for treatment of solid tumours. In addition, IgE lacks inhibitory Fc receptors that could cause immunosuppression such as FcγRIIb in the case of IgG[37]. Consequently, use of IgE antibodies in cancer therapy has been tested both *in vitro* and in *in vivo* mouse models, using transgenic hFceRI mice[38] as well as rats.

Side to side studies demonstrated that an IgE mAb targeting tumour-associated antigen was superior to

its IgG1 counterpart in terms of survival and reduction of tumor growth[38–40]. Furthermore, it was found that the main anti-tumour effector function of IgE antibodies was mediated by myeloid cells[40,41], and*in vitro* experimental data showed that monocytes can mediate IgE tumour killing via both ADCC through FceRI as well as ADCP through FceRII[42]. Remarkably, the IgE antibodies both recruited tumour-associated macrophages (TAM) for ADCC and ADCP, but also differentiated them towards activated M1-like phenotype characterised by upregulation of a TNFα/MCP-1/IL-10 cytokine signature, suggesting a potential role of IgE in TME modification[43]. Furthermore, IgE has been shown to facilitate DC cross-presentation of IgE IC-derived antigens, further supporting the anti-tumour effect by inducing a T cell based anti-tumour response[44–46]. An intrinsic concern regarding IgE therapy is the risk of inducing potentially life-threatening anaphylaxis triggered by degranulation of MC or basophils. Nevertheless, MC are predominantly localised within the peri-tumoural surrounding of most types of tumour and rarely within the tumours themselves. Since MC are not expected to be found in close proximity to tumour-bound IgE, their degranulation appears less likely. Furthermore, tumour antigens released into the blood as monomers are not expected to induce crosslinking of FceR-bound IgE required for degranulation[44]. Consistently, no signs of anaphylaxis were found in preclinical models and safety data was satisfactory in both rodents and monkeys[44,47,48].

In conclusion, the experimental data suggest that IgE might be a rather attractive Ig isotype to improve the clinical efficacy of tumour-depleting mAbs and one clinical trial using a tumour-targeting IgE mAb has already been initiated (NCT02546921).

**IgA**

Another rather promising Ig isotype for tumour-depleting mAbs is IgA, which mediates its effector functions through FcαRI. The FcαRI induces activating signals when IgA is encountered as an immune complex, however, induces inhibitory signals upon monomeric binding[49,50]. FcαRI is highly expressed on polymorphonuclear cells (PMNs), making neutrophils the most relevant cell type for IgA mAb therapy. Neutrophils represent the most abundant cytotoxic cell type in humans. They are armed with a variety of potent cell destruction mechanisms, including the release of cytotoxic molecules, induction of apoptosis and necrosis. Furthermore, they are well established for their recruitment of other immune cells and phagocytosis[49,51]. Importantly, it has been shown that, compared to cross-linking of FcγR, FcαRI cross-linking is far more efficient in the activation of neutrophils[51–53].

However, *in vivo* studies are still scarce, largely due to the fact that mice lack a FcαR homolog. Creation of a transgenic human FcαR mouse strain[54] allowed *in vivo* studies in which the anti-tumoural effect of IgA antibodies was demonstrated[55,56]. Surprisingly, macrophages were shown to be the crucial effector population for anti-EGFR IgA *in vivo* , leaving the role of neutrophils unclear. Unfortunately, the transgenic hFcαR mouse only partially resolved the lack of a useful model, as human IgA has a very short half-life in mice. Therefore, hIgA mouse pharmacokinetics and exposure were enhanced by attaching an albumin-binding domain to improve FcRn binding and thus the recycling of the antibody[57]. Furthermore, by Fc-engineering the clearance by the asialoglycoprotein receptor in the liver could be reduced[58]. In both cases, increased IgA half-life translated into improved anti-tumour efficacy in mouse models.These strategies may direct more extensive exploration of IgA-based cancer therapies in murine models and might be considered for extending the relatively short serum half-life of IgA mAbs in humans.

Similar to IgG1/IgG3 chimeras, IgG1/IgA chimera has been constructed with the intention of combining the advantages of the two different isotypes. Its binding to FcRn, FcγRs and C1q provided the IgG1/IgA chimera with an extended half-life and the capability to activate macrophages and complement, respectively. Furthermore, the additional FcαR binding of this chimeric antibody construct initiated recruitment of neutrophils, resulting in an overall improved cytotoxicity[59]. Thus, the combined effector functions of such chimeric isotype mAb construct may further improve the clinical efficacy of tumour-targeting mAbs.

**Isotypes and patient-tailored medicine**

With so many different strategies to improve the downstream effector functions of tumour-targeting antibodies, the question arises which approach to follow. The answer may depend on patient-related factors

and tumour intrinsic characteristics. For example, two FcγR polymorphisms that affect the binding of IgG antibodies have been described: H131R in FcγRIIa and V158F in FcγRIIIa. The R131 variant shows lower affinity for IgG2 while the F158 variant shows lower affinity for IgG1 and IgG3. The clinical implication of these variants has not yet been fully resolved, with some studies finding negative correlation with therapeutic efficacy while others do not[60,61]. Nevertheless, if larger and better designed studies confirm the negative correlation between lower affinity FcRs variants and response to IgG antibody treatment, these patients may benefit more from IgE, IgA or mAbs optimised for complement activation. When it comes to complement optimised mAbs it may further be important to consider tumour microenvironment (TME) factors such as pH that can affect CDC[62] or the expression level of complement regulatory proteins which allow complement evasion by cancer cells[63]. Furthermore, it has been shown that C reactive protein (CRP) shares its binding site on FcγRs (I and II) and FcαRI with IgG and IgA, respectively, whereas it can also bind to C1q[64,65]. Whether CRP can act as a competitive inhibitor for FcRs and complement binding of those antibodies *in vivo* has not been studied yet, but it could have important implications. For instance, patients suffering from chronic inflammatory and neurodegenerative diseases, such as atherosclerosis, type 2 diabetes mellitus or Parkinson's disease have chronically elevated CRP levels[66] which may interfere with antibody treatment. Thus, for those patients, IgE-based antibody treatment might be an attractive choice. In conclusion, antibody engineering offers a wide range of opportunities to improve effector functions of mAbs. but patient-related factors should also be taken into consideration for optimal isotype selection. This multilevel approach could result in a more effective personalised treatment.

**Antibodies targeting immunological checkpoint proteins**

A recently identified class of mAbs for cancer therapy are the so-called checkpoint inhibitors. These antibodies do not target the tumour directly but enhance anti-tumour immune responses by targeting immunological checkpoint proteins, such as PD-1 or CTLA-4, or their ligands such as PD-L1. These checkpoint proteins are expressed on activated T cells and limit excessive T cell responses. As a means of immune resistance, the ligands of PD-1 are often expressed by tumour cells[67] as well as by myeloid cells infiltrating the TME[68,69]. Checkpoint blockade leads to enhanced T cell activation[67,70] and, consequently, the clinical introduction of checkpoint inhibitors led to a tremendous improvement of cancer therapy for several different types of cancers.

In theory, checkpoint blocking antibodies do not require Fc-mediated effects, as their main effector function is expected to be derived from blocking the receptor-ligand interaction (Fab-mediated). However, it was found that a functional Fc tail contributed to the therapeutic efficacy of anti-CTLA4 checkpoint inhibitors in mouse models[71,72]. These studies revealed that whereas both effector T cell (Teff) and regulatory T cell (Treg) populations were increased in lymph nodes after therapy, within tumours, specifically the Treg but not the Teff population was decreased. This decrease was only observed with anti-CTLA4 of the IgG2a isotype (the isotype with highest A/I ratio in mouse) and appeared mFcγRIV dependent. The underlying mechanism was found to be caused by a selectively high abundance of macrophages expressing high levels of FcγRIV in tumours but not in lymph nodes[71]. Furthermore, Tregs express much higher levels of CTLA4 than Teff cells and were therefore preferentially depleted[13]. These findings point to the importance of the TME for therapeutic mAbs efficacy.

There are indications that anti-CTLA4 mAbs show the same effect in humans. A recent study confirmed the importance of Treg depletion for human anti-CTLA4 antibody in a hFcγR mouse model[73]. In addition, in advanced melanoma patients with high neoepitope burden the authors found a positive correlation between the presence of the high-affinity V158 FcγRIII allele and increased response to the CTLA-4 targeting antibody ipilimumab, providing further clinical evidence for the importance of Fc-mediated function. These findings may be relevant to explain why only some patients respond to anti-CTLA4 therapy and provide further rationale to optimise CTLA4 mAbs by improving their A/I ratio[74] or switching to IgA or IgE isotypes given the microenvironmental requirements for selective tumour Treg depletion are met.

Similarly, it was shown that the binding of anti-PD-L1 mAb to activating FcγRs enhances its therapeutic efficacy in mouse models, due to Fc-mediated depletion of immunosuppressive myeloid cell subsets in the

TME[68]. However, although another study confirmed that Fc-mediated depletion of myeloid cells in the TME contributes to the therapeutic effect of anti-PD-L1 antibodies, this effect was found to be dependent on the mouse genetic background as it occurred in CT26 tumours transplanted in BALB/c but not MC38 tumours in C57BL/6 mice[69]. The depleted myeloid cell subset was the one with the highest PD-L1 expression whereas PD-L1 expression on the tumour cells did not contribute to the therapeutic effect of anti-PD-L1 antibody[69]. Currently, there are three clinically approved anti-PD-L1 mAbs, two of which have a mutated Fc tail with abrogated FcγR binding (atezolizumab, durvalumab) and one is a wild-type IgG1 (avelumab). Since hundreds of clinical trials with these antibodies are currently ongoing, future results might help to resolve the question whether a functional Fc tail improves clinical efficacy of PD-L1 targeting antibodies in humans. If so, a further Fc-effector function optimisation might be an appealing step forward.

In contrast to anti-CTLA4 and anti-PD-L1, a functional Fc tail compromised the activity of anti-PD-1 mAbs *in vivo*. The underlying mechanism of this detrimental effect was the depletion of tumour-infiltrating CD8+ T cells, which are characterised by high PD-1 expression[68]. Not surprisingly, two clinically approved anti-PD-1 mAbs are of the IgG4 subclass, the human IgG subclass with the lowest A/I ratio. However, since IgG4 still binds to activating FcγRs to some extent, it would be interesting to compare its therapeutic efficacy with that of a mutated mAb with completely abolished FcγR binding[75]. Similarly, antibodies targeting CD47, a 'don't eat me signal' often upregulated by tumour cells to avoid elimination by myeloid cells as part of CD47/SIRP-α checkpoint pathway, do not require Fc-effector function either[76].

In conclusion, these findings strongly suggest that the cellular composition of the TME as well as the relative expression of the target molecule on different immune cell populations can greatly affect the outcome of checkpoint blocking mAb therapy. These factors dictate the need for Fc-mediated mechanisms for an optimal therapeutic effect and, thus, the isotype selection for checkpoint inhibitors. (**fig.4b** ).

**TNFR family targeting agonistic antibodies**

Recently, the Fc tail of agonistic mAbs that target specific members of the Tumour Necrosis Factor Receptor (TNFR) family has been shown to play a critical role in their therapeutic efficacy. This class of mAbs is designed to either activate death receptors such as DR4, DR5 and FAS on cancer cells in order to induce cell death, or to activate co-stimulatory receptors such as CD40, 41BB, OX40, GITR and CD27 on immune cells in order to improve anti-tumour immune responses.

TNFRs require trimerisation in order to initiate their associated signalling cascade[77]. Therefore, bivalent engagement of these receptors with Fab arms is usually not sufficient for their activation and additional cross-linking is required. For these antibodies, the interaction with FcγRs functions as an effective scaffold for clustering. Specifically, it has been shown that FcγRIIb represents a dominant scaffold for antibody mediated TNFR crosslinking and activation of downstream signalling because of its relatively high expression[78,79]. Consequently, *in vivo*agonistic antibody activity was found to be highly dependent on successful FcγRIIb engagement[80,81] and Fc-engineered antibodies with improved FcγRIIb binding showed stronger anti-tumour activity[82,83]. However, the expression of FcgRIIb is dynamic and can be downregulated by particular cytokines[84], leaving the success of FcγRIIb-mediated cross-linking for receptor clustering unpredictable. In addition, effective FcR-engagement by agonistic antibodies was found to be associated with serious hepatotoxicity[85–87], which could potentially be explained by the high expression of FcγRIIb on certain subsets of liver cells[88]. Therefore, new strategies have been explored to improve the agonistic activity of these mAbs independent of FcγR engagement. One of these strategies is the use of hIgG2(B), whose unique disulphide bonds rearrangement in the hinge region[89] provides it with a compact and highly agonistic conformation[90]. In line with this finding, the agonistic effect of anti-CD40 hIgG2 antibodies was demonstrated to be FcγR-independent both *in vitro* and *in vivo* , confirming that the use of hIgG2(B) is a viable strategy for improving the agonistic activity of mAbs targeting TNFR family members[91]. Furthermore, isotype switching from hIgG1 to hIgG2 was sufficient to convert an immunosuppressive anti-CD40 antagonistic antibody into a potent agonist with anti-tumour activity[92]. These findings constitute one of the most striking examples of how the choice of the isotype can completely change the activity of a mAb.

Another approach to improve the agonistic activity of TNFR family targeting mAbs, independent of FcγR engagement, is the recently developed HERA platform. HERA is an artificial chimeric molecule which, instead of Fab-arms, has two trimeric TNFR binding domains, fused to an IgG1 Fc backbone with abrogated FcγR binding. The resulting hexavalent molecule is capable of exerting its agonistic activity without FcγR-mediated crosslinking. So far, two HERA molecules targeting CD27 and CD40 have shown promising anti-tumour activity, without significant toxicological signs in pre-clinical mouse models[93,94]. These findings suggest that agonistic HERA molecules may offer improved safety combined with unaltered efficacy and thus an advantageous clinical profile.

The strategies described to improve agonistic activity in a FcγR-independent manner could have an additional advantage as they prevent unwanted depletion of immune cells expressing the target molecule. However, experiments in mice suggest that the therapeutic effect of some TNFR family targeting agonistic antibodies (such as anti-GITR[95], anti-OX40[96] or anti-4-1BB[97]) also involved Treg depletion, suggesting that, analogous to anti-CTLA4, a functional Fc tail might be advantageous. Similarly, some Fc-mediated downstream effector functions may be useful for agonistic mAbs targeting death receptors on cancer cells, as Fc-mediated cytotoxicity and ADCP would act as an additional tumour cell depleting mechanism and might facilitate cross-presentation inducing an adaptive anti-tumour response.

A few solutions have been proposed to combine the divergent properties, as mentioned above, in a single Ig molecule. For instance, a pentameric IgM antibody with high complement activation capacity has been used to successfully induce DR5 clustering via multivalent interaction, inducing tumour regression in preclinical models[98]. An alternative approach which takes advantage of Ig multimerisation, but avoids IgM manufacturing issues, is the so called HexaBody technology. It is based on a single point mutation (E430G) in the Fc domain of IgG1 that enhances Fc-Fc interactions upon binding to membrane-bound targets[99]. Consequently, these antibodies have a strong tendency to form hexamers on the target cell, ultimately leading to both high agonistic activity and improved CDC[100]. A combination of different HexaBodies targeting two different epitopes on DR5 is currently in an early clinical testing (NCT03576131). Given this enhanced complement activation of HexaBodies, this antibody form could furthermore be attractive whenever tumour cell lysis is intended, such as for classical tumour antigen-targeting antibodies, such as anti-CD20; suggesting for the design of an entirely novel type of tumour antigen-targeting antibodies.

In addition to HexaBodies, a highly agonistic anti-4-1BB recombinant Ig with potent Fc-effector function was achieved by combining human IgG2 CH1 and hinge locked in B conformation, with murine IgG2a CH2 and CH3 (the IgG subclass with the highest A/I ratio in the mouse)[97]. In mice, tumour treatment with this chimeric construct induced both Teff stimulation in lymph nodes (strong 4-1BB agonism) and Fc-mediated Treg depletion within tumours, leading to increased intra-tumoural Teff/Treg ratio and enhanced survival compared to a wild-type mIgG2a construct[97]. By analogy with the mouse example, a chimera of hIgG2(B) and hIgG1 might be applicable in humans.

In conclusion, important breakthroughs have been made in the design of TNFR agonistic antibodies by making their activity FcγR independent. It is precisely the FcγR independency that may overcome initial problems seen in the clinic such as severe toxicity and modest efficacy. However, the contribution of Fc-mediated cell-depletion to the therapeutic efficacy represents an important consideration for the optimal design of a specific agonistic antibody (**fig.4c** ).

## Conclusion

The introduction of mAbs into the clinic has fundamentally changed cancer therapy. Nevertheless, it has increasingly become apparent that mAbs mediate their effects via a multitude of different mechanisms of action. Since the selection of the correct Ig isotype was recognised as crucial, much effort was put into understanding the Fc-mediated effects of different antibody isotypes as well as into Fc-modifications for further improvement of mAbs efficacy. Consequently, several strategies have been developed in order to optimise Fc-mediated effector functions, opening entirely novel opportunities to improve mAbs-based cancer therapy. Furthermore, by considering patient-related factors such as their immune status, characteristics of

the TME or FcγR polymorphism, the isotype selection may either allow for the development of antibodies that are active in a wider range of patients or may allow for the selective use of antibodies tailored towards the individual's needs. Such considerations may lead us one step further to patient-tailored medicine and more effective mAb treatment in the future.

**Competing Interests**

The authors claim no competing interests.

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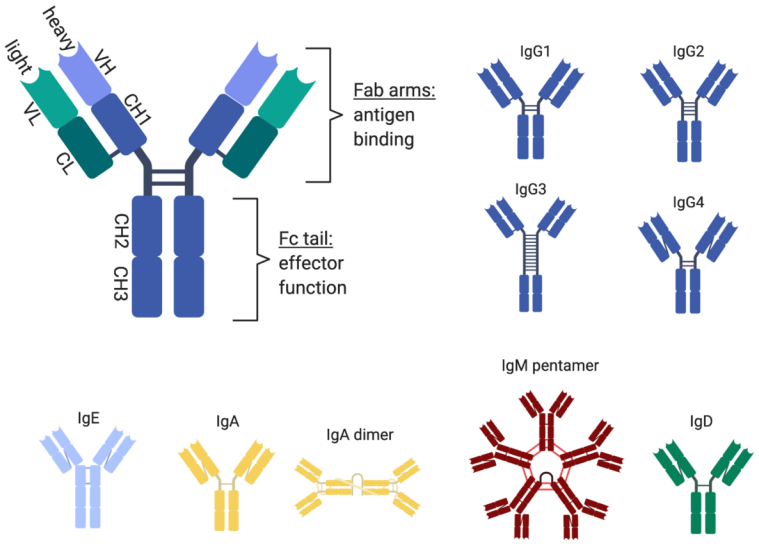


**Fig 1. Antibody structure and isotypes**

Human antibodies can be classified into five main isotypes - IgG, IgA, IgM, IgE and IgD, with IgG and IgA being further divided into the subclasses IgG1, IgG2, IgG3, IgG4, and IgA1 and IgA2, respectively. Overall structural organisation of an antibody molecule is similar for all isotypes. It consists of two heavy and two light chains joined by disulphide bonds. Both the heavy and light chain have a highly diverse variable domain (VH, VL respectively) and one or more constant domains (CH1 CH2, CH3, and CL, respectively). The constant domains of heavy chain are identical for all antibodies of the same isotype/subclass. Antibodies can also be divided into two functional subunits: (1) Fab arm, responsible for the specific binding to the antigen, (2) Fc tail, responsible for the activation of antibody effector functions (CDC, ADCP, ADCC, antigen cross-presentation) through interaction with the complement system and binding to Fc receptors present on immune and other cells. A graphical overview of different isotypes and subclasses is shown.

**Table 1.** Ig isotypes mediate their Fc effect via different receptors and activate different immune cells

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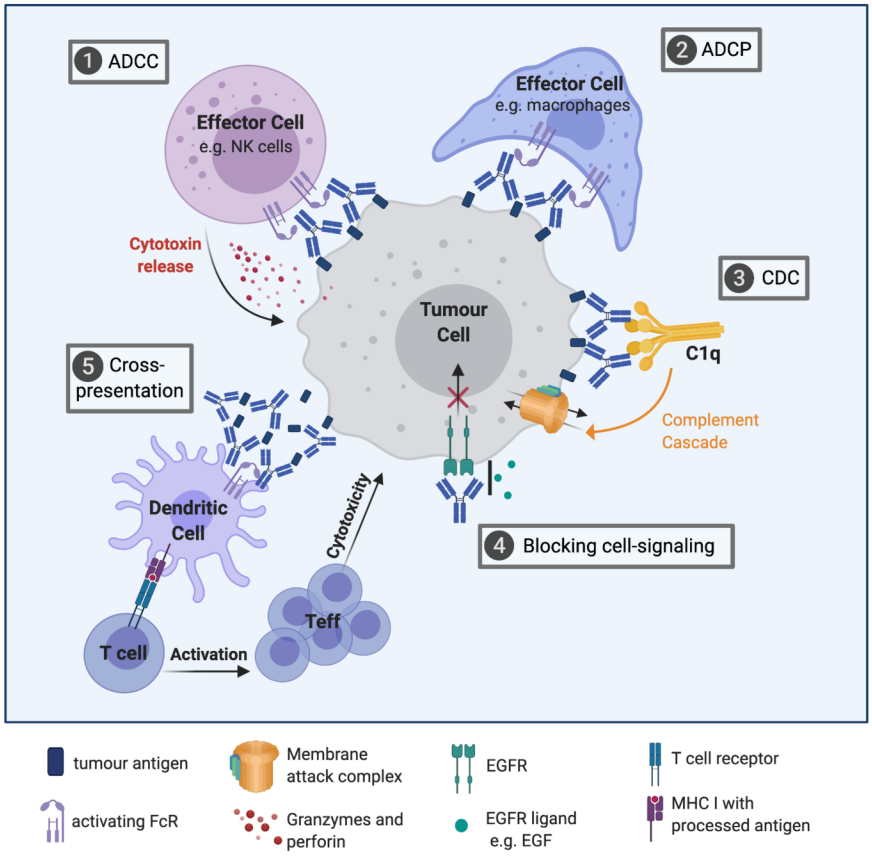


**Fig 2. Mechanisms of action of tumour antigen-targeting antibodies**

Tumour antigen-targeting (tumour-depleting) antibodies mediate tumour cell-killing via different mechanisms: (1,2) activation of immune effector cells (ADCC, ADCP), (3) initiation of complement cascade (CDC), (4) blocking important signalling pathways in tumour cells and (5) formation of immune complexes inducing enhanced tumour antigen cross-presentation by DCs, leading to adaptive immune response.

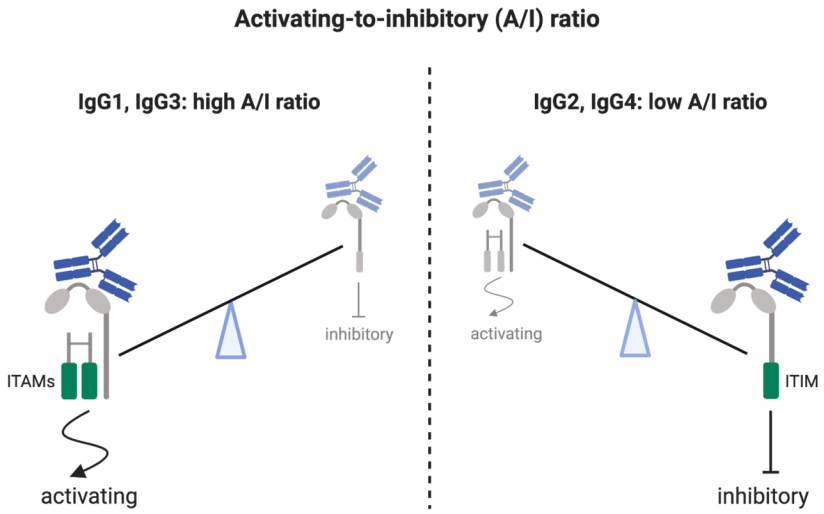


**Fig 3. A/I ratio dictates the outcome of Fc-effector function of IgG antibodies**

FcγRs can be either inhibitory (FcγRIIb) with immunoreceptor tyrosine-based inhibition motif (ITIM) or activating (FcγRI, FcγRIIa/IIc, FcγRIIIa, FcγRIIIb)[11]. Activating FcγRI and IIIa are associated with the common FcR gamma chain dimer containing two ITAMs (immunoreceptor tyrosine-based activating motifs); FcγRIIa and IIc are not associated with the gamma chain, but contain their own ITAM motif, whereas FcγRIIIb does not contain an ITAM motif and is not always considered as an activating receptor[11]. Expressed mostly on neutrophils, FcγRIIIb has been shown to favor phagocytosis (ADCP) in cooperation with FcγRIIa, but, on the other hand, it has a negative impact on neutrophilic ADCC by acting as a decoy receptor for IgG, thus competing with FcγRIIa for antibody binding[10].

Crosslinking of an activating receptor with the inhibiting receptor results in downregulation of the activating signal. Therefore, all activating FcγRs are counter-balanced by one inhibiting receptor FcγRIIb. Differential affinity of IgG for FcRs is defined as activating-to-inhibitory (A/I) ratio and varies across IgG subclasses. IgG subclasses with high A/I ratio (IgG1, IgG3) exert potent effector functions desirable for depleting antibodies, whereas low A/I ratio (IgG2, IgG4) is preferred when Fc-mediated cell depletion is unwanted.

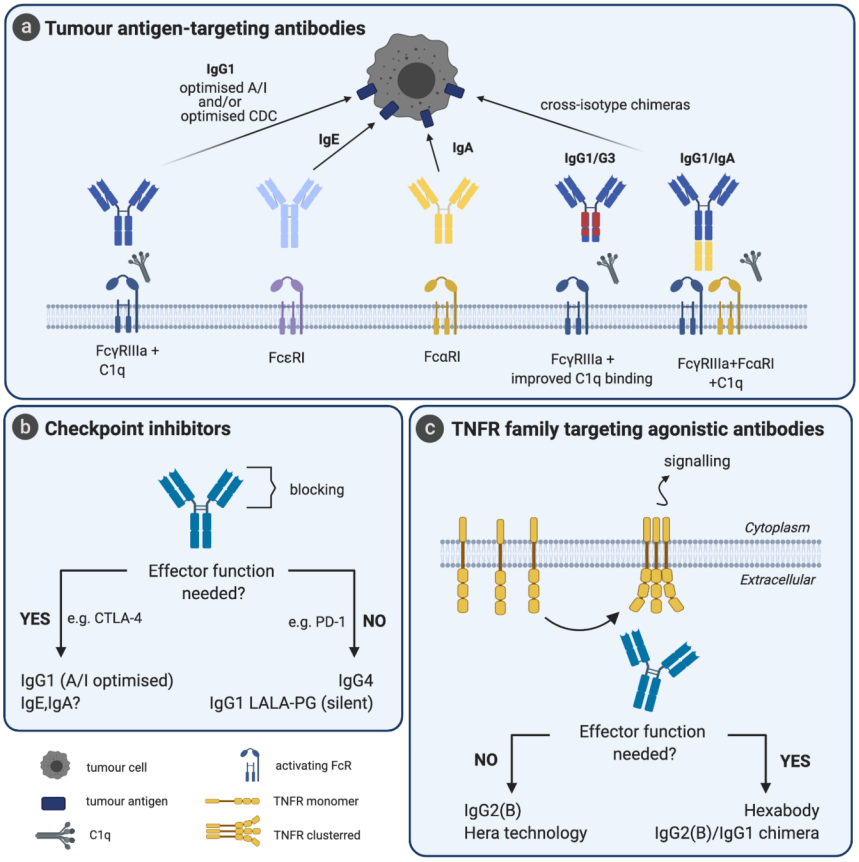


**Fig 4. Optimal Ig isotypes for therapeutic mAbs**

1. Tumour antigen-targeting antibodies are mostly of IgG1 isotype which can be further improved by optimising their A/I ratio and complement activation through Fc- and glyco-engineering. Recently, interest in IgE, IgA and cross-isotype chimeras is rising as they can offer alternative immune responses against tumor cells and the chimeras combine the advantages of two different isotypes.
2. For checkpoint inhibitors, a functional Fc tail may be either beneficial (anti-CTLA-4) or detrimental (anti-PD-1). If Fc-effector function is needed, isotype selection is similar to depleting antibodies (a). If Fc-mediated effects are unwanted, optimal isotypes are IgG4 (low A/I ratio) or IgG1 with abrogated FcR and C1q binding, for instance via LALA-PG mutation[75].
3. Agonistic antibodies target different receptors of the TNFR family, which require receptor clustering for initiation of their signaling cascade. Different strategies for achieving receptor clustering are available. In addition, Fc-mediated cell depletion plays an important role in some cases. Prior to selecting the optimal isotype, the need for a functional Fc tail should be considered.