



Isotype selection for antibody-based cancer therapy

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Introduction

Monoclonal antibodies (mAbs) have become an increasingly important class of drugs, with a global market comprising a total of 93 mAbs with marketing approval [1], with cancer being their most prevalent target disease [2]. Significant breakthroughs made in the areas of hybridoma technology, phage display and recombinant antibody production have enabled the development of a large variety of specific mAbs of any isotype.

In the case of cancer therapy, most therapeutic mAbs have been designed to interfere with the biological function of their target molecules and Fab arm specificity is, therefore, extremely important for mAb therapeutic efficacy. In addition, the Fc tail, which dictates downstream effector functions of an antibody, also plays an important role. Therefore, the final outcome of the binding of an antibody to its target is also influenced by the chosen isotype (Fig. 1, Table 1). Moreover, Fc- or glyco-engineering of the chosen isotype can be used to

Summary

The clinical application of monoclonal antibodies (mAbs) has revolutionized the field of cancer therapy, as it has enabled the successful treatment of previously untreatable types of cancer. Different mechanisms play a role in the anti-tumour effect of mAbs. These include blocking of tumour-specific growth factor receptors or of immune modulatory molecules as well as complement and cell-mediated tumour cell lysis. Thus, for many mAbs, Fc-mediated effector functions critically contribute to the efficacy of treatment. As immunoglobulin (Ig) isotypes differ in their ability to bind to Fc receptors 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 optimization 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: cancer, effector functions, Fc tail, isotype, mAbs

further optimize 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) tumour necrosis factor receptor (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

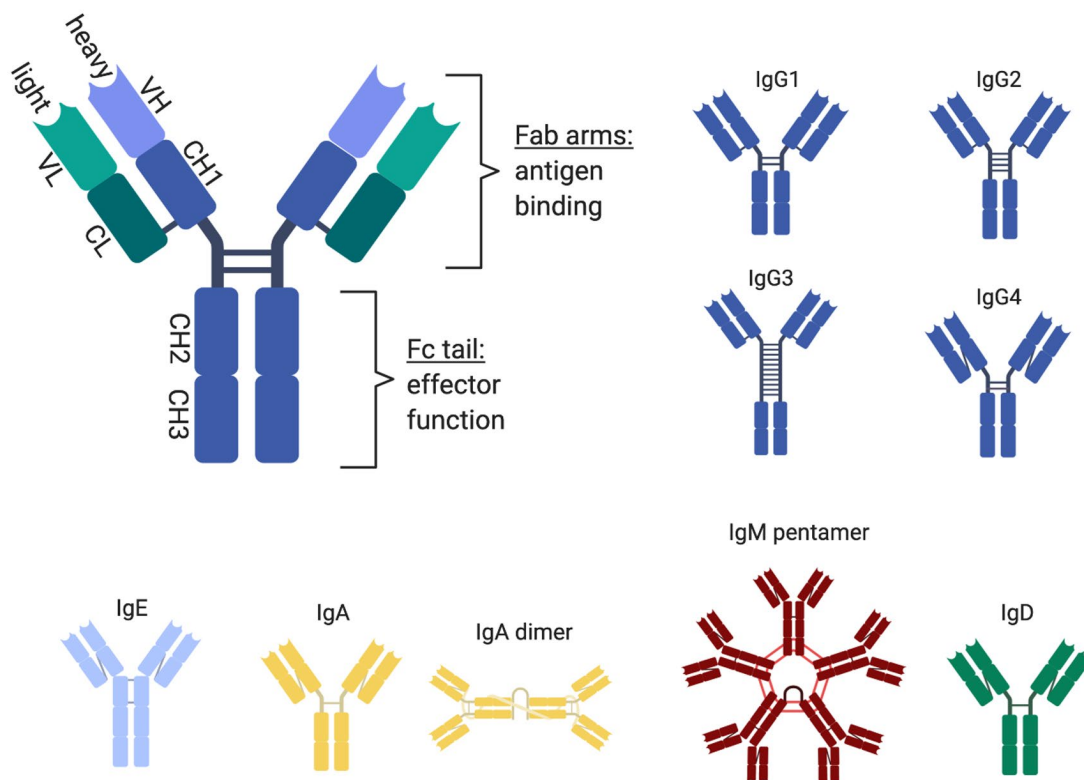


Fig. 1. Antibody structure and isotypes. Human antibodies can be classified into five main isotypes – immunoglobulin (Ig)G, IgA, IgM, IgE and IgD, with IgG and IgA being further divided into the subclasses IgG1, IgG2, IgG3, IgG4, and IgA1 and IgA2, respectively. Overall structural organization of an 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 chains have a highly diverse variable domain (VH and 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 [complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated phagocytosis (ADCP), antibody-dependent cell-mediated cytotoxicity (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.

to tumour cell death [e.g. anti-human epidermal growth factor receptor 2 (HER2), anti-epidermal growth factor receptor (EGFR)] [8,9]. 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 [9,10] (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 [10]. 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 [11]. These include the formation of the membrane attack complex (MAC), that directly induces the lysis of target

cells [complement-dependent cytotoxicity (CDC)], or the attraction of immune cells through the chemoattractive activity of the complement components C3a and C5a. Furthermore, the opsonization 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 [11]. Antibody-mediated cell death also leads to the release of tumour antigens and the formation of immune complexes (IC) which facilitate the initiation of anti-tumour T cell responses, sustaining the tumour control and rejection. During this process, binding to Fc γ Rs 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* [12,13].

In conclusion, in addition to blocking important signalling pathways in tumour cells with their Fab arm,

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

Antibody isotype	IgG1, IgG3*	IgE	IgA1, IgA2**	IgM
Main effector Fc receptor/ Clq	FcγRIIIa	Clq	FcαRI	Clq
Activated immune cells [3–5]	Macrophages, monocytes, NK cells	FcεRI Mast cells, granulocytes, macrophages, monocytes, DCs	FcγRII Eosinophils, neutrophils, macrophages***, monocytes, DCs***	-

*IgG2 and IgG4 are generally considered to have poor Fc effector function, although IgG2 can activate myeloid cells via FcγRIIa [6]; **IgA2 seems to be more proinflammatory [7]; ***some subpopulations.

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 the highest clinical efficacy. We will discuss different strategies to improve immunoglobulin (Ig)G Fc-effector functions, as well as the potential use of alternative isotypes such as IgE and IgA (Fig. 4a).

Optimizing IgG effector function

IgG Fc-effector functions are mediated via complement and FcγRs which are either activating (FcγRI, FcγRIIa/IIc, FcγRIIIa, FcγRIIIb [14]) or inhibitory (FcγRIIb) [15]. As 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 affinity of an antibody for its receptors is defined as the activating-to-inhibitory (A/I) ratio [16] (Fig. 3). The concept of the A/I ratio is based on observations in mice which show a high A/I for mIgG2a, a low A/I for mIgG1 and an intermediate A/I for mIgG2b [16]. As a consequence, therapeutic antibodies of the mIgG2a subclass have been shown to clear the tumours more efficiently in many *in-vivo* model systems [17]. Although the differences in the A/I ratio are less pronounced between human IgG subclasses, they also differ in their capability to induce immune responses due to their different FcR binding profile [18,19]. IgG1 and IgG3 bind to all FcRs, but show higher affinity for the activating ones. Thus, they are defined as highly activating with strong Fc-effector function. Conversely, IgG4 binds with similar affinities to most activating FcRs and inhibitory FcγRIIb and is considered as poorly activating. Finally, IgG2 shows overall poor binding to most FcRs, with the exception of the high-affinity H131 FcγRIIa allele (as discussed later), and has limited Fc-effector function. Therefore, IgG1 and IgG3 are capable of exerting potent effector functions desirable for depleting antibodies, whereas IgG2 and IgG4 are preferred when Fc-mediated cell depletion is to be avoided.

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 [20]. FcRn also binds albumin with similar effects [20] which can be exploited for mAb engineering, as will be discussed later.

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* [21]. IgG1 achieves most of its Fc-effector functions via FcγRIIIa present on macrophages and NK cells (ADCC, ADCP), as well as via complement activation [22].

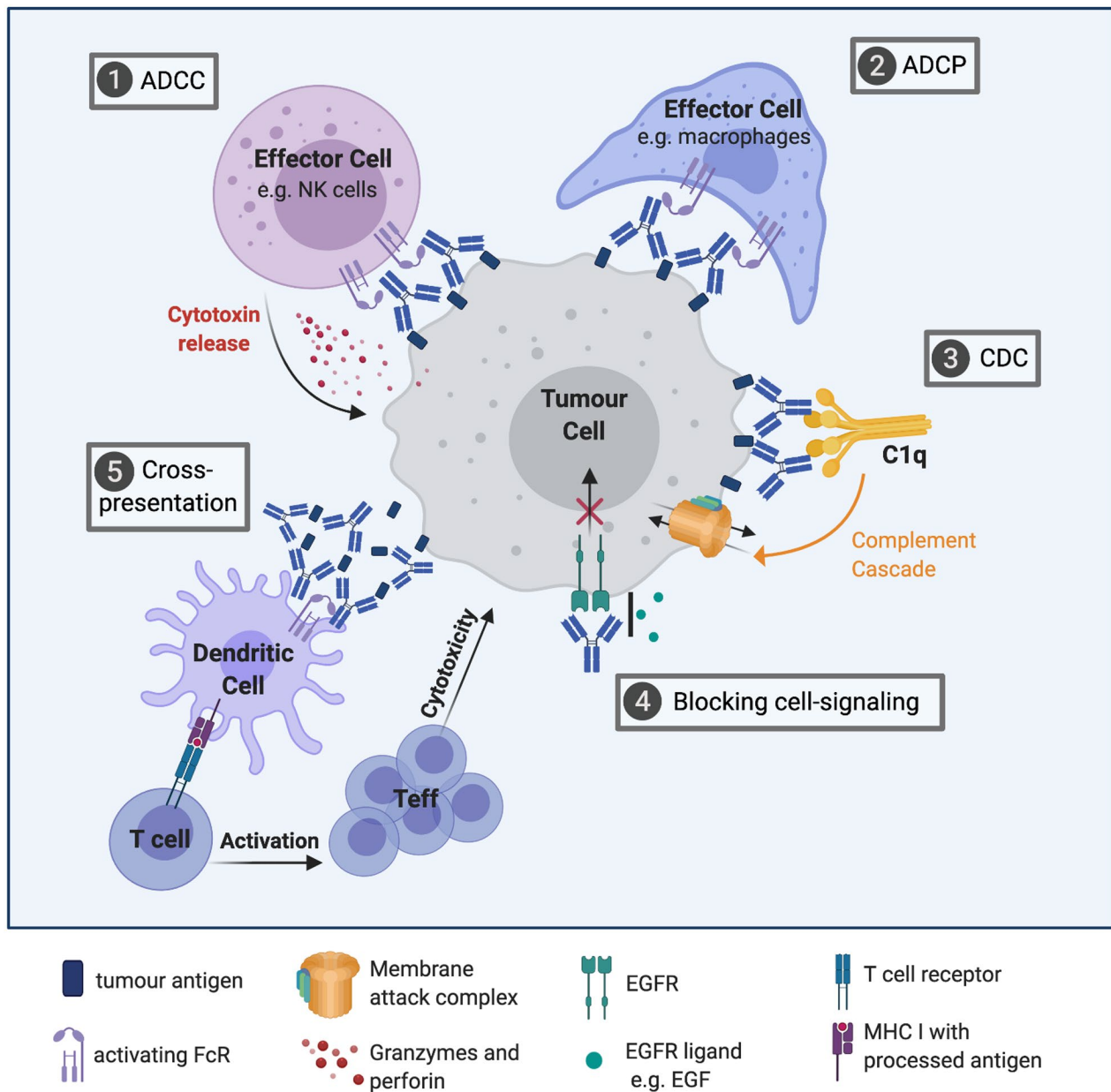


Fig. 2. Mechanisms of action of tumour antigen-targeting antibodies. Tumour antigen-targeting (tumour-depleting) antibodies mediate tumour cell-killing via different mechanisms: (1, 2) activation of immune effector cells [antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated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

Furthermore, IgG1 shows favourable biopharmaceutical characteristics with regard to production and purification. However, independently of their specificity, all tumour-targeting IgGs used hitherto in the clinic displayed a therapeutic effect only in a subset of patients. Therefore, over the years different strategies have been explored to further optimize tumour-targeting mAbs, many of which focused upon improving Fc-mediated functions.

Improving the A/I ratio. One of the common approaches to improve the IgG Fc-effector functions is to optimize the A/I ratio by increasing affinity for the activating FcγRs, on one hand, and decreasing binding to the inhibitory FcγRIIb on the other hand. One approach to improve the 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

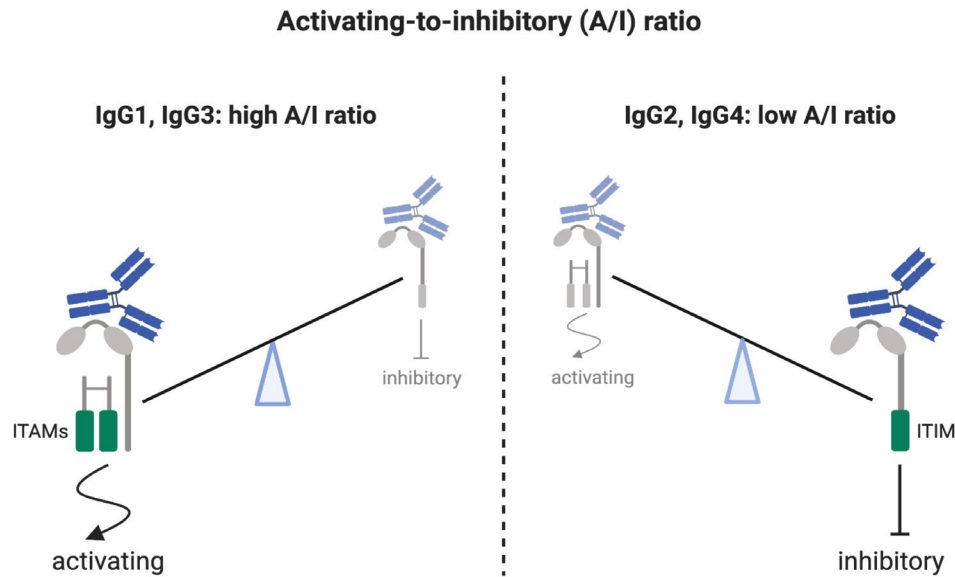


Fig. 3. T. A/I ratio dictates the outcome of Fc-effector function of immunoglobulin (Ig)G antibodies. Fc γ R_s can be either inhibitory (Fc γ R_{IIb}) with immunoreceptor tyrosine-based inhibition motif (ITIM) or activating (Fc γ R_I, Fc γ R_{IIa/IIc}, Fc γ R_{IIIa}, Fc γ R_{IIIb}) [15]. Activating Fc γ R_I and IIIa are associated with the common FcR gamma chain dimer containing two immunoreceptor tyrosine-based activating motifs (ITAMs); Fc γ R_{IIa} and IIc are not associated with the gamma chain, but contain their own ITAM motif, whereas Fc γ R_{IIIb} does not contain an ITAM motif and is not always considered as an activating receptor [15]. Expressed mainly on neutrophils, Fc γ R_{IIIb} has been shown to favour phagocytosis (ADCP) in co-operation with Fc γ R_{IIa} but, conversely, it has a negative impact on neutrophilic antibody-dependent cell-mediated cytotoxicity (ADCC) by acting as a decoy receptor for IgG, thus competing with Fc γ R_{IIa} for antibody binding [14]. Cross-linking of an activating receptor with the inhibiting receptor results in down-regulation of the activating signal. Therefore, all activating Fc γ R_s are counterbalanced by one inhibiting receptor Fc γ R_{IIb}. Differential affinity of IgG for FcR_s is defined as activating-to-inhibitory (A/I) ratio.

ADCC effect *in vitro* [23], which was mirrored by improved *in-vivo* anti-tumour responses in mouse models [24]. Two afucosylated mAbs have already received marketing approval [mogamulizumab (anti-CCR4) [25], obinituzumab (anti-CD20)] [26], and several others are currently in clinical trials [27]. Obinituzumab was found to be superior to rituximab (non-glycoengineered anti-CD20) in terms of complete response rate and progression-free survival in various clinical settings [28–30]. Another commonly used strategy to improve the A/I ratio is the introduction of point mutations in the Fc tail [22]. The most promising mAb in this group is margetuximab, an anti-HER2 antibody featuring five point-mutations in its Fc tail resulting in improved binding to Fc γ R_{IIIa} and Fc γ R_{IIa}, as well as a decreased Fc γ R_{IIb} binding [31]. These modifications translated into improved ADCC *in vitro* [31], enhanced anti-tumour activity in mice [31] and higher response rate and progression-free survival in HER2-positive metastatic breast cancer when compared to its non-Fc-engineered analogue trastuzumab [32]. In conclusion, glyco- and Fc- engineered IgG1 mAbs with an optimized A/I ratio appear superior to non-engineered IgG1, due most probably to enhanced ADCC.

Optimizing CDC. CDC has been recognized as an important mechanism of action for some therapeutic mAbs,

such as anti-CD20 [33–35]. Thus, strategies to optimize 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 received little attention in therapeutic mAbs development, and only a few tumour-targeting IgM mAbs have been evaluated in clinical trials [36], most prominently with PAT-SM6 receiving orphan drug designation by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) for multiple myeloma [37,38].

Among IgG subclasses, IgG1 and IgG3 are good complement activators, with IgG3 appearing to be the more potent isotype. Nevertheless, although IgG3 intrinsic problems, such as a short *in-vivo* half-life, have successfully been addressed [39], specific manufacturing issues 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 [40]. The optimal construct, called 113E, combined the CH1 and the hinge of IgG1 with the CH2 of IgG3 and a CH3 which was partly of

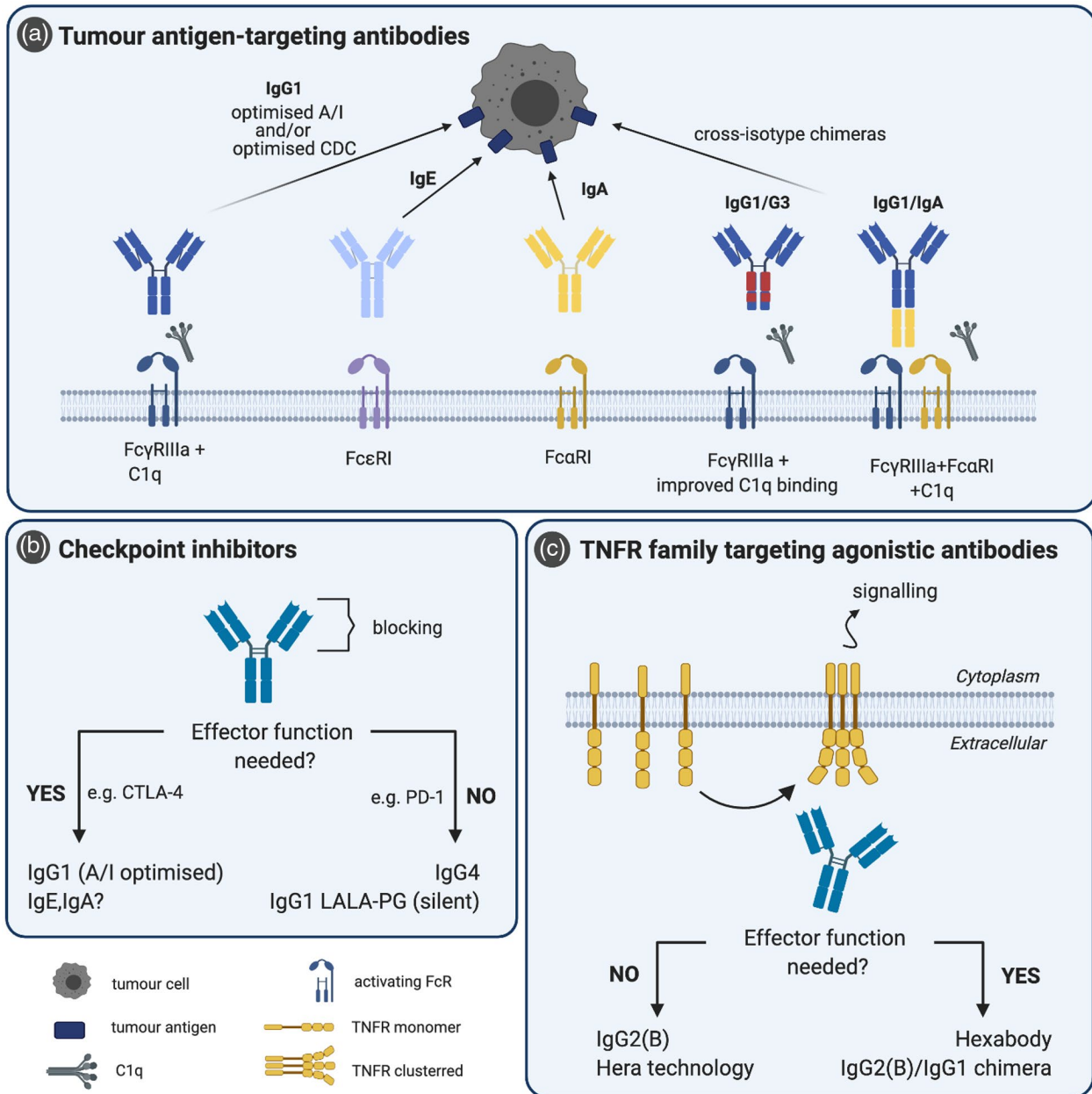


Fig. 4. Optimal immunoglobulin (Ig) isotypes for therapeutic monoclonal antibodies (mAbs). (a) Tumour antigen-targeting antibodies are mainly of IgG1 isotype, which can be further improved by optimizing their A/I ratio and complement activation through Fc- and glyco-engineering. Recently, interest in IgE, IgA and cross-isotype chimeras is rising, as they can offer alternative immune responses against tumour cells and the chimeras combine the advantages of two different isotypes. (b) For checkpoint inhibitors, a functional Fc tail may be either beneficial [anti-cytotoxic T lymphocyte antigen (CTLA-4)] or detrimental [anti-programmed cell death 1 (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 (poor Fc effector function) or IgG1 with abrogated FcR and C1q binding, for instance via LALA-PG mutation [86]. (c) Agonistic antibodies target different receptors of the tumour necrosis factor receptor (TNFR) family, which require receptor clustering for initiation of their signalling 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.

IgG3 and partly of IgG1 origin. The non-fucosylated version of this chimeric antibody showed enhanced CDC and ADCC comparable to non-fucosylated 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 non-fucosylated for improved ADCC). This study indicates that the combination of optimized 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 [22], 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 [41], thus broadening the effector function of these antibodies. Finally, the mutations that favour IgG hexamer formation also significantly enhance C1q fixation and thus CDC [42,43]. However, currently it remains to be seen whether or not these Fc mutations that enhance CDC in *in-vitro* and *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 [44,45], 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, dendritic cells (DCs) and myeloid cells, although 10–100-fold lower than on fully matured and activated MC [46]. 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 [47]. Because of such a high FcεRI affinity, IgE is locally retained on the cells expressing FcεRI 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 [47]. Consequently, the use of IgE antibodies in cancer therapy has been tested both in *in-vitro* and *in-vivo* mouse models, using transgenic hFcεRI mice [48] 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 tumour growth [48–50]. Furthermore, it was found that the main anti-tumour effector function of IgE antibodies was mediated by myeloid cells [50,51], and *in-vitro* experimental data showed that monocytes can mediate IgE tumour killing via both ADCC through FcεRI as well as

ADCP through FcεRII [52]. Remarkably, the IgE antibodies both recruited tumour-associated macrophages (TAM) for ADCC and ADCP, but also differentiated them towards activated M1-like phenotype characterized by up-regulation of a tumour necrosis factor (TNF)α/monocyte chemoattractant protein-1 (MCP)-1/interleukin (IL)-10 cytokine signature, suggesting a potential role of IgE in tumour microenvironment (TME) modification [53]. 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 [54–56].

An intrinsic concern regarding IgE therapy is the risk of inducing potentially life-threatening anaphylaxis triggered by degranulation of MC or basophils. Although tumour antigens released into the blood as monomers are not expected to induce cross-linking of FcεR-bound IgE required for degranulation [54], circulating tumour cells expressing multiple copies of targeted antigen would have the potential to induce degranulation. However, no signs of anaphylaxis were found in preclinical models and safety data were satisfactory in both rodents and monkeys [54,57,58], supporting the initiation of the first clinical trial using a tumour-targeting anti-folate receptor alpha IgE mAb MOv18 (NCT02546921). Interim Phase I data from 24 patients support the safety and potential efficacy of MOv18 IgE [59]. Potential to develop systemic allergic toxicity was evaluated by pretreatment skin prick test and *ex-vivo* basophil activation test (BAT). Readily manageable urticaria was the most common side effect. Only one patient experienced anaphylaxis – the only patient with a positive BAT test – indicating that the BAT test might be an important tool to exclude patients with potential risk of anaphylaxis. In addition, although the primary objective was to evaluate the safety and low doses of MOv18 IgE were used, anti-tumour effect was observed in one patient.

In conclusion, the experimental data suggest that IgE might be an attractive Ig isotype to improve the clinical efficacy of tumour-depleting mAbs. Upcoming results of the MOv18 IgE clinical trial will be a major step forward in evaluating IgE-based therapy in human settings.

IgA. Another 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, it induces inhibitory signals upon monomeric binding [3,60]. 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 [60,61]. Importantly, it has been shown that, compared to cross-linking of Fc γ R, Fc α RI cross-linking is far more efficient in the activation of neutrophils [61–63].

However, *in-vivo* studies are still scarce, due largely to the fact that mice lack a Fc α R homologue. Creation of a transgenic human Fc α R mouse strain [64] allowed *in-vivo* studies in which the anti-tumour effect of IgA antibodies was demonstrated [65,66]. 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 hFc α R 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 [67]. Furthermore, the clearance by the asialoglycoprotein receptor in the liver could be reduced by Fc-engineering [68]. 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, attempts to construct IgG1/IgA chimeras have been made with the intention of combining the advantages of the two different isotypes [69,70]. Binding to FcRn, Fc γ Rs and C1q with the IgG1 part as well as to Fc α R with the IgA part, has been successfully achieved [70]. It provided the IgG1/IgA chimera with an extended half-life, the capability to activate macrophages and complement and initiate recruitment of neutrophils, respectively, resulting in an overall improved cytotoxicity [70]. Thus, the combined effector functions of such a 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 as to which approach to follow. It is tempting to speculate that personalized medicine approach may give an answer, by taking into consideration patient-related factors and tumour intrinsic characteristics. For example, two Fc γ R polymorphisms that affect the binding of IgG antibodies have been described: H131R in Fc γ RIIa and V158F in Fc γ RIIIa. 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 a negative correlation with therapeutic efficacy while others do not [71,72]. 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 optimized for complement activation, given that these are proven to be effective anti-tumour treatments in the future. With regard to complement optimized mAbs, it may further be important to consider tumour microenvironment (TME) factors, such as pH, that can affect CDC [73] or the expression level of complement regulatory proteins, which allow complement evasion by cancer cells [43]. Furthermore, it has been shown that C-reactive protein (CRP) shares its binding site on Fc γ Rs (I and II) and Fc α RI with IgG and IgA, respectively, whereas it can also bind to C1q [74,75]. Whether CRP can act as a competitive inhibitor for FcRs and complement binding of those antibodies *in vivo* has not yet been studied, 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 [76] 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 multi-level approach could result in a more effective personalized 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 programmed cell death 1 (PD-1) or cytotoxic T lymphocyte antigen 4 (CTLA-4), or their ligands, such as programmed cell death ligand 1 (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 [77] as well as by myeloid cells infiltrating the tumour microenvironment (TME) [78,79]. Checkpoint blockade leads to enhanced T cell activation [77,80] 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-CTLA-4 checkpoint inhibitors in mouse models [81,82]. These studies revealed that whereas both effector T cell (T_{eff}) and regulatory T cell (T_{reg}) populations were increased in lymph nodes after therapy within tumours, specifically the T_{reg} but not the T_{eff} population was decreased. This decrease was only observed with anti-CTLA-4 of the IgG2a isotype (the isotype with the highest A/I ratio in mouse) and appeared mFc γ RIV-dependent. The underlying mechanism was found to be caused by a selectively high abundance of macrophages expressing high levels of Fc γ RIV in tumours but not in lymph nodes [81]. Furthermore, T_{regs} express much higher levels of CTLA-4 than T_{eff} cells and were, therefore, preferentially depleted [83]. These findings point to the importance of the TME for therapeutic mAb efficacy.

There are indications that human anti-CTLA-4 mAbs show the same effect. A recent study confirmed the importance of T_{reg} depletion for human anti-CTLA-4 antibody in a hFc γ R mouse model [84]. In addition, in advanced melanoma patients with a high neopeptide burden the authors found a positive correlation between the presence of the high-affinity V158 Fc γ RIIIa 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-CTLA-4 therapy and provide further rationale to optimize CTLA-4 mAbs by improving their A/I ratio [85] or switching to IgA or IgE isotypes, given that the microenvironmental requirements for selective tumour T_{reg} depletion are met.

Similarly, it was shown that the binding of anti-PD-L1 mAb to activating Fc γ Rs enhances its therapeutic efficacy in mouse models, due to Fc-mediated depletion of immunosuppressive myeloid cell subsets in the TME [78]. 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 upon the mouse genetic background, as it occurred in CT26 tumours transplanted in BALB/c but not MC38 tumours in C57BL/6 mice [79]. 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 the anti-PD-L1 antibody [79]. Currently, there are three clinically approved anti-PD-L1 mAbs, two of which have a mutated Fc tail with

abrogated Fc γ R binding (atezolizumab, durvalumab) and one is a wild-type IgG1 (avelumab). As 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 optimization might be an appealing step forward.

In contrast to anti-CTLA-4 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 characterized by high PD-1 expression [78]. Not surprisingly, two clinically approved anti-PD-1 mAbs are of the IgG4 subclass with poor Fc effector functions. However, as IgG4 still binds to activating Fc γ Rs to some extent, it would be interesting to compare its therapeutic efficacy with that of a mutated mAb with completely abolished Fc γ R binding [86]. Similarly, antibodies targeting CD47, a ‘don’t eat me signal’ often up-regulated by tumour cells to avoid elimination by myeloid cells as part of CD47/SIRP- α checkpoint pathway, do not require Fc-effector function either [87].

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).

Tumour necrosis factor receptor family targeting agonistic antibodies

Recently, the Fc tail of agonistic mAbs that target specific members of the 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 trimerization in order to initiate their associated signalling cascade [88]. 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 Fc γ Rs functions as an effective scaffold for clustering. Specifically, it has been shown that Fc γ RIIb represents a dominant scaffold for antibody-mediated TNFR cross-linking and activation of downstream signalling because

of its relatively high expression [89,90]. Consequently, agonistic antibody activity was found to be highly dependent upon successful FcγRIIb engagement in mice [91,92], and Fc-engineered antibodies with improved FcγRIIb binding showed stronger anti-tumour activity [93,94]. However, the expression of FcγRIIb is dynamic and can be down-regulated by particular cytokines [95], rendering 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 [96–98], which could potentially be explained by the high expression of FcγRIIb on certain subsets of liver cells [99]. Therefore, new strategies have been explored to improve the agonistic activity of these mAbs independently of FcγR engagement. One of these strategies is the use of hIgG2(B). This compact and highly agonistic conformation of hIgG2 [100] is a consequence of a unique disulphide bonds rearrangement in the hinge region [101]. Compared to hIgG2(A), whose Fab arms are not linked to the hinge via disulphide bonds, hIgG2(B) presents with two disulphide bonds between each Fab arm and hinge, making them more rigid and potentially able to pack TNFR molecules closer together [102]. 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*. Importantly, it is possible to lock hIgG2 in B conformation via a specific cysteine mutation in CH1 region which allows its recombinant production [100]. Thus, the use of hIgG2(B) is a viable strategy for improving the FcγR-independent agonistic activity of mAbs targeting TNFR family members [103]. 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 [104]. 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, independently 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 cross-linking. So far, two HERA molecules targeting CD27 and CD40 have shown promising anti-tumour activity, without significant toxicological signs in pre-clinical mouse models [105,106]. 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 GITR [107], anti-OX40 [108] or anti-4-1BB) [109] also involved T_{reg} depletion, suggesting that, analogous to anti-CTLA-4, 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 multi-valent interaction, inducing tumour regression in preclinical models [110]. An alternative approach which takes advantage of Ig multimerization, 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 [111]. Consequently, these antibodies have a strong tendency to form hexamers on the target cell, ultimately leading to both high agonistic activity and improved CDC [112]. A combination of different HexaBodies targeting two different epitopes on DR5 is currently in early clinical testing (NCT03576131). Given the 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 (e.g. anti-CD20), suggestive 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) [109]. In mice, tumour treatment with this chimeric construct induced both T_{eff} stimulation in lymph nodes (strong 4-1BB agonism) and Fc-mediated T_{reg} depletion within tumours, leading to an increased intratumoral T_{eff}/T_{reg} ratio and enhanced survival compared to a wild-type mIgG2a construct [109]. 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 Fc γ R-independent. It is precisely the Fc γ R-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 recognized as crucial, much effort has been 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 optimize 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 Fc γ R 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.

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The authors have no competing interests.

Data Availability Statement

Data availability are not applicable to this review article.

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