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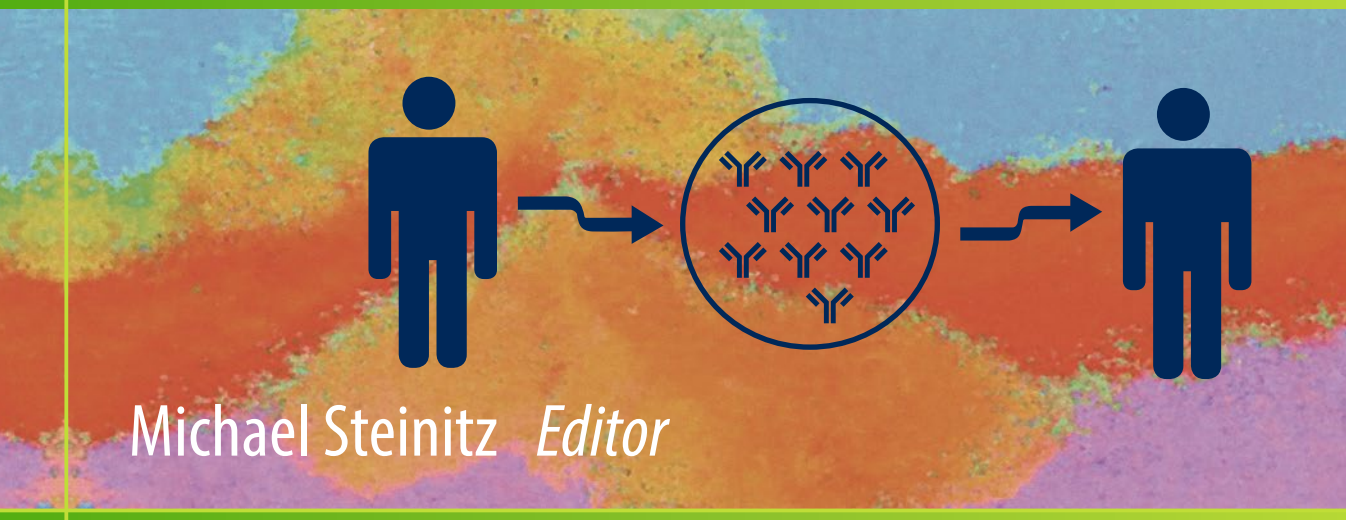

Michael Steinitz *Editor*

Human Monoclonal Antibodies

Methods and Protocols

Second Edition

Humana Press

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Second Edition

Edited by

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Dedication

This book is dedicated to George Klein, 1925–2016, a giant creative and stimulating scientist of infinite knowledge, friend, and mentor. His legacy is a never-fading light house to hundreds of former students, co-workers, colleagues, and friends.

Preface

A second edition of Springer Protocols book *Human Monoclonal Antibodies* is a natural outcome of the rapid developments in the area and the remarkable interest attracted by our 2014 first edition of the book.

It is amazing what a rapid and dramatic development has occurred in the field of human monoclonal antibodies since the first time these were produced in the laboratory. It was the pioneering study initiated by Professor George Klein in Stockholm in 1977 [1] that showed the feasibility of making such antibodies from immortalized peripheral blood antigen-committed human B lymphocytes.

The extensive basic immune research which has taken place during the last years rapidly converged into the clinic. The Fast and dramatic development of novel immune treatments in the clinic using human monoclonal antibodies urged the improvements of traditional and also completely new techniques involved in the production of the antibodies. The introduction of monoclonal antibodies directed against lymphocyte cell-surface costimulatory/immune checkpoint receptors that mediate the immune response has been revolutionary. These antibodies enable overcome immune unresponsiveness (i.e., tolerance) of T cells. Antibodies against costimulatory lymphocyte receptors which now are used in the clinic proved very beneficial for tumor patients at least in relation to some types of cancer.

Continuous progress in molecular techniques enables genuine management of antibody molecules. Such reagents introduced into cytotoxic T cells promise new horizons for the treatment of cancer patients.

The present Springer Protocols book reflects some of the recent developments in the area. It includes several completely new chapters related to topics that were not discussed in the first edition. In addition, some chapters from the first edition are updated with necessary revisions. Similar to the first edition, besides the detailed specific technical protocols, there are a few review manuscripts too.

Jerusalem, Israel *Michael Steinitz*

Reference

1. Steinitz M, Klein G, Koskimies S, Mäkelä O (1977) EBV virus induced B lymphocyte cell lines producing specific antibody. Nature 269:420–422

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Chapter 1

Human Monoclonal Antibodies: The Benefits of Humanization

Herman Waldmann

Abstract

The major reasons for developing human monoclonal antibodies were to be able to efficiently manipulate their effector functions while avoiding immunogenicity seen with rodent antibodies. Those effector functions involve interactions with the complement system and naturally occurring Fc receptors on diverse blood white cells. Antibody immunogenicity results from the degree to which the host immune system can recognize and react to these therapeutic agents. Thus far, there is still no generally applicable technology guaranteed to render therapeutic antibodies antigenically silent. This is not to say that the task is impossible, but rather that we need to train the immune system to help us. This can be achieved if we take advantage of natural mechanisms by which an individual can be rendered tolerant of "foreign" antigens, and as a corollary minimize the potential immunogenicity of any contaminating protein aggregates, or "aggregates" arising from antibodies complexing with their antigen. I here summarize our efforts to engineer antibodies to harness optimal effector functions, while also minimizing their immunogenicity. Potential avenues to achieve the latte are predicted from classical work showing that monomeric "foreign" immunoglobulins are good tolerogens, while aggregates of immunoglobulins ate intrinsically immunogenic. Consequently, I argue that one solution to the immunogenicity problem lies in ensuring a temporal quantitative advantage of tolerogenic non-cell-bound monomer over the cell-binding immunogenic form.

Key words Therapeutic antibodies, Complement system, Fc receptors, Immunogenicity, Adjuvanticity, High dose tolerance, Humanized and human antibodies

1 Introduction

Although monoclonal antibodies were first described in 1975 [1], their potential as therapeutic agents was not properly appreciated until technology evolved to replace, to different degrees, the original rodent forms with human equivalents [2–8]. The reasons for this are complex, but relate to a combination of perceptions related to patentability, immunogenicity, effector function, and wish to avoid undesirable side effects. Undoubtedly, the terms *human* or *humanized* (Fig. 1) carried some emotive advantage over *rodent*, *murine*, or *rat* in giving comfort that agents close to the human form were

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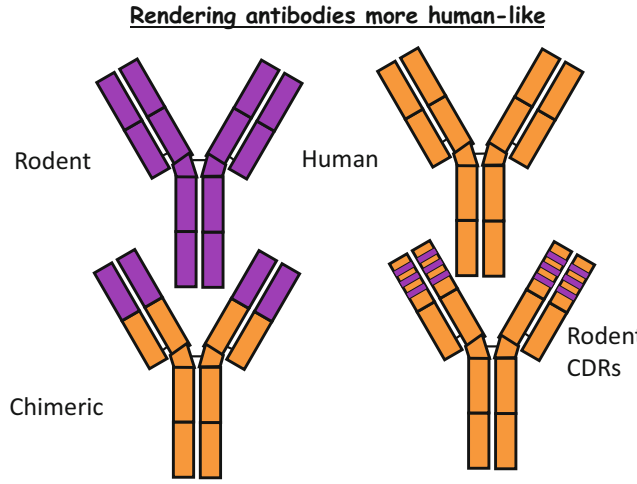

Fig. 1 The construction of chimeric and humanized antibodies. The bulk of the humanized Ab (so-called frameworks-in orange) is tolerated as if "self." The complementarity determining regions (CDRs) which bind antigen will however be regarded by the immune system as foreign

somehow preferable, even before all the evidence was in [9]. That emotive argument has even been extended to comparisons between *fully human* as opposed to more *humanized* antibodies, as if there were some important and significant functional difference. Undoubtedly though, the commercially driven demand for human antibodies has, to its credit, catalyzed technologies related to antibody engineering and manufacture which have aided commercialization in a very productive way. The basic human constructs and expression vectors generated for the purpose have also served as templates to enable generation of antibody variants designed to deliver improved therapeutic performance [10].

In this short chapter I will discuss our past work assessing the ability of human immunoglobulin subclasses to harness natural effector mechanisms, and the extent to which engineering therapeutic antibodies to human forms has provided solution to the "immunogenicity" problem.

2 Antibody Effector Functions

Early work with rodent "therapeutic" monoclonal antibodies taught us that choice of antibody class and subclass were important in harnessing therapeutic effector mechanisms in vitro and in vivo [11–13]. In the first steps toward derivation of engineered "human" antibodies reagents chimeric for human Fc (constant) regions with rodent variable regions (Fig. 1) proved invaluable in driving decisions as to which human Fc region was best suited to

achieve a desired therapeutic effect [14]. In one of the first of such in vitro studies [15, 16] a series of chimeric antibodies were constructed all having identical variable regions binding to a defined hapten (4-hydroxy-3-nitrophenacetyl). Whereas IgM, IgG1, and IgG3 constant regions bound C1q efficiently, and could mediate lysis with human complement, IgE, IgG2, and IgG4 were very weak in that regard. In cell mediated lysis studies IgG1 and IgG3 were efficient, while IgM, IgG2, IgG4, IgA, and IgE were very weak. This hierarchy of IgG subclasses in mediating effector functions was also demonstrated with the humanized antibody CAMPATH-1H [5] (Fig. 1). In the case of a humanized anti-CD3 antibody we wished to find an immunoglobulin Fc region that would not allow mitogenicity and cytokine release [17], as these had been associated with significant toxicity due to a cytokine storm in patients treated with the original rodent forms of anti-CD3 antibodies. We observed that all of the IgG subclasses, IgE and IgA were mitogenic in vitro, whereas a mutated IgG1 constant region mutated to lose the glycosylation site (Asn297) by changing to alanine, was non-mitogenic. The aglycosyl IgG1 form was less able to generate cytokine release in hCD3 transgenic mice [17, 18], unlike the parental IgG1 form. However, the mutant form, although non-lytic, has proven immunosuppressive both in vitro and in vivo [17–20]. Although the native hIgG1 form of humanized anti-CD3 antibody failed to activate human complement lysis in vitro, an engineered monovalent form was able to do so [21].

In. summary then, these studies taught us that selected human IgG isotypes, both natural and engineered, could be adopted for desired effector activity in therapeutic application. In more recent times, especially in the arena of checkpoint blockade, selection of human immunoglobulin isotypes in relation to binding particular Fc receptors, may be of great importance [22].

3 Immunogenicity

It has long been known that “foreign” polyclonal antibodies are potentially immunogenic in humans and in experimental animals. Seminal studies from Chiller and Weigle, and Dresser indicated that even though human immunoglobulins were foreign to mice, when given as monomers, they were tolerogenic rather than immunogenic [10]. However, given as heat induced aggregates, they were obligate immunogens. At high doses the monomers could tolerize both T-helper cells and B-cells, but at low doses would only tolerize the T-helper cells [10]. As therapeutic antibodies tended to target antigens within the body, it was likely that, when bound to cell surface antigens, they would be generating “immunogenic” aggregates within the treated hosts. Whereas polyclonal antibodies might

bind to multiple epitopes within the antigen, monoclonal antibodies would be restricted to just one or very few such targets.

In 1986, we examined a series of rat antibodies that were directed toward mouse leucocyte antigens, and found that virtually all proved immunogenic, except antibodies to the CD4 molecule [23].

In contrast, monomeric rat monoclonal immunoglobulins that did not bind to leucocytes, proved non-immunogenic, but were actually tolerogenic, in markedly reducing the antibody response to cell-binding antibodies given at a later time. It also emerged that the anti-CD4 antibodies were indeed directing the immune system to regard them as tolerogens, as well as other proteins that might be given under the umbrella of the anti-CD4 therapy. This observation formed the basis for many subsequent studies on therapeutic reprogramming of the immune system through recruitment of host tolerance mechanisms [24]. These findings suggested that antibodies binding to leucocytes simulated the Chiller-Weigle aggregates in generating sufficient adjuvanticity to evoke immune responses, but also left some questions about what target cell type or antigen was needed for that purpose. To this day, there has been very little attention to this question. For example, what if the target antigen was a monomer in solution, or a trimer (such as TNF)? Would therapeutic antibodies to these be immunogenic? As mentioned earlier, tolerogenicity can be quite dose dependent, and therapeutic doses of antibodies may not always achieve the level required to tolerize both T- and B-cells.

As humans are largely tolerant of the constant regions of their own antibodies (self-tolerance), it was assumed that human antibodies, or engineering of antibodies to a human form, would bypass the immunogenicity problem. The concept was supported by evidence that the closer a monoclonal immunoglobulin was engineered toward host-type, then the less immunogenic it proved [3]. In a study comparing a humanized anti-CD52 antibody with a previous administration of the rodent form, the humanized version appeared far less immunogenic after a single course [25].

However, the humanization approach depended on retention of the original murine CDRs within the new human framework, and so eventual immunogenicity was still a potential issue. The notion of fully human antibodies implied that humans would be tolerant to the CDRs and framework-overlapping regions of antibodies derived from a human repertoire. This cannot be the case [9]. We know from past work that anti-idiotype responses can be generated to one's own antibodies [26]; and we also know that in the evolution of an antibody response, VJ and VDJ recombinations as well as somatic hypermutation can change the CDRs away from their germ line configuration. Consequently, there is still no evidence-based argument that would make the general case for fully human antibodies being less immunogenic than humanized

antibodies. In a published study of the humanized CD52 antibody (CAMPATH-1H or alemtuzumab) the majority of patients treated with a second course of antibody made strong anti-idiotypic responses to the humanized therapeutic [27]. This teaches us that the CDRs can remain a focus of the host immune response to humanized (and probably also human) monoclonal antibodies.

4 Overcoming the Immunogenicity Problem

The current portfolio of antibody therapeutics comprises members for whom immunogenicity has yet to be identified as a problem, and others where immunogenicity is well documented. In some scenarios the use of a synergistic immunosuppressive drug may not only benefit the target disease, but also mask the extent of antibody immunogenicity [28]. Where immunogenicity has arisen, options may be available to switch to a different agent serving the same purpose, or even to a different antibody target, as in anti-TNF therapy. Where immunogenicity has not occurred, we may not always be able to establish why. In other words, is lack of immunogenicity a feature of the target antigen, the dose, or some unique feature of the therapeutic agent?

Nevertheless, when all the information from clinical studies is made available, there will surely be examples of human antibodies where immunogenicity will have been shown to limit clinical utility. What can be done to more effectively control immunogenicity? There are a number of directions that might be considered.

First, and not insignificant, is the issue of natural aggregates resulting from the biopharmaceutical processing. Somehow these can create immunogenicity in their own right, irrespective of the therapeutic antibody binding to its target. A discussion of such natural aggregates is beyond the scope of this article, but the reader is directed to a few recent reviews dealing with this complex problem. Some of the solutions may involve approaches discussed below, but others may require attention to the bioprocessing and formulation of given products [29–33].

When it comes to immunogenicity of the desired drug product, then one needs to recognize that in order for T-cells to recognize the "foreign" determinants it is essential that the antibody is processed into peptides that can bind to MHC Class II [34–37] while B-cells may have special requirements for recognition of conformational epitopes [36, 37]. By scanning the primary sequence of antibody heavy and light chains for potential antigenic epitopes, it has been claimed that one can purge the therapeutic of T-cell epitopes, and reduce the number of B-cell epitopes [36, 37]. The success of this depends upon such drugs being manufactured and assessed in clinical trials, as there really is no in-vitro system that can replace the in-vivo assessment. Until that is achieved in a head to

head comparison with a conventional antibody, we cannot be certain that this will eliminate the problem.

Another route to eliminate immunogenicity is to find a route to tolerize the patient to the therapeutic antibody, so that any immune response to T-cell or B-cell would be rendered impossible [38]. This may sound counterintuitive, but we know from Chiller and Weigle that this ought to be possible. In principle a tolerogenic form of the therapeutic antibody might be generated if one could produce a limited number of mutations in the key CDRs concerned with antigen binding. A few mutations that could drastically reduce binding might provide a tolerogenic version which would be given ahead of the non-mutated therapeutic form of the antibody. The feasibility of this approach has been demonstrated in mice transgenic for the human CD52 antigen [39]. A human IgG1 antibody to CD52 was used to ablate mouse T-lymphocytes. This ablation was associated with immunogenicity of the foreign antibody. In contrast, mice that had previously received single or double mutant forms of the antibody which were markedly reduced in their binding (Fig. 2), could not be immunized to either the tolerogen nor to

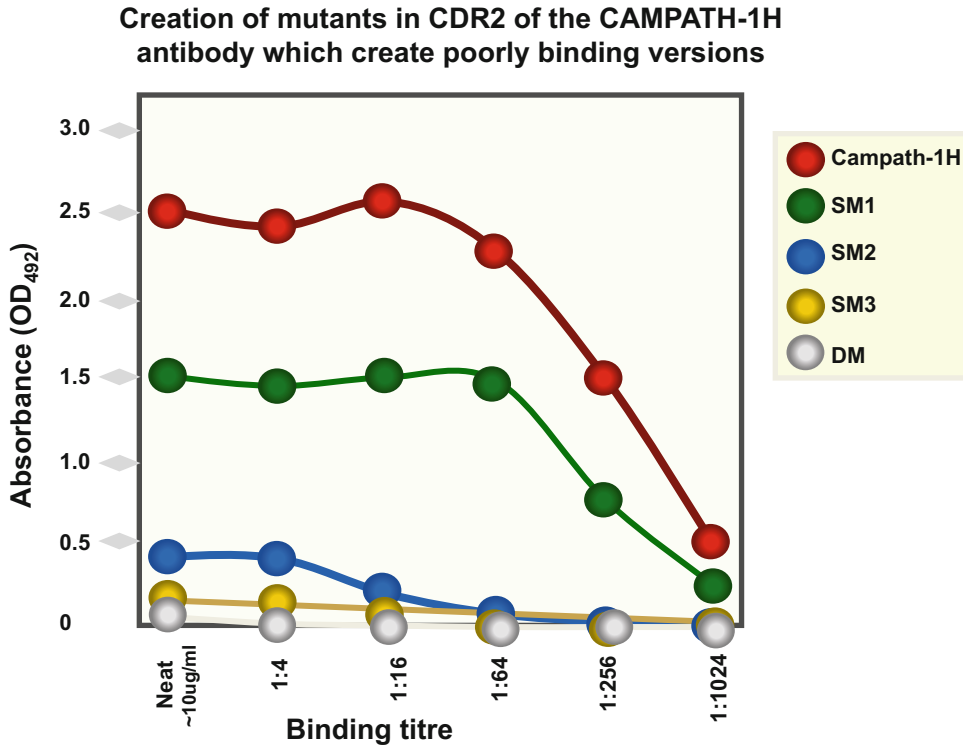

Fig. 2 Mutants can be created in the CDR regions which render the antibody far less able to bind to cells. In this case mutants were created in CDR2 of the heavy chain of the CAMPATH-1H anti-CD52 antibody. *SM* single mutations, *DM* double mutants. Binding to antigen is substantially reduced by three (blue, yellow, and gray symbols) of the four mutant antibodies compared to the original therapeutic (red) (Adapted from Gilliland et al. [39])

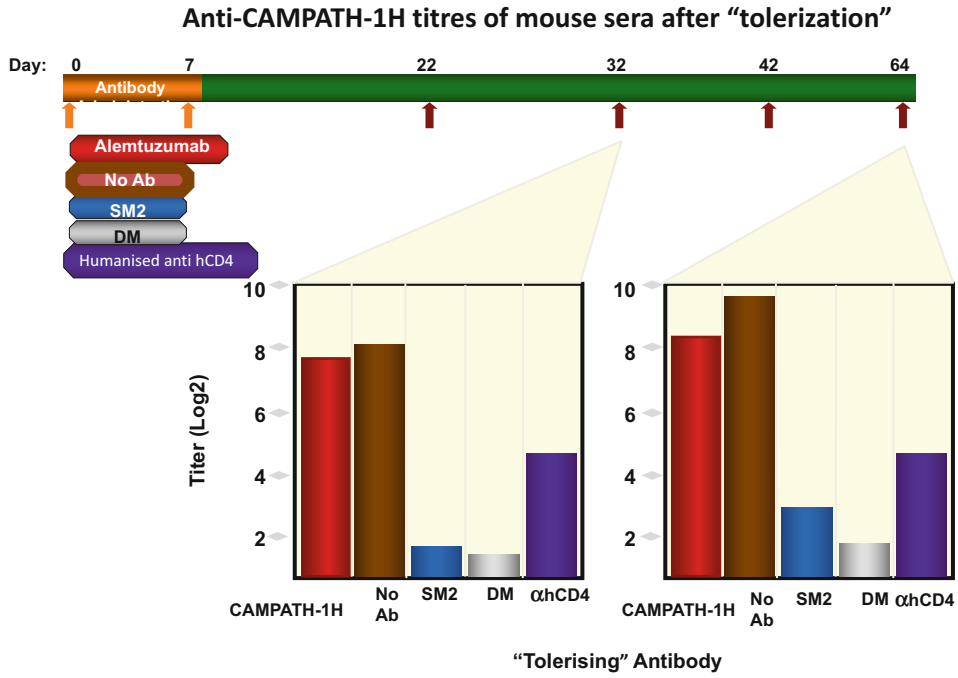

Fig. 3 Poorly-binding mutants tolerize CD52-transgenic mice to subsequent CAMPATH-1H treatment. CD52 transgenic mice were pretreated with two injections of 1 mg of a poorly binding mutant in CDR2 of the heavy chain of CAMPATH-1H, or a control anti-human CD4 antibody from 22 days onwards mice were given multiple challenges with the wild-type therapeutic antibody. "Tolerogen" pretreated mice made negligible antibody responses to the therapeutic (Adapted from Gilliland et al. [39])

challenge with the therapeutic form (Fig. 3). This provides a clear demonstration that high dose tolerance to the mutant prevented a response to the therapeutic version.

This two stage tolerizing protocol was applied in a small scale clinical study in patients given the IgG1 CD52 antibody, alemtuzumab, as a treatment for multiple sclerosis. A mutant "tolerogen" given before treatment substantially diminished the antibody response to a primary course of the therapeutic, as well as a second course given one year later [27].

Although impressive the disadvantage of this tolerizing approach is the need to manufacture and utilize two antibody forms. Thus far, no pharmaceutical company has made use of this strategy.

Is it possible that one could produce a version of the therapeutic antibody which can serve both as a tolerogen, yet still be able to exert its functional effect on cells? Such a one-step strategy has been achieved by engineering a covalently attached antigen mimotope into the antibody-binding site [40]. As the blocker mimotope renders the major proportion of antibody molecules "non-binding" at the time of infusion, it allows tolerogenesis before the bulk

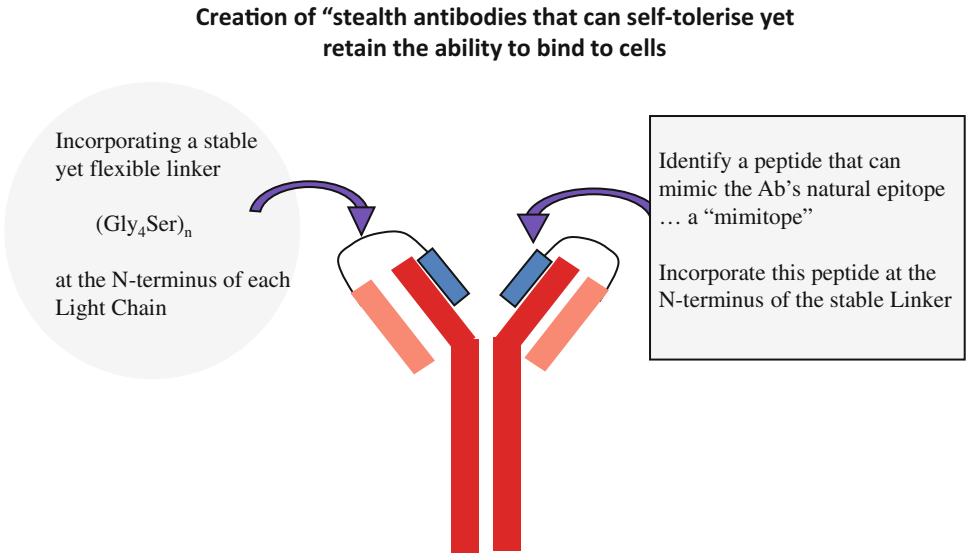

Fig. 4 Creation of "stealth antibodies that can self-tolerize yet retain the ability to bind to cells. (From issued patent US 7465,790B2-Therapeutic Antibodies). A peptide mimotope of the CD52 epitope is covalently bound into the CAMPATH-1H-binding site. This severely impairs binding, allows tolerogenesis but still retains, in the antibody, a capacity for cell-lysis

cell-binding consequences become effective. This sort of "stealth" antibody (Fig. 4), although tolerogenic in the mouse model, has not yet been subject to a clinical test. By reducing the pace of the lytic effect of the drug, it has also been possible to diminish some of the "cytokine" release-dependent side-effects of CD52 antibody therapy. There are obvious variations of this approach that could include concomitant administration of reversible "chemical" blockers of the antigen-binding site given together with the therapeutic, creating an initial "blocked" tolerogen whose cell binding eventually returns once the blocker is cleared.

It should be noted that the experimental models shown above both used human antibodies given to mice. Tolerization was achieved despite the extensive degree of "foreigness." However, even if humans prove tolerizable to rodent antibodies, it is obvious that one should apply "tolerization" approaches using antibodies whose constant regions are human, so making the task of tolerization easier. Moreover, as human constant regions are likely to be subject to various engineering strategies to optimize function, then one should regard the human rather than rodent frameworks as the template for such improvements.

5 Prospects and Conclusions

Thus far human antibodies seem to have satisfied the requirements of the biopharmaceutical industry even with antibodies where immunogenicity has been established. The need to do more to prevent this has become an issue of investment against likely demand, and at this stage of the therapeutic antibody experience, the need for active tolerization to the therapeutic has, sadly, not become a priority. I would venture that for some antibodies immunogenicity will never be a problem, but for others it may substantially enhance the longevity of the antibody as a drug. Given this, we should continue to evolve methodologies that can guarantee elimination of immunogenicity.

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Chapter 2

Cancer Immunotherapy: The Dawn of Antibody Cocktails

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Abstract

Since the approval of the first monoclonal antibody (mAb), rituximab, for hematological malignancies, almost 30 additional mAbs have been approved in oncology. Despite remarkable advances, relatively weak responses and resistance to antibody monotherapy remain major open issue. Overcoming resistance might require combinations of drugs blocking both the major target and the emerging secondary target. We review clinically approved combinations of antibodies and either cytotoxic regimens (chemotherapy and irradiation) or kinase inhibitors. Thereafter, we focus on the most promising and currently very active arena that combines mAbs inhibiting immune checkpoints or growth factor receptors. Clinically approved and experimental oligoclonal mixtures of mAbs targeting different antigens (hetero-combinations) or different epitopes of the same antigen (homo-combinations) are described. Effective oligoclonal mixtures of antibodies that mimic the polyclonal immune response will likely become a mainstay of cancer therapy.

Key words Antibody mixtures, Cancer, Chemotherapy, Immune checkpoints, Immunotherapy

1 The Power of Drug Combinations: A Systems Biology Perspective

It is worthwhile considering the evolution of biological systems and networks as a prelude for discussing pharmacological attempts to block pathological versions of biological networks. Viewed from an evolutionary perspective, the two genome duplications that created all metazoans generated families of four genes and laid the cornerstone for the modular structure of biological networks, a key feature of robustness [1]. Robustness was further boosted by means of training to overcome internal (mutational) and external (environmental) perturbations. However, due to low frequency, perturbations were introduced one at a time [2]. Hence, when challenged by two or more simultaneous perturbations, networks often expose remarkable fragilities [3]. This fundamental attribute of network training translates to high efficacy of pharmacological strategies utilizing drug combinations (poly-pharmacology). For example, kinome-wide profiling and *Drosophila* genetics showed that concurrent inhibition of three pathways, Ret and Raf, Src and ribosomal S6-kinase, was required for optimal survival of a Ret-driven

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fly model of multiple endocrine neoplasia [4]. Accordingly, combining targeted therapies (TTs), such as protein kinase inhibitors (PKIs) and monoclonal antibodies (mAbs), is considered a major future arena of medical oncology [5, 6]. It is notable that the ability of biological networks to resist common perturbations is greatly enhanced by their capacity to rewire information and metabolic circuitries [7]. This non-mutational mechanism of adaptation to a changing environment underlays many mechanisms that confer resistance to TTs [8, 9]. A similarly important mode of resistance entails genetic aberrations: either emergence of pre-existing mutant-expressing clones (tumor heterogeneity) [10] or de novo amplification/mutagenesis of drug targets [11]. Herein we review the short history of TT, with a focus on mAbs, their various modes of action and mechanisms underlying emergence of resistance to antibodies. Following brief descriptions of the main therapeutic antibodies, we review several efficacious combinations of antibodies with chemotherapy (CT), radiotherapy (RT), and PKIs. Lastly, we focus on the emerging, highly successful trend of combining several different antibodies to the same antigen (homo-combinations) and other mixtures of antibodies, including immune checkpoint inhibitors, recognizing distinct protein targets (hetero-combinations).

2 An Introduction to Cancer Therapy, Including Molecular Targeted Therapy

Cancer treatment depends on several factors: type of tumor, stage of the disease (local or metastatic), patient's age, and health status. Surgery, radiation therapy, and chemotherapy represent the primary modalities of cancer treatment [12]. The dawn of the third millennium witnessed the birth of molecular targeted therapy, which includes the use of antibodies specific to either cell surface molecules or soluble antigens. Surgery is the oldest treatment and, if cancer has not spread, this approach can completely cure a patient. For example, the excision of primary melanomas is sufficient to cure this type of cancer in 90% of cases. In some other cases, surgery can be employed to reduce the bulk of tumor prior to treatment of the residual cancer. Surgery is not only an important treatment modality but it can also be used for prevention, such as prophylactic mastectomy in women with BRCA mutations. Resection of primary solid tumors, when these are confined to the anatomic side of origin, is the first application of surgery in cancer.

Radiation therapy (RT) is used as an initial treatment, alone or in combination with other treatments, in 30–50% of all cancer patients [13]. In many patients this translates to high-energy external-beam photon therapy. Ionizing radiation affects normal cell division, causes DNA damage, and finally induces cell death. Electrons can be used to treat superficial tumors (for instance, skin and breast cancer) since they can penetrate up to 6-cm of tissue. In the

case of deeper tumors photons are used because they spare the skin and deposit dose along the path until the beam leaves the body. Radiation can be used alone, when the tumor is localized and surgery is not an option, or it can be associated with either chemotherapy or TT in case of locally advanced or aggressive cancers. RT causes side effects in normal tissues because ionizing radiation is unable to discriminate between cancer and healthy tissues. The tissues most affected by RT are those that depend on rapid self-renewal, such as skin and mucosal surfaces (e.g., organs of the gastrointestinal tract). In addition, a decrease in lymphocyte count is observed following irradiation [13].

Chemotherapy (CT) is the most widely used approach in cancer treatment. In principle, CT may cure even advanced cancers. However, the major issues are raised by drug toxicity toward normal tissues and emergence of resistance. CT is used as a primary treatment in patients with advanced cancers that cannot receive other types of treatment, or it is used as neoadjuvant treatment aimed at reducing tumor mass before proceeding with local therapy (i.e., surgery or RT). Chemotherapy can also be combined with RT or with TT. The main classes of chemotherapeutic drugs include alkylating agents, platinum compounds, antimetabolites like 5-fluorouracil, topoisomerase inhibitors, such as irinotecan, and anti-microtubule agents, like paclitaxel. Similar to RT, organs with rapid self-renewal are damaged by CT. Toxicity to the bone marrow with consequent leukopenia and increased risk of infections is a common side effect of most of the chemotherapeutic drugs.

Treatment of cancer has profoundly changed since the introduction of TT and the birth of precision medicine, namely pharmacological interventions able to specifically block mutation-bearing drivers of cancer or signaling pathways essential for survival of tumor cells [14]. Targeted therapies include small-molecule drugs and mAbs, which will be the focus of this review. The first small molecule to be approved for cancer treatment was the BCR-ABL protein kinase inhibitor (PKI) called imatinib, which was approved in 2001 for the treatment of chronic myeloid leukemia (CML) [15]. Since then many other small molecules have been approved. For example, PKIs specific to the epidermal growth factor receptor (EGFR), such as erlotinib, afatinib, and osimertinib, are commonly used to treat lung cancer tumors harboring mutant forms of EGFR [16]. Similarly, mAbs to the vascular endothelial growth factor (VEGF), EGFR, and its closely related protein, called HER2 or ERBB2, entered clinical applications around the turn of the millennium [17]. Accordingly, their first biosimilars are becoming available worldwide. Notably, these and additional antibodies are often combined with CT or RT. In this review we argue that mixtures of mAbs (oligoclonal antibodies) might be the welcome swallows of a spring of synergistic anti-cancer antibodies.

3 A Primer to Therapeutic Antibodies

The immune system plays a general defensive role against infectious agents, such as bacteria and viruses, which present a threat to human health. This system consists of two arms: the innate immune response, which engages soluble proteins, cytokines and physical barriers, recognizes many invaders without any specificity, whereas the adaptive immune response shows high target specificity and can be divided into humoral (antibody-mediated) and cellular (cell-mediated) responses [18]. The first use of antibodies as therapeutic tools dates back to the late nineteenth and the early twentieth centuries when infectious diseases were treated with serum from patients who had recovered from that specific disease.

Antibodies, also called immunoglobulins (Ig), are proteins consisting of four polypeptide chains, two heavy chains, and two light chains, which interact with each other through disulphide bonds and globally form a typical "Y" shape [19]. The light and heavy chains of a mAb contain variable (V_H and V_L) and constant (C_H and C_L) regions. The constant region determines the mechanism responsible for the destruction of the antigen (e.g., recruitment of macrophages, natural killer cells, or neutrophils). Based on the structure of the constant regions and thus on the immune function, immunoglobulins are divided into five classes: IgM, IgG, IgA, IgD, and IgE. The most common isotype of immunoglobulins used as therapeutic antibodies is IgG. The variable regions of both heavy and light chains present hypervariable amino acid sequences, called CDRs (complementarity determining regions), which are responsible for the interaction and specificity toward different antigens. One IgG molecule contains three pairs of different CDRs: CDR1, CDR2, and CDR3, with CDR3 showing the highest variability. The first mAbs were isolated from hybridoma cells by Cesar Milstein and Georges Kohler in 1975 [20]. The first therapeutic antibody, muromonab (OKT-3), was a murine antibody directed against the CD3 receptor expressed on the surface of T cells. Muromonab was approved in 1986 for organ acute rejection [21]. Notably, this antibody was not very effective in preventing organ rejection, mainly because it induced a strong human anti-mouse antibody (HAMA) response in treated patients [22]. For this reason and due to the introduction of alternative treatments, OKT-3 was discontinued in 2010. In general, the use of murine antibodies in the clinic is limited because of the differences between the rodent and human immune system and the HAMA-mediated allergic response [23].

Even though the hybridoma technology instigated an enormous step forward, similar to muromonab, the initial mAbs were murine and immunogenic when injected into humans, which limited their clinical use. This problem was initially overcome by the

replacement of the murine *C* (constant) chains with the human constant sequences (i.e., chimerization) [24]. Notably, the first murine/human chimeric mAb, abciximab [25], was approved in 1994 for hemostasis. Typically, 65% of the sequence of chimeric antibodies is derived from human sequences, which reduces HAMA responses. Rituximab and cetuximab are examples of chimeric antibodies approved in 1997 and in 2004, respectively, for the treatment of non-Hodgkin lymphoma (rituximab) and colorectal cancer (cetuximab; see below). Another attempt to overcome the rodent origin of murine antibodies has been the introduction of humanized antibodies, in which the mouse hypervariable regions are grafted onto the human IgG backbone (humanization). In this case the human sequence represents about 95% of the entire molecule. Daclizumab (Zenapax), the first humanized mAb, was approved in 1997 for kidney transplant rejection [26]. Later, the introduction of transgenic mice and phage display platforms allowed the production of fully human antibodies [27]. Adalimumab was the first fully human mAb to be approved, for the treatment of rheumatoid arthritis. Similarly, the fully human anti-EGFR antibody panitumumab was first approved in 2006 for the treatment of metastatic colorectal cancer [28]. The use of mAbs in cancer therapy has been particularly productive [29]. These molecules display high specificity and thus can be used to target with high selectivity specific antigens, which are mainly expressed by tumors (targeted therapy). As a result, the number of clinically approved therapeutic antibodies has steadily increased in the last decade (*see* Table 1), and many more are in clinical trials for cancer and other diseases. The targeted antigens of cancer-specific mAbs include surface glycoproteins playing roles in growth or differentiation, such as CD20, which has been successfully targeted by rituximab [30]. Other antigens that can be targeted in cancer are growth factor receptors. The humanized anti-HER2 antibody, trastuzumab, was the first antibody to be successfully used in the treatment of solid tumors [31]. In addition, antibodies can bind and neutralize soluble antigens. Bevacizumab, a humanized antibody that effectively binds with the vascular endothelial growth factor (VEGF), has been approved for several types of cancer.

4 Mechanisms of Action of Therapeutic Antibodies

In general, the mechanisms enabling therapeutic antibodies to inhibit growth of, or kill, cancer cells can be divided into two categories: immune-mediated mechanisms (e.g., ADCC, antibody-dependent cellular cytotoxicity and CDC, complement-dependent cytotoxicity) and mechanisms that interfere with pathways of tumorigenesis (e.g., triggering apoptosis, inhibiting cell proliferation or blocking angiogenesis). In order to trigger

Table 1
Monoclonal antibodies currently approved for use in oncology

Antibody	Brand name (company)	Target	Type	Year of first FDA/EMA approval	Therapeutic indication
Rituximab	Rituxan/MabThera (Genentech)	CD20	Chimeric IgG1k	1997/1998	NHL, CLL
Trastuzumab	Herceptin (Genentech)	HER2	Humanized IgG1k	1998/2000	HER2-overexpressing breast cancer, HER2-overexpressing metastatic gastric or GEJ adenocarcinoma
Ibritumomab tiuxetan	Zevalin (Spectrum Pharms)	CD20	Murine IgG1k (ARC)	2002/2004	NHL
Cetuximab	Erbitux (ImClone)	EGFR	Chimeric IgG1	2004/2004	HNSCC, CRC
Bevacizumab	Avastin (Genentech)	VEGF-A	Humanized IgG1	2004/2005	Metastatic CRC, NSCLC, metastatic breast cancer, glioblastoma multiforme; metastatic RCC
Panitumumab	Vectibix (Amgen)	EGFR	Human IgG2	2006/2007	Metastatic CRC
Ofatumumab	Arzerra (Glaxo Grp Ltd)	CD20	Human IgG1	2009/2010	CLL
Denosumab	Xgeva (Amgen)	RANK Ligand	Human IgG2	2010/2011	Giant cell tumors of the bone, prevention of SREs in patients with MM and bone metastases from solid tumors
Ipilimumab	Yervoy (Bristol Myers Squibb)	CTLA-4	Human IgG1	2011/2011	Melanoma, RCC
Brentuximab vedotin	Adcetris (Seattle Genetics)	CD30	Chimeric IgG1 (ADC)	2011/2012	HL, systemic ALCL

Pertuzumab	Perjeta (Genentech)	HER2	Humanized IgG1	2012/2013	HER2+ metastatic breast cancer
Ado-Trastuzumab emtansine	Kadcyla (Genentech)	HER2	Humanized IgG1 (ADC)	2013/2013	HER2+ metastatic breast cancer
Obinutuzumab	Gazyva/Gazyvaro (Genentech)	CD20	Humanized IgG1	2013/2014	CLL
Ramucirumab	Cyramza (Eli Lilly)	VEGFR2	Human IgG1	2014/2014	Gastric cancer, NSCLC, CRC
Pembrolizumab	Keytruda (Merck Sharp Dohme)	PD-1	Humanized IgG4	2014/2015	Melanoma, NSCLC, HNSCC, HL, large B-cell lymphoma, urothelial carcinoma, gastric cancer, cervical cancer
Blinatumomab	Blincyto (Amgen)	CD19/CD3	BiTE	2014/2015	Precursor cell lymphoblastic leukemia-lymphoma
Nivolumab	Opdivo (Bristol Myers Squibb)	PD-1	Human IgG4	2014/2015	Urothelial carcinoma, NSCLC, RCC, HL, melanoma, CRC, HNSCC, hepatocellular carcinoma
Dinutuximab	Unituxin (United Therapeutics)	GD2	Human IgG1/κ	2015/W	Neuroblastoma
Necitumumab	Portrazza (Eli Lilly)	EGFR	Human IgG1	2015/2016	NSCLC
Elotuzumab	Empliciti (Bristol Myers Squibb)	SLAMF7	Humanized IgG1	2015/2016	MM
Daratumumab	Darzalex (Janssen Biotech)	CD38	Human IgG1/κ	2015/2016	MM
Olaratumab	Lartruvo (Eli Lilly)	PDGFR-α	Human IgG1	2016/2016	Soft tissue sarcoma
Atezolizumab	Tecentriq (Genentech-Roche)	PD-L1	Humanized IgG1	2016/2017	

(continued)

Table 1
(continued)

Antibody	Brand name (company)	Target	Type	Year of first FDA/EMA approval	Therapeutic indication
					Metastatic NSCLC, urothelial carcinoma
Durvalumab	Imfinzi (Astrazeneca UK)	PD-L1	Human IgG1/κ	2017/NA	Metastatic urothelial carcinoma, NSCLC
Avelumab	Bavencio (EMD Serono INC.)	PD-L1	Human IgG1/κ	2017/2017	Metastatic Merkel cell carcinoma, urothelial carcinoma
Inotuzumab ozogamicin	Besponsa (Wyeth Pharmaceuticals Inc.)	CD22	Humanized IgG4/k (ADC)	2017/2017	B-cell precursor ALL
Gemtuzumab ozogamicin	Mylotarg (Wyeth Pharmaceuticals Inc.)	CD33	Humanized IgG4 (ADC)	2017/2018	CD33-positive AML
Bevacizumab-awwb	Mvasi (Amgen/Allergen)	VEGF-A	Biosimilar to Bevacizumab	2017/2018	CRC, NSCLC, glioblastoma,RCC, cervical cancer
Trastuzumab-dkst	Ogivri (Mylan GMBH)	HER2	Biosimilar to Trastuzumab	2017/NA	HER2-overexpressing breast or metastatic gastric or GEJ adenocarcinoma

Listed are all clinically used mAbs, their immunoglobulin types, year of first approval by the Food and Drug Administration (FDA; United States) or by the European Medicines Agency (EMA), as well as the respective major therapeutic indications. Note that gemtuzumab ozogamicin was first approved in 2000, but in 2010 it was withdrawn. ADC, antibody-drug conjugate; ALCL, anaplastic large cell lymphoma; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ARC, antibody-radionuclide conjugate; BiTE, bispecific T-cell engager; CLL, chronic lymphocytic leukemia; CRC, colorectal cancer; GEJ, gastroesophageal junction; HL, Hodgkin lymphoma; HNSCC, head and neck squamous cell cancer; MM, multiple myeloma; NA, not approved; NHL, non-Hodgkin lymphoma; NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma; SREs, skeletal-related events; W, withdrawn

ADCC, the antibody has to bind a specific antigen expressed on the surface of a cancer cell. This event leads to the recruitment of immune effector cells, such as natural killer (NK) cells, macrophages, or neutrophils. Subsequently, the F_C region of the antibody interacts with an F_C receptor on an effector cell, which enables lysis of the target tumor cells [32]. For example, one mechanism of action of the anti-CD20 antibody, rituximab, harnesses ADCC. Two lines of evidence exemplify ADCC involvement: Firstly, rituximab was not effective when tested in $F_{C\gamma}R^{-/-}$ mice, which do not express the stimulatory Fc-gamma receptor type III, and conversely, it exhibited enhanced activity when tested in mice lacking the inhibitory $F_{C\gamma}R$ type IIb [33]. Secondly, polymorphisms in the FcRIIIa gene affect the response rates of NHL patients to rituximab: in humans, a polymorphism in FcRIIIa places either a valine (V) or a phenylalanine (F) at position 158. Several studies have shown that patients with receptor homozygous for V in position 158 (158V/V) respond better to rituximab as compared with patients displaying the 158V/F or the 158F/F receptor [34]. This has been attributed to higher in vitro affinity of 158V/V FcRIIIa toward IgG1 compared to the other isoforms, 158V/F or 148F/F [35]. Similarly, polymorphisms in Fc receptors have been associated with the efficacy of cetuximab in colorectal cancer [36]. Interestingly, cetuximab is an IgG1 antibody, which induces ADCC, while another anti-EGFR antibody, panitumumab, which is an IgG2 molecule, is unable to trigger immune responses because IgG2 molecules do not recognize $F_{C\gamma}$ receptors on immune effector cells [37]. CDC starts when the antibody-antigen complex interacts with C1q, thereby forms the membrane attack complex (MAC). This results in the activation of the complement cascade, which is regulated by several zymogens (C1–C9) and inhibitory proteins, such as CD35, CD46, CD55, and CD59 [38]. As a final result, the target cell undergoes lysis. Several studies in mice suggest that CDC is one of the mechanisms of action of rituximab: mice lacking C1q display reduced sensitivity to rituximab and complement inhibitory proteins are able to inhibit cell death induced by rituximab [39, 40]. In addition, whereas blocking the inhibitory proteins enhances rituximab-induced CDC.

The non-immune modes of actions of anti-cancer mAbs may be exemplified by bevacizumab, an anti-VEGF antibody, which binds and inactivates the soluble growth factor with no known involvement of immune mechanisms [41]. Other antibodies may bind a receptor on the target cell surface, and this often leads to blocking ligand binding or receptor dimerization. Alternatively, by virtue of their bivalence, mAbs may induce internalization and degradation of oncogenic/survival receptors. As a result, mAbs may modulate signaling pathways controlling important cellular processes such as apoptosis, proliferation, and angiogenesis. Apoptosis is a programmed cell death process involving activation of several

proteases, known as caspases, and occurring via two pathways: the intrinsic pathway, activated by intracellular signals, such as stress, which leads to release of cytochrome C from the mitochondria, and the extrinsic pathway, activated by the binding of extracellular cytokines to death receptors localized at the cell surface, and formation of a complex that activates the caspase cascade. Binding of rituximab to CD20, a modulator of calcium channels [42], may induce apoptosis via accelerating calcium fluxes. Similarly, the anti-HER2 antibody trastuzumab may induce apoptosis by inhibiting the AKT and the mitogen-activated protein kinase (MAPK) pathways, as well as by enhancing the TRAIL- (tumor necrosis factor-related apoptosis-inducing ligand) mediated apoptosis pathway [43]. Alternatively, the anti-EGFR antibody, cetuximab, induces an increase in the expression levels of the apoptotic protein BAX and a decrease in the levels of the anti-apoptosis protein BCL-2 [44]. Another common mechanism used by antibodies to inhibit cell proliferation is the modulation of key proteins of the cell cycle [45]. Trastuzumab induces upregulation of the cyclin-dependent kinase inhibitor (CDKI) p27kip1, which arrests cancer cells in the G1 phase of the cell cycle [45]. Another important mechanism of action of therapeutic antibodies is the inhibition of angiogenesis. It has been reported that trastuzumab inhibits angiogenesis in different cancer models [46]. Antibodies targeting growth factor receptors, such as cetuximab or trastuzumab, may also exert their antitumor activity by blocking the mitogenic signaling pathways downstream of the respective receptors. Cetuximab prevents binding of ligands to EGFR and inhibits receptor dimerization [47, 48]. It has been shown that anti-HER2 mAbs induce internalization and degradation of HER2 through the activation of the Cbl ubiquitin ligase [49]. In addition, a specific subset of anti-HER2 mAbs inhibits formation of heterodimers containing HER2 [50]. It was later reported that certain anti-HER2 mAbs, such as pertuzumab, bind to subdomain II of HER2 and inhibit formation of heterodimers containing other EGFR family members, thereby inhibit the ability of HER2 to enhance downstream signaling [51].

5 Resistance to Therapeutic Antibodies

Since mAbs were introduced in oncology wards, they have significantly improved the treatment of cancer. For example, rituximab in combination with chemotherapy (CHOP; cyclophosphamide, doxorubicin, vincristine and prednisone) has significantly improved the overall survival of patients with non-Hodgkin lymphoma (NHL) within the first five years after approval of the mAb [52]. This anti-CD20 antibody has also modified the treatment of patients with follicular lymphoma (FL), even though it has not changed the final patient outcome. Every other patient with

relapsed/refractory FL shows no response to rituximab, and in about 60% of cases that show initial positive response, patients stop responding to a second treatment [53]. Similarly, the introduction of trastuzumab has clearly improved the outcome in breast cancer patients, but the median response to this treatment is still modest [54]. In general, the failure of mAb therapy might be due to several resistance mechanisms. The resistance can be intrinsic or acquired: in intrinsic mechanisms the antibody is not effective, even if the antigen is present on tumor cells. By contrast, in acquired resistance, tumor cells display initial sensitivity to the treatment, but after a variable period of time they stop responding. The underlying mechanisms of resistance include heterogeneity of HER2 downregulation in the tumor, signaling pathway promiscuity [55], as well as immune escape due to impairment of ADCC or CDC [6]. Loss or modifications of the antigens can be responsible for resistance to mAb treatment. Loss of CD20 expression on the cell surface is one of the mechanisms responsible for resistance to rituximab [56]. Likewise, expression of the truncated p95-HER2 isoform is related to diminished sensitivity to trastuzumab in breast cancer patients [57]. In addition to loss of the target antigen, or its masking by other molecules, such as MUC4 [58], one common resistance mechanism to mAbs is the presence of mutations in downstream signaling molecules or compensatory activation of other receptors. Patients with advanced colorectal cancer do not respond to anti-EGFR therapy (cetuximab or panitumumab) if KRAS mutations are present in the tumors [59]. Likewise, evidence from cellular and animal models indicate that treatment of EGFR-mutated lung cancer cells with an anti-EGFR antibody causes upregulation of other members of the EGFR family of receptors, primarily HER2 and HER3, with consequent hyper-activation of ERK-MAPK, but co-treatment with anti-EGFR, anti-HER2 and anti-HER3 antibodies completely abrogated activation of the compensatory pathway [60]. Similarly, upregulation of the receptors for the insulin-like growth factor 1 (IGF1R) and the hepatocyte growth factor (MET) has been related to resistance to trastuzumab in models of breast cancer [61, 62]. In a number of patients, mutations in PIK3CA or low expression of PTEN, which lead to activation of the PI3K-to-AKT pathway, are associated with poor prognosis after trastuzumab treatment [63].

6 Examples of mAbs Employed to Treat Cancer

Cancer treatment has dramatically changed since 2000, primarily due to the clinical approval of recombinant mAbs and kinase inhibitors. Remarkably, the new agents significantly enrich the armamentarium of clinical oncology rather than replace the relatively nonspecific cytotoxic treatments, such as radiotherapy and

chemotherapy. It is also notable that in comparison to small-molecule drugs, like PKIs, mAbs display very narrow target selectivity, hence toxicity due to off-target interactions is less common in immunotherapy.

6.1 mAbs for Hematological Tumors

Hematological malignancies comprise several different types of blood cancers, which are divided into four groups: leukemia, Hodgkin lymphoma, non-Hodgkin lymphoma (NHL), and myeloma. Rituximab, the first antibody to be approved for hematological tumors, is still the most widely used mAb for treatment of B cell-NHL and for chronic lymphocytic leukemia (CLL). Rituximab is a chimeric IgG1 antibody that binds to CD20, a B-lymphocyte transmembrane antigen, which is expressed on the surface of both non-neoplastic B cells (pre-, immature, mature, and activated) and malignant B cells [64]. Given that CD20 is not expressed on the surface of hematopoietic stem cells, normal B cells can regenerate after stopping treatment with rituximab. The mechanisms of action of rituximab include ADCC, CDC, and induction of apoptosis. The antibody was first approved 1997 for NHL and subsequently, in 2009, for the treatment of CLL patients. Later, rituximab became a standard component of the treatment of follicular lymphoma, diffuse large B-cell lymphoma, CLL, and mantle cell lymphoma. Importantly, several clinical trials have shown that rituximab is able to extend the time to disease progression and also the overall survival rates [65]. Following 20 years of post-marketing surveillance, the major side effects to rituximab in B-cell malignancies are quite well known, with the most common being infusion-related reactions (IRRs), the cytokine release syndrome, bronchospasm, and hypotension. In the majority of cases, IRRs occur after the first injection of rituximab, but later their incidence decreases. Other common adverse events to rituximab include cardiovascular events, infections, and hematological side effects, like neutropenia. Despite the clear efficacy of rituximab in patients with B-cell malignancies, some patients do not respond to the first-line therapy and some other patients become resistant following an initial response. Resistance to rituximab involves loss of CD20 expression [66] and impairment of either CDC (because of complement protein depletion), ADCC (polymorphism of the FcRIIIa on immune effector cells may alter the sensitivity of the tumor cells to ADCC), or apoptosis (upregulation of antiapoptotic proteins) [67]. The enormous success of anti-CD20 treatment in B-cell malignancies led to the development of additional antibodies against this antigen. Ofatumumab is a fully human anti-CD20 antibody, which was approved in 2009 for CLL. Similarly, obinutuzumab, a humanized glycoengineered anti-CD20 antibody, was approved in 2013 for the treatment of CLL and later also for FL. This antibody, as a result of posttranslational glycoengineering modification, lacks a fucose residue in the IgG oligosaccharides of the Fc region, resulting in

enhanced binding affinity to the FcRIII on the surface of immune effector cells [68]. Antibodies directed against other antigens (e.g., anti-CD19, inebilizumab, and anti-CD22, epratuzumab) have been investigated, but they did not show promising results in clinical trials. Furthermore, two mAbs were recently approved for clinical use, namely the humanized anti-SLAMF7 mAb called elotuzumab and daratumumab, an anti-CD38 mAb.

6.2 mAbs for Solid Tumors

The approval of trastuzumab, an anti-HER2 antibody, in 1998, for the treatment of patients with HER2-positive metastatic breast cancer instigated the era of solid tumor treatment. The receptor tyrosine kinase (RTK) called HER2 is overexpressed in 15–20% of invasive breast cancers, and high HER2 expression levels are correlated with poor prognosis [69]. While anti-HER2 mAbs were able to inhibit growth of HER2-overexpressing mammary cancer cells, both in vitro and in vivo, the parent of trastuzumab, the murine antibody MuMAb4D5, showed no inhibitory activity toward normal cells or toward tumor cells devoid of HER2. Thus, because HER2 is mainly overexpressed in tumor cells, there is only low risk of toxicity associated with anti-HER2 treatments. The parent anti-HER2 murine antibody was humanized in order to obtain a recombinant antibody comprising almost 95% of human sequence. Trastuzumab is directed against the extracellular subdomain IV of the receptor [70]. Trastuzumab decreased receptor signaling, while increasing HER2 internalization and subsequent degradation [49]. In addition, trastuzumab induces apoptosis and inhibits angiogenesis. Because response rates to trastuzumab administered alone (monotherapy) are relatively low, usually this mAb is administered together with chemotherapeutic drugs, such as paclitaxel or docetaxel, with consequent improvement in response rates, overall survival, and disease progression [71]. Despite initial responses to therapy based on this mAb, within 12–18 months many patients become resistant to the drug. The proposed resistance mechanisms include expression of a truncated isoform of HER2, overexpression of other RTKs, and loss of PTEN, a lipid phosphatase [72]. Another approved anti-HER2 humanized recombinant antibody is pertuzumab. Pertuzumab binds to the extracellular dimerization domain II of HER2, which is distinct from the epitope recognized by trastuzumab [73].

Other important mAbs used for treatment of solid tumors include the anti-EGFR antibodies cetuximab, panitumumab, and necitumumab. The EGFR signaling pathway is involved in processes crucial for tumor growth and progression, cell proliferation, angiogenesis, and inhibition of apoptosis [74]. Cetuximab was first approved in 2004 for the treatment of metastatic colorectal cancer, either alone, after failure of chemotherapy, or in combination with chemotherapy as first-line treatment. Because it was later observed that this mAb confers no benefit when used to treat colorectal

tumors harboring KRAS mutations [75], the antibody is not indicated for the treatment of tumors with RAS mutations. In 2011 cetuximab was also approved for head and neck cancer, in combination with radiotherapy or with chemotherapy, or as a single agent after failure of chemotherapy [76]. Cetuximab is a chimeric mAb which binds with extracellular domain III of EGFR and blocks the binding of the ligand, thereby inhibits activation of the receptor and all downstream signaling pathways. In addition, the antibody induces ADCC and promotes receptor internalization and degradation [77]. The anti-EGFR human mAb called panitumumab was approved in 2006 for the treatment of KRAS wild type metastatic colorectal cancer. Another human anti-EGFR antibody, necitumumab, was approved in 2015 for clinical application in combination with two chemotherapeutic drugs (gemcitabine and cisplatin), because it offers a survival advantage for patients with advanced squamous NSCLC [78].

Given the essential roles played by VEGF-A and VEGFR-2 in tumor angiogenesis, the blockade of this pathway represents a suitable strategy to inhibit cancer progression [79]. Bevacizumab, a recombinant humanized anti-VEGF-A monoclonal antibody, was first approved in 2004 for the treatment of metastatic colorectal cancer, in combination with chemotherapy. The binding of bevacizumab to VEGF-A prevents its interaction with the receptors and their activation, which leads to regression of immature tumor vasculature and inhibition of angiogenesis. In 2006, bevacizumab was also approved for the treatment of metastatic HER2-negative breast cancer, in combination with chemotherapy, based on preliminary analysis of the E2100 clinical trial. However, approval for this breast cancer treatment was revoked in 2011, following completion of additional trials [80]. Currently, bevacizumab is approved for the treatment of colorectal cancer, non-small cell lung cancer, glioblastoma, renal cell carcinoma, cervical cancer, ovarian cancer, fallopian tube cancer, and peritoneal cancer.

7 Immune Checkpoint Inhibitors

The immune response to a specific antigen requires interactions among antigen-presenting cells, T cells, and target cells. The activation of T cell requires a first signal, which involves the interaction of the antigen, bound to the MHC (major histocompatibility complex), with the T-cell receptor, and a second signal, the binding of the T-cell activator CD28 to a member of the B7 co-stimulatory molecules (CD80 or CD86). These events lead to autocrine production of interleukin-2 (IL-2) and subsequent T-cell activation. Tumors can escape the immune response by means of several mechanisms [81], for example by activating immunoregulatory mechanisms, also known as immune checkpoints. Targeting the

immune checkpoints with antibodies represents an effective way to enhance the anti-tumor immune response.

Cytotoxic T lymphocyte-associated protein 4 (CTLA-4) potently regulates T-cell responses [82]: after engagement of the T-cell receptor (TCR) and a co-stimulatory signal mediated by CD28, CTLA-4 outcompetes CD28 for binding to CD80 and CD86, two co-stimulatory proteins. This interaction arrests both proliferation and activation of T cells [83]. Hence, blocking CTLA-4 translates to enhanced T-cell activation. Two mAbs against CTLA-4 were developed: ipilimumab and tremelimumab. The success of a large phase III clinical trial, which compared ipilimumab with standard dacarbazine chemotherapy [84], which showed that the antibody improved overall survival of patients with metastatic melanoma, led to the approval of this mAb, in 2011, for the treatment of unresectable or metastatic melanoma. Unfortunately, given the mechanism of action of this antibody, namely T-cell proliferation and activation, the toxicity to this drug is common and it mainly involves inflammatory side effects, mostly confined to skin and to the gastrointestinal tract. However, in some cases it can affect also liver and endocrine glands [85].

Another key immune checkpoint of T cells is the programmed death 1 (PD-1) molecule, which is expressed not only on T cells, but also on natural killer (NK) cells and B cells. When PD-1 binds to its ligand, PD-L1, which is broadly expressed by tumor cells and also by many somatic cells upon exposure to proinflammatory cytokines, it causes inhibition of T-cell proliferation, cytokine production, and cytotoxicity, as well as elevated apoptosis [86]. Hence, blocking PD-1 is another approach that has been used in order to enhance the immune response against cancer cells. In vivo studies confirmed that the blockade of PD-1 with antibodies led to enhancement of anti-tumor immunity [87]. Subsequently, several clinical trials in patients with advanced melanoma [88], NSCLC [89], renal cell carcinoma [90], bladder cancer [91], and Hodgkin's lymphoma [92], led to the approval of nivolumab, an anti-PD1 mAb, in all the aforementioned indications. Currently, five antibodies that target the PD-1/PD-L1 axis have been approved: nivolumab and pembrolizumab, which target PD-1, and three anti-PD-L1 antibodies, atezolizumab, avelumab, and durvalumab [93].

8 Combinations of Antibodies and Cytotoxic Treatments (*See* Fig. 1)

It is important noting that the labels of many clinically approved mAbs indicate combinations with cytotoxic regimens, namely chemotherapy or, in a fewer cases, radiotherapy. For example randomized clinical trials comparing CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) regimen alone or in combination with rituximab (R-CHOP) in NHL showed that

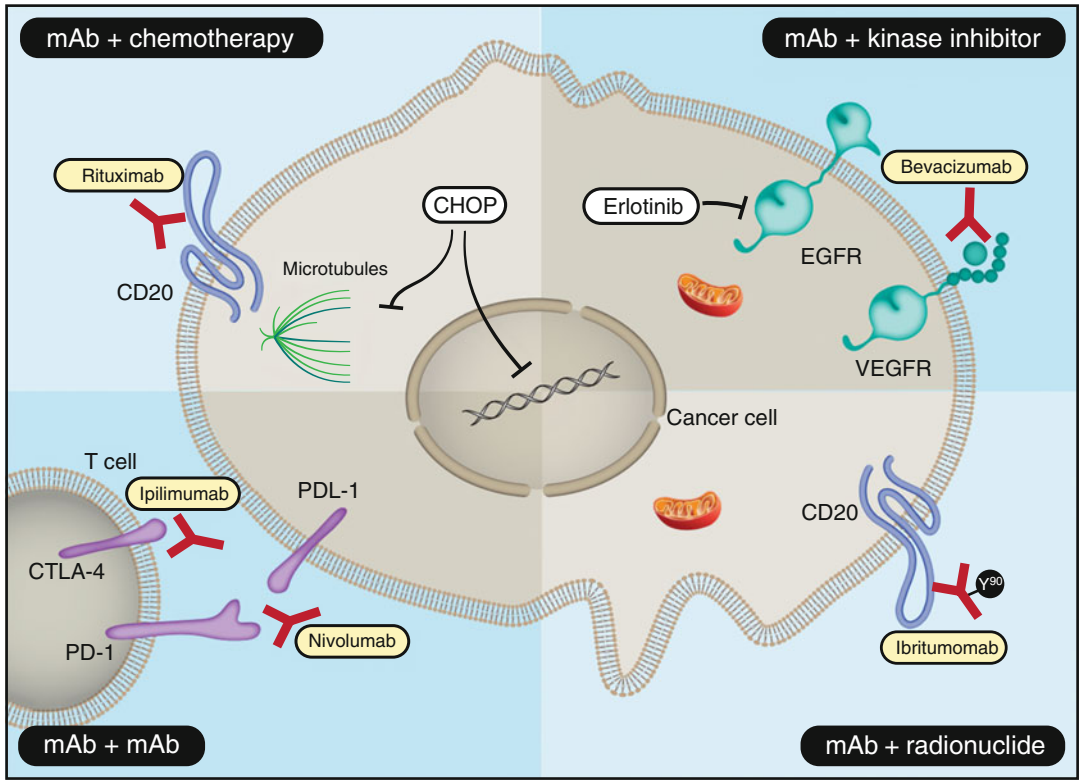

Fig. 1 Clinically approved combinations of antibodies and other pharmacological approaches. Four different examples of pharmacological strategies are presented. The upper left corner presents a combination of a monoclonal anti-CD20 antibody (rituximab) and chemotherapy (CHOP; cyclophosphamide, doxorubicin, vincristine and prednisone), which is approved for non-Hodgkin lymphoma. The upper right corner presents a combination of a monoclonal anti-VEGF antibody (bevacizumab) and an EGFR-specific kinase inhibitor (erlotinib). This combination has been approved as first-line treatment of patients with unresectable metastatic (or recurrent) NSCLC presenting EGFR mutations. The lower left corner presents a combination of two monoclonal antibodies recognizing T-cell antigens: CTLA-4 (ipilimumab) and PD-1 (nivolumab). This combination has been approved for patients with unresectable or metastatic melanoma, as well as some types of kidney and colon tumors. The lower right corner presents treatment with an anti-CD20 antibody (ibritumomab), which is conjugated to a radioactive isotope, Yttrium-90 (Y-90). This construct is approved for patients with relapsed or refractory low-grade CD20-positive B-cell non-Hodgkin lymphoma

co-treatment with the mAb and chemotherapy significantly increased the overall survival, as well as progression-free survival of treated patients [94]. Likewise, a phase III combination trial comparing chemotherapy alone (two regimens: anthracycline plus either cyclophosphamide or paclitaxel) and in combination with trastuzumab showed that patients receiving paclitaxel and trastuzumab displayed a median time to progression of 6.9 months, as compared to 3.0 months in the group treated only with paclitaxel, while patients treated with anthracycline, cyclophosphamide, and trastuzumab showed a median time to progression of 7.8 months,

compared to 6.1 months in the chemotherapy regimen [95]. More lately, trastuzumab has also been approved for adjuvant treatment of HER2-overexpressing breast cancer, both node positive and node negative, in combination with chemotherapy (e.g., doxorubicin, cyclophosphamide, and paclitaxel/docetaxel). Similarly, the anti-EGFR antibody cetuximab was approved in metastatic colorectal cancer in combination with different chemotherapeutic regimens. This antibody was also approved with radiotherapy in head and neck cancer since it has been shown that the combination reduces mortality without increasing the toxic effects of radiotherapy treatments [96].

mAbs can be used to deliver radiation or cytotoxic drugs directly to the tumor site. In the radioimmunotherapy approach radionuclides, typically β-emitters, are conjugated to an antibody. Ibritumomab tiuxetan (an anti-CD20 antibody labeled with the radionuclide ^{90}Y) and tositumumab (an anti-CD20 antibody labeled with ^{131}I) were approved in 2002 and 2003, respectively, for the treatment of NHL. Two antibody-drug conjugates (ADC) have been approved for clinical use: brentuximab vedotin (BV, in 2011) and ado-trastuzumab emtansine (TE, in 2013). BV consists of an anti-CD30 antibody conjugated to mono-methyl auristatin E. BV was approved for relapsed Hodgkin lymphoma based on a phase II trial which showed overall response rate of 75%, with complete remission in 34% of patients [97], and for systemic anaplastic large-cell lymphoma (ALCL) on the basis of another phase II trial, in which brentuximab vedotin induced objective responses in the majority of patients and complete responses in more than half of patients with recurrent systemic ALCL [98]. Ado-trastuzumab emtansine was prepared by conjugating maytansinoid DM1 to trastuzumab. Its approval in 2013 was based on a phase III study which showed that this ADC prolonged overall survival compared to lapatinib plus capecitabine in patients with HER2-positive metastatic breast cancer previously treated with trastuzumab and a taxane [99].

9 Combinations of mAbs and Protein Kinase Inhibitors (PKIs; *See* Fig. 1)

Imatinib, a BCR-ABL tyrosine kinase inhibitor, was the first PKI to be approved, in 2001, for the treatment of chronic myeloid leukemia (CML). The introduction of this PKI into the clinic has turned CML, a fatal cancer, into a manageable disease. Since then several other PKIs have been successfully used to treat cancer, including several generations of EGFR-specific PKIs (e.g., erlotinib, gefitinib, afatinib, and osimertinib), which are used to treat NSCLC [100]. Currently, the major issue with EGFR PKIs and similar drugs is the inevitable emergence of resistance after a variable period of time [5]. The most common mechanism of resistance to

PKI therapy is the appearance of point mutations within the kinase domain, resulting in decreased affinity of the inhibitor to the ATP-binding site. For example, resistance to first generation EGFR-PKIs, erlotinib and gefitinib, is mainly due to a secondary point mutation in the kinase domain of the receptor, namely the T790M mutation [101]. Another mechanism of resistance entails gene amplification, or other modes that upregulate expression levels of compensatory RTKs. Amplification of MET has been found in 20% of cases of resistance to first generation EGFR-PKIs in patients with NSCLC [102]. In addition, emergence of resistance to PKIs can be due to alterations in intracellular signaling pathways. Thus, resistance to erlotinib in EGFR-mutated lung cancer has been shown to be related to PTEN loss and consequent activation of AKT [103]. Considering all possible mechanisms leading to PKI resistance, several attempts to combine PKIs and mAbs have been conducted. Combining cetuximab and erlotinib or gefitinib caused inhibition of tumor growth and induction of apoptosis in head and neck and lung cancer cell lines [104]. Likewise, specific combinations of EGFR-specific mAbs and EGFR-PKIs showed a synergistic effect in terms of reducing cell proliferation and inhibiting the RAS signaling in triple-negative breast cancer cell lines [105]. The combination of three monoclonal antibodies (anti-EGFR, anti-HER2, anti-HER3) with osimertinib, a third generation EGFR-PKI, was highly effective in reducing tumor growth in NSCLC xenografts [60, 106]. Interestingly, when applied in vitro and in animals, the PKI induced apoptosis whereas the triple combination of mAbs induced senescence of EGFR-driven tumor cells. Several clinical trials examined combinations of PKIs and mAbs. A combination of an anti-MET antibody, onartuzumab, and erlotinib was tested in a phase III clinical study in NSCLC patients presenting MET amplification. However, this study showed that adding onartuzumab to erlotinib did not improve clinical outcome [107]. Another study, the JO25567 trial, analyzed a combination of erlotinib and bevacizumab in EGFR-driven lung cancer patients [108]. This study showed a clear improvement in progression-free survival. Another phase II trial confirmed the efficacy of combining erlotinib with bevacizumab in NSCLC patients with activating EGFR mutations [109], which led to clinical approval in 2016, in Europe. Lastly, a dual-specificity PKI, lapatinib, which blocks both EGFR and HER2, showed synergistic effects when mixed with trastuzumab and applied on HER2-overexspressing breast cancer cell lines [110]. Two explanations for the synergistic in vitro effect could be downregulation of survivin and enhanced tumor cell apoptosis. However, although several clinical studies showed that the combination of lapatinib and trastuzumab has better efficacy in comparison to the respective single agent treatments in metastatic HER2-positive breast cancer, the combinations also induced relatively high toxicity [111].