Chapter 16
Trifunctional Triomab® Antibodies for Cancer Therapy

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16.1 History of Bispecific Antibodies

In the 1980s, two groups described in parallel and for the first time T-cell-redirecting bispecific antibodies (bsAbs) for the elimination of tumor cells (Perez et al. 1985; Staerz et al. 1985). In vitro, this approach was extremely potent, highlighting the superiority of T-cell-induced compared to accessory cell-mediated killing. But two major drawbacks hampered the rapid transition of this new approach from preclinical investigations to successful clinical trials and market approval.

First, at the beginning of the 1990s, production and purification of bsAbs was a real challenge. Bispecific antibodies were produced using quadroma technology, i.e., the fusion of two different hybridoma cell lines. However, quadroma cells derived from two different mouse hybridomas produced mainly nonfunctional mismatched heavy (H)/light (L) chain variants (De Lau et al. 1991). Purification of the functional bsAbs from among the many mismatch variants was difficult and hardly manageable for clinical application. Second, regulation of T cells is complex, requiring two independent stimulation signals for physiological activation. Obviously, this outcome could hardly be accomplished using a single bispecific molecule delivering only one signal, e.g., via CD3 binding to the T cell (Jung et al. 1991).

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To address this situation, we developed rat/mouse bsAbs with species-restricted H/L chain pairing, reducing the mismatch variants from about 70% to <10% (Lindhofer et al. 1995). Moreover, because of the different binding affinities of rat and mouse IgG isotypes for protein A matrices, we found a solution to the production and purification problem. Of importance, we also discovered a preferential binding of the hybrid Fc portion to human-activating Fc-gamma (γ) receptors (R) I/IIa and III when we used the isotype combination of rat IgG2b and mouse IgG2a (see Sect. 16.3.1). This characteristic led to a solution for the second drawback of T-cell-redirecting bsAbs, the lack of a co-stimulatory signal. With activation of FcγR-positive cells such as macrophages, dendritic cells (DCs), or natural killer (NK) cells, co-stimulatory molecules such as CD40 or CD80/CD86 are upregulated on the surface, allowing delivery of the important second stimulation signal to the T cell (see Sect. 16.3).

Others suggested a different approach, such as Weiner et al. (1994), who presented pre-clinical data demonstrating strong release of cytokines with whole IgG T-cell-engaging bsAbs. In their conclusion, they recommended removal of the Fc region to reduce the risk of cytokine-mediated negative side effects. Various groups adopted this approach, which was promoted by the advent of recombinant antibody technology. As a result, a plethora of different bsAb formats lacking the Fc region have been developed (Müller and Kontermann 2010). In contrast, our group pursued the whole-IgG approach, developing the concept of trifunctional Triomab® antibodies as outlined in detail in the following sections.

16.2 Purification of Quadroma Supernatants

The quadroma technology, i.e., the somatic hybridization of two different hybridomas to hybrid-hybridoma (quadroma) cells, represents one of the first techniques for the production of bsAbs. However, single-species quadroma cell lines such as mouse/mouse or human/human hybrid-hybridomas often showed random H/L chain pairing with up to ten possible assembling variants (Smith et al. 1992; Tiebout et al. 1987). As a result, the output of functional bsAbs was low and the purification process complex. In contrast, Triomab® antibodies are produced using hybrid mouse/rat quadroma cell lines. Here, we present an overview of the manufacturing and purification processes, identifying three essential features in the biology of rat/mouse quadromas and in the structural nature of produced Triomab® antibodies.

First, stable mouse/rat quadroma cell lines secrete Triomab® antibodies into the culture supernatant. Cells can be grown in defined protein-free medium with sufficient post-production stability using cell factory systems and batch fermentation. Then, antibodies are purified from the cell-free culture supernatant by means of protein A affinity and ion-exchange chromatography, followed by diafiltration, nanofiltration, and final formulation (Fig. 16.1). Structural and functional characterization achieved by applying liquid chromatography, mass spectrometry and

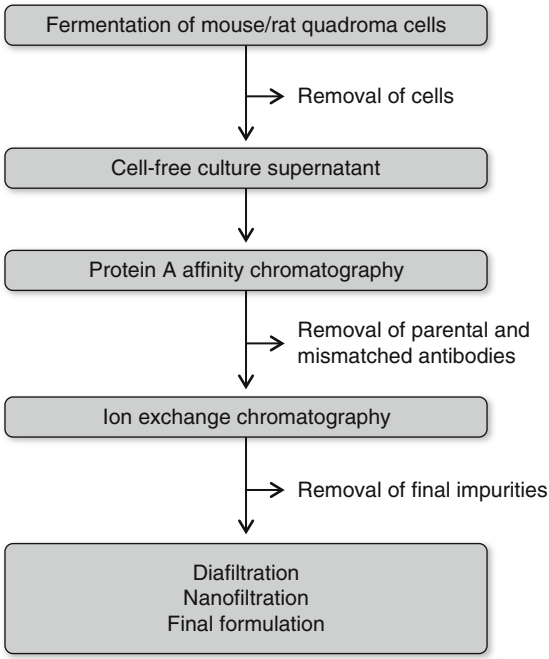

Fig. 16.1 Overview of the manufacturing and purification process of Triomab® antibodies

various immunological methods and bioassays confirmed that Triomab® antibodies of high integrity, purity, and quality result from this downstream processing technology (Chelius et al. 2010).

The high yield of functional Triomab® antibodies and the simple purification process are based on special characteristics of mouse IgG2a/rat IgG2b quadroma cell lines distinct from conventional single-species hybrid-hybridomas.

16.2.1 Preferential Intra-species H/L Chain Pairing

In a comprehensive study, De Lau et al. (1991) investigated H and L chain pairing in eight different mouse/mouse quadromas. Surprisingly, in only 4 of 16 combinations did the L chains associate preferentially with their homologous H chain partners. In other words, the majority of mouse/mouse quadromas displayed a random association of H and L chains with the consequence of a low yield of functional bsAbs. Theoretically, only 12.5% of produced antibodies are of a bispecific nature if all H and L chains combine stochastically (Milstein and Cuello 1983). In contrast, Triomab® antibodies produced by mouse/rat quadromas show a preferential pairing of the mouse H with the mouse L chain on the one side and of the rat H with the rat L chain on the other side. With this strategy, the amount of correctly paired bsAbs is significantly increased, as we have demonstrated

(Lindhofer et al. 1995). In three different mouse IgG2a/rat IgG2b and in one mouse IgG2a/rat IgG2a quadromas analyzed, the bsAbs fractions accounted for 30–49% of whole produced IgG. The major impurities detected were parental mouse and parental rat IgG, whereas H/L mismatch variants generally occurred to a minor degree (4–10%).

16.2.2 Efficient Heterologous H/H Chain Pairing

Because of an approximately 25% difference in the amino acid sequence of the Fc region between mouse IgG2a and rat IgG2b (http://imgt.cines.fr), a preferential homologous H/H chain pairing might be expected; however, this pairing has not been observed. In contrast to H/L chain pairing, H/H chain assembly is apparently not species restricted. Otherwise, the high yield of up to 49% heterologous bsAbs, which approaches the theoretically expected proportion of 50%, cannot be explained. However, a closer look at the hinge region of mouse IgG2a and rat IgG2b reveals a crucial homology. This flexible inter-domain region located between C_{H1} and C_{H2} is considered most important for H chain assembly because of the formation of inter-chain disulfide bonds. Interestingly, the hinge regions of mouse IgG2a and rat IgG2b are of comparable lengths, and three matched cysteine residues easily allow the covalent interaction of the H chains. In this way, inter-species H chain pairing between mouse IgG2a and rat IgG2b may not be hindered but instead be equally effective.

16.2.3 Different Affinities of Mouse and Rat IgG for Protein A

Whole IgG antibodies can be simply purified by affinity chromatography using protein A, which binds to the C_{H2}–C_{H3} interface of the Fc region (Deisenhofer 1981). However, different IgG isotypes bind with different affinities. In contrast to mouse IgG2a, which is a strong binder, rat IgG2b shows only a weak affinity for protein A (Burton 1985). This fact was exploited for the efficient separation of heterologous mouse IgG2a/rat IgG2b from homologous parental IgG antibody variants: With application of a decreasing pH gradient, the IgG produced by a mouse/rat quadroma cell line was eluted in three main peaks, as demonstrated in Fig. 16.2. In accordance with the increasing binding affinity for protein A, the peak analysis revealed mainly parental rat IgG2b in the first fraction, rat/mouse bispecific IgG in the second fraction, and parental mouse IgG2a in the third fraction. Peak integration indicated about 35% bsAbs produced. Because some bsAbs had already co-eluted with parental rat IgG in the first peak, the real production rate was even higher.

In summary, the combination of mouse IgG2a and rat IgG2b is characterized by intra-species restricted H/L chain pairing and by non-restricted H/H chain

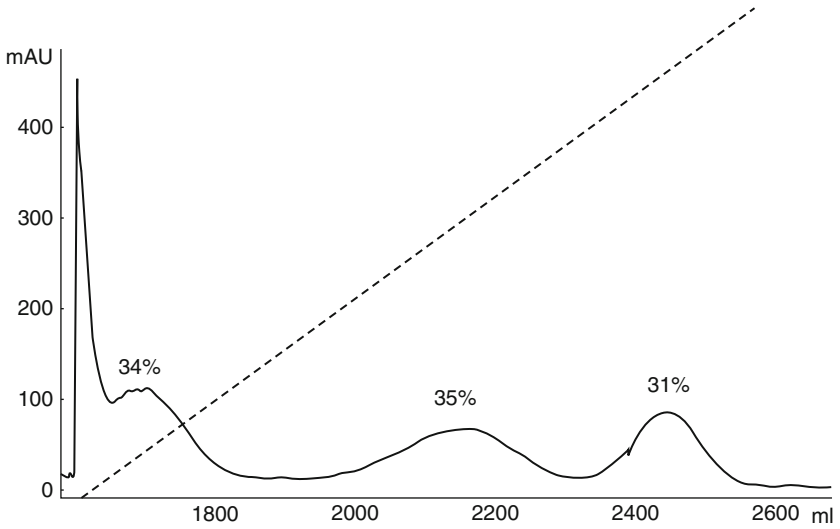

Fig. 16.2 Purification of Triomab® antibody by protein A affinity chromatography. A total of 500 ml of cell-free culture supernatant were loaded onto a 19.7-ml protein A column (MabSelectSure, GE Healthcare), and bound antibodies were eluted by decreasing the pH gradient (pH 8.5 to 3.5 in 75 column volumes), indicated by the *dotted line*. The UV_{280nm} elution profile showed three main peaks with 34%, 35%, and 31% area under the curve distribution. The first fraction contained mainly parental rat IgG, the second one bispecific rat/mouse IgG, and the third parental mouse IgG as analyzed by isoelectric focusing, mass spectrometry, and flow cytometry (data not shown). The first sharp peak was excluded from peak integration because it contained no protein but was the result of an absorbing media component

assembly, resulting in a high yield of 30–50% of correctly paired functional Triomab® antibodies. The main impurities consist of parental rat and mouse IgG, which can be efficiently removed by sequential pH elution/protein A affinity chromatography. Based on these features, TRION Pharma (Munich, Germany) developed a global unique platform technology for the cost-effective and cGMP-compliant production of Triomab® antibodies, a prerequisite for clinical development and commercialization.

16.3 The Mode of Action of Triomab® Antibodies in Tumor Therapy

Triomab® antibodies are bispecific and trifunctional, consisting of a tumor-associated antigen (TAA)-specific binding arm, a second binding arm specific for CD3 expressed on T cells, and a chimeric mouse IgG2a × rat IgG2b Fc region that preferentially binds to activating Fcγ receptors present on accessory cells such as macrophages, DCs, or NK cells. More than 12 years of intensive work has led to the proposed mode

of action of Triomab® antibodies in tumor therapy. The first crucial step in this mode of action is thought to be redirection of T cells to the tumor via the bispecific Triomab® antibody-mediated crosslink of a TAA with CD3 (Fig. 16.3, upper). Antibody-mediated engagement of CD3 as a component of the T-cell receptor complex is a powerful first stimulus to activate T cells in a major histocompatibility complex (MHC)-independent manner, accompanied by TNF-α and IFN-γ secretion (Jacobs et al. 1997). However, the physiological activation of T cells requires a second signal. Attracted by opsonized T cells and tumor cells as well as proinflammatory cytokines, FcγR-positive immune cells are additionally engaged via the Fc region of Triomab® antibodies. A cluster of different immune cell types is formed at the tumor cell (Fig. 16.3, upper). This tri-cell complex formation consisting of tumor cells, T cells, and FcγR-positive accessory immune cells suggests several important consequences: First, there is mutual stimulation of accessory immune cells and T cells. Triomab® antibody-triggered interaction of T cells and CD14-positive monocytes results in the upregulation of CD83, CD86, and CD40 (Riechelmann et al. 2007; Stanglmaier et al. 2008; Zeidler et al. 2000). Thus, T cells receive a second co-stimulatory signal in the form of CD40/CD40L or CD80-CD86/CD28 interaction. As a consequence, they are profoundly and physiologically activated, as characterized by high secretion of IL-2 and strong proliferation with detection of the proliferation marker Ki-67 (Riesenberg et al. 2001; Ruf et al. 2004; Zeidler et al. 1999). Additionally, the T-cell activation markers CD25 and CD69 are upregulated (Riechelmann et al. 2007). Conversely, accessory immune cells are stimulated by interaction with T cells and the FcγR crosslink. This stimulation is manifested as high levels of proinflammatory cytokines such as IL-6 and IL-12 are measured, which are mainly secreted by accessory cells (Hirschhaeuser et al. 2009; Jäger et al. 2009b; Zeidler et al. 1999). Furthermore, the cross-talk between accessory and T cells is indicated by the release of Th1-biased cytokines, especially IL-2 and IFN-γ (Hirschhaeuser et al. 2009; Jäger et al. 2009b; Zeidler et al. 1999). Finally, the targeted tumor cells are efficiently destroyed by the concerted attack of different types of immune effector cells, as shown in allogeneic settings as well as in autologous human ex vivo systems (Gronau et al. 2005). Necrotic and apoptotic tumor cells and particles are phagocytosed (Riesenberg et al. 2001; Zeidler et al. 2000) and may be processed and presented by professional antigen-presenting cells in a stimulatory context, the ideal prerequisite for anti-tumor immunization (Fig. 16.3, *bottom*). In this regard, the elevated expression of the DC-specific cytokine DC-CK1 in the presence of DCs, targeted tumor cells, and Triomab® antibodies was an important finding that indicated the specific activation of professional antigen-presenting cells (Zeidler et al. 1999).

The observed Fc-mediated nonspecific activation of immune cells off-site of the tumor was regarded as a major obstacle to previous clinical approaches with intact bispecific antibodies. Uncontrolled systemic release of cytokines that causes severe adverse events is a serious concern (Weiner et al. 1994). Therefore, it was an important finding that Triomab® antibodies reach their full activating efficacy only at the tumor cell when all three binding partners are present (Ruf et al. 2004). The relevance of this feature holds especially true for the production of IL-2, the most

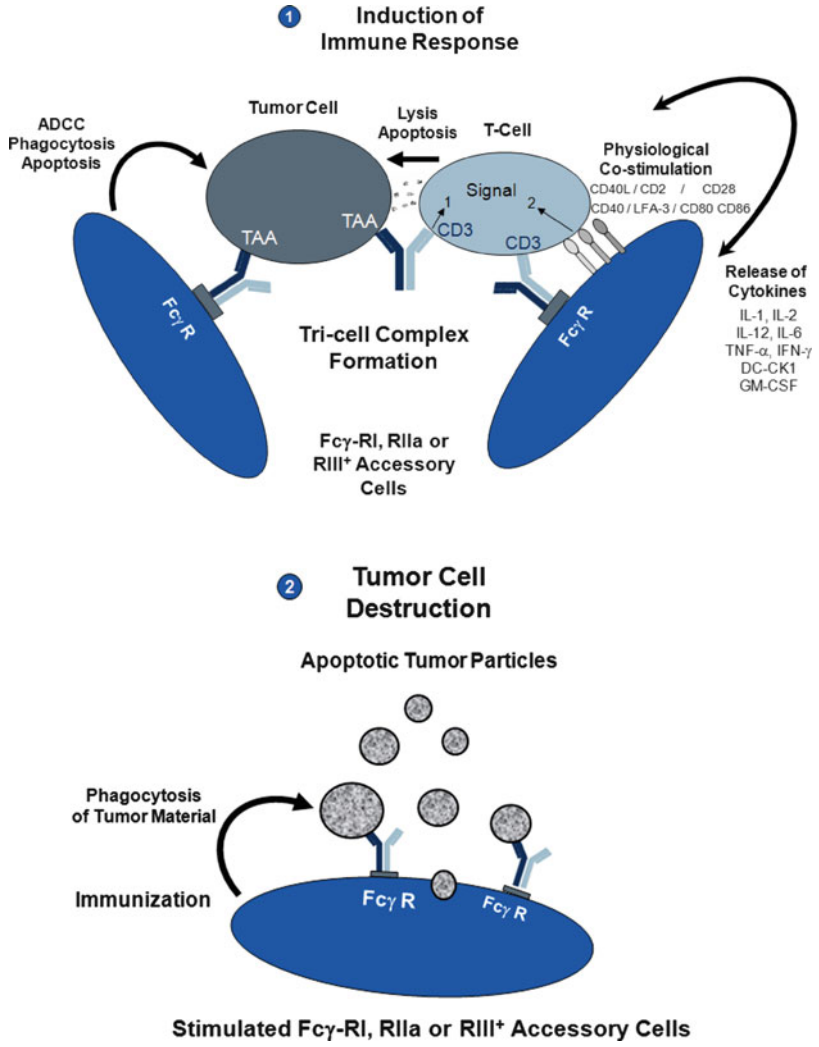

Fig. 16.3 Stepwise mode of action of Triomab® antibodies. In a first step for this process, (1) T cells are redirected to and activated at the tumor cells in an MHC-independent manner by Triomab® antibody-mediated crosslink of TAA with CD3 (Fig. 16.3, *upper*; signal 1). Simultaneously or subsequently, accessory cells can be additionally bound via the Fc region of the trifunctional antibody (Fig. 16.3, *upper*). A process of mutual cross-talk between T cells and accessory immune cells (macrophages, DCs and NK cells) is initiated. T cells are strongly activated in a physiological manner, receiving a second co-stimulatory signal (signal 2), while accessory immune cells are stimulated via FcγR crosslinking leading to the release of proinflammatory cytokines. As a result, tumor cells are effectively destroyed by a concerted attack of T cells and accessory immune cells applying different killer mechanisms like antibody-dependent cellular cytotoxicity (ADCC), phagocytosis, or perforin/granzyme-mediated lysis and apoptosis induction. Finally, T cell proliferation occurs as well as necrotic or apoptotic tumor particles are phagocytosed, processed, and presented by stimulated professional antigen-presenting cells (macrophages, DCs), a prerequisite for the induction of long-term anti-tumor immunization (2) (Fig. 16.3, *bottom*)

important autocrine growth factor for T cells. Significant amounts of IL-2 are induced only when trifunctional binding is enabled (Stanglmaier et al. 2008; Zeidler et al. 1999; Ruf et al. 2004). It should be emphasized that IL-2 is absolutely needed for self-supporting proliferation of T cells upon activation (Robb et al. 1981).

Triomab® antibodies cannot be substituted by a combination of both parental, monospecific antibodies because the antigen-binding functions must be closely linked to achieve the immunostimulatory capacity required. In different in vitro and in vivo models, cytokine secretion and tumor cell elimination are significantly less efficient with an equimolar combination of both parental antibodies (Ruf and Lindhofer 2001; Zeidler et al. 1999). Thus, the excellent efficacy of Triomab® antibodies in the destruction of tumor cells allows the identification of a therapeutic window with very low (microgram range) but effective drug concentrations and an acceptable safety profile. This possibility has been impressively demonstrated in 2009 by the first approval of a trifunctional Triomab® antibody (catumaxomab, Removab®) for the intraperitoneal (i.p.) treatment of malignant ascites (MA) in the European Union (Heiss et al. 2010; Linke et al. 2010). Several clinical studies involving different Triomab® candidates and showing promising results indicate that both locoregional (Heiss et al. 2010; Sebastian et al. 2009a) and systemic Triomab® antibody administration are feasible (Buhmann et al. 2009; Kiewe et al. 2006; Sebastian et al. 2007; Stemmler et al. 2005).

16.3.1 The Role of the Fc Region

The Fc region is of exceptional importance for the therapeutic concept and mode of action of Triomab® antibodies. Cross-talk between and activation of different types of immune effector cells in close proximity to the tumor cells can be accomplished only by intact Fc-containing bsAbs. Recent findings have recalled the important role of the Fc region of conventional monospecific antibodies in tumor therapy. Fc-mediated killing mechanisms such as ADCC, antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC) are essential in tumor models and in the clinic. Prominent examples are trastuzumab (Herceptin®) and rituximab (Rituxan®) (Clynes et al. 2000; Racila et al. 2008; Weng and Levy 2003). Consequently, much effort has focused on improving therapeutic monoclonal antibodies (mAbs) by means of Fc engineering (Desjarlais et al. 2007; Moore et al. 2010). The selectively enhanced binding of mAbs to the activating isoform CD32a but not to its inhibitory counterpart CD32b of FcγRII (CD32) turned out to be of special interest: An improved CD32a/CD32b binding ratio strongly increases ADCP (Richards et al. 2008) and favors activation and maturation of DCs with a positive effect on the induction of tumor immunity (Boruchov et al. 2005; Kalergis and Ravetch 2002).

The Fc region of Triomab® antibodies consists of mouse IgG2a and rat IgG2b half antibodies, each mediating strong effector functions such as ADCC as homodimers (Chassoux et al. 1988; Larson et al. 1988). Moreover, it is worth

mentioning that the constant Fc regions of mouse and rat H chain isotypes IgG2a and IgG2b have an amino acid homology of only approximately 75%, giving rise to the truly complex FcγR-binding behavior of this heterodimeric entity with its underlying immunostimulatory features. Interestingly, the IgG2a and IgG2b isotypes of mouse and rat also demonstrate efficacious activation of human effector cells such as monocytes via FcγRI and IIa interaction (Haagen et al. 1995; Koolwijk et al. 1991). Thus, a chimeric mouse IgG2a × rat IgG2b Fc region might interact equally well with human FcγR expressed on different immune effector cells. This idea has been investigated in detail, as follows. First, the binding of Triomab® antibodies to purified NK cells ($CD56a^+/CD3^-$) verified recognition of FcγRIII (CD16), the only FcγR expressed on NK cells (Zeidler et al. 2000). Moreover, interaction with the high-affinity FcγRI (CD64) was analyzed by surface plasmon resonance. With this approach, the Triomab® antibody catumaxomab showed binding kinetics comparable to the humanized alemtuzumab (hIgG1) with Ka values of 2.46×10^7 and 3.18×10^7 [1/M], respectively (Table 16.1).

Finally, preferential binding to the activating FcγRIIa (R131) in comparison to the inhibitory FcγRIIb has been observed (Table 16.2). Monomeric catumaxomab bound about 2.1 times more strongly to FcγRIIa than to FcγRIIb. However, when pre-aggregated catumaxomab was used instead of the monomeric form, the FcγRIIa/IIb binding ratio increased up to 19 times (Fig. 16.4). This increase resulted from

Table 16.1 Characterization of the binding of Triomab® antibody catumaxomab to human FcγRI by surface plasmon resonance

Antibody	Fc	ka[a] [1/Ms]	kd[b] [1/s]	Ka[a] [1/M]	Kd[b] [M]
Catumaxomab	mIgG2a × rIgG2b chimeric	$2.35 \pm 0.096 \times 10^4$	$9.59 \pm 0.936 \times 10^{-4}$	$2.46 \pm 0.146 \times 10^7$	$4.08 \pm 0.252 \times 10^{-8}$
Alemtuzumab	Human IgG1	3.5×10^4	1.08×10^{-3}	3.18×10^7	3.26×10^{-8}

The experiments were performed by BioAnaLab (Oxford, UK) Abbreviations: *m* mouse; *r* rat
[a]Association rates (ka) and constants (Ka) as well as [b]dissociation rates (kd) and constants (Kd) (± standard deviation), respectively, were determined by Biacore analysis using Langmuir 1:1 binding model fits. Mean values of three individual catumaxomab and two alemtuzumab batch runs are shown. $F(ab')_2$ fragment controls of catumaxomab displayed no specific binding

Table 16.2 Binding of monomeric and aggregated catumaxomab to human FcγRI, FcγRIIa, and FcγRIIb

Catumaxomab status	FcγRI[a]	FcγRIIa[a] R131	FcγRIIb[a]	IIa/IIb[b] Ratio
Monomeric	0.34 ± 0.01	26.56 ± 1.48	54.45 ± 5.22	2.1
Aggregated	–	1.66 ± 0.04	31.79 ± 1.66	19.2
Fold	–	16	1.7	–

Abbreviation: *EC50* effective concentration at half maximal binding signals
[a]EC50 values indicated in μg/ml (± standard error) were obtained from GraphPadPrism one site specific binding with Hill slope fit of ELISA data. Mean data of two independent experiments are shown
[b]IIa/IIb ratio = EC50 (FcγRIIb)/EC50 (FcγRIIa)
[c]Fold equals EC50 (FcγRII, catumaxomab monomeric)/EC50 (FcγRII, catumaxomab aggregated)

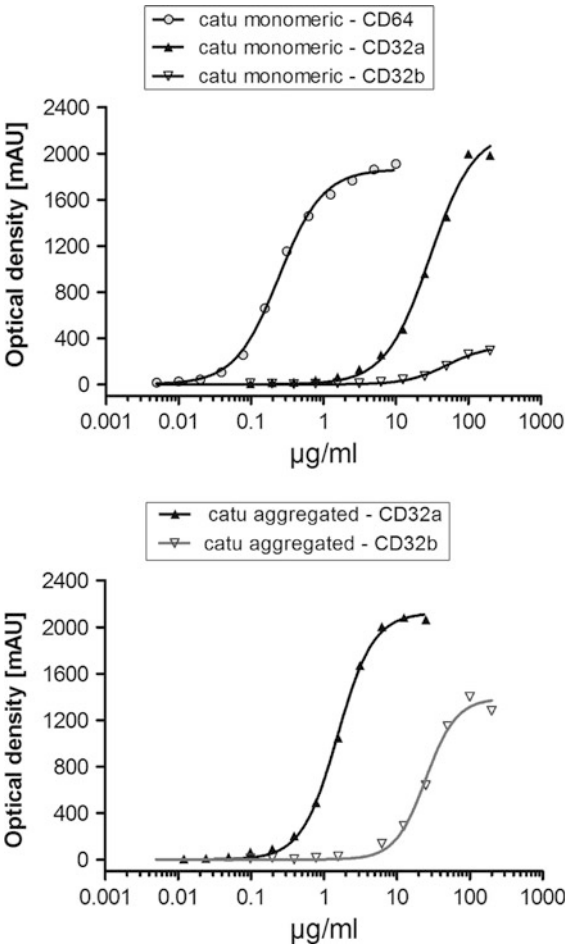

Fig. 16.4 Binding of monomeric catumaxomab to FcγRI, IIa (R131), and IIb (*upper*), and of aggregated catumaxomab to FcγRIIa (R131) and IIb (*bottom*). Binding was measured by ELISA, coating the corresponding FcγRs on the plate and detecting bound catumaxomab using a mixture of biotinylated F(ab′) 2-fragments of goat anti-mouse and anti-rat IgG, both F(ab′)2-fragment specific. For complex formation, catumaxomab was cross-linked by equimolar amounts of biotin-labeled anti-mouse and anti-rat IgG beforehand. Nonspecific binding of the $F(ab')_2$ fragment of catumaxomab was subtracted

the fact that the avidity of aggregated catumaxomab for FcγRIIa increased greatly in comparison to the binding to FcγRIIb (Table 16.2). Of importance, the use of aggregated antibodies clearly better resembles the physiological situation of an immune-complexed or cell-bound antibody while monomeric antibodies will hardly bind to the low-affinity FcγRII at therapeutic concentrations. In summary, the chimeric mouse IgG2a × rat IgG2b Fc region of Triomab® antibodies preferentially binds to activating FcγRI, IIa, and III. Thus, different immune effector cells are involved and activated by trifunctional Triomab® antibodies. Taken together, these findings indicate that by chance, the heterodimeric structure of Triomab® antibodies may already fulfill the balanced interplay with activating Fcγ receptors that is considered to be the next important step in optimizing therapeutic antibody properties by means of Fc engineering.

16.3.2 The Different Killer Mechanisms of Involved Immune Cells

According to the postulated mode of action, Triomab® antibodies induce a concerted attack of different types of immune effector cells against the tumor. Based on this orchestrated tumor defense, Triomab® antibodies are superior to conventional therapeutic antibodies. This superiority also holds true for recognition of tumor cells showing only low surface expression of the relevant target antigen, such as CD20 or HER2/neu (Jäger et al. 2009b; Stanglmaier et al. 2008). This important hallmark of Triomab® antibodies has been shown, for instance, by the capacity of anti-HER2/neu × anti-CD3 ertumaxomab to mediate in vitro killing of tumor target cells that express only low HER2/neu levels (scored 1+) (Jäger et al. 2009b). In contrast, trastuzumab absolutely failed to eliminate low HER2/neu-expressing tumor cell lines in the presence of peripheral blood mononuclear cells (PBMCs), reflecting the disadvantage of the monospecific recognition mode in the case of rare surface antigen expression. Below, the different cytotoxic mechanisms of recruited immune cells are described.

16.3.2.1 T Lymphocytes

T cells are the most potent effector cells of the immune system with regard to tumor therapy. Bispecific antibodies that crosslink TAA with the trigger molecule CD3 recruit cytotoxic T cells while bypassing natural T cell receptor specificity. In this way, all mature CD3-positive T cells are in principle available for an anti-tumor attack, although different T-cell subpopulations demonstrate unequal cytotoxic potential: CD8 T cells of the memory phenotype (RO^+) expand rapidly and are most efficacious with a combination of anti-CD3/CD30 and anti-CD28/CD30 bispecific antibodies for T cell stimulation (Renner et al. 1997). In this context, it should be noted that cytotoxic CD4 T effector cells may also contribute to tumor cell cytotoxicity mediated by bispecific antibodies with anti-CD3 stimulation function, as others have shown (Porakishvili et al. 2004).

T cells targeted by Triomab® antibodies are physiologically stimulated within the tri-cell complex, receiving anti-CD3 and additional co-stimulatory signals from accessory immune cells. Along this stimulation pathway, the activation markers CD25 and CD69 are upregulated, and cytokines such as IFN-γ and TNF-α that indicate T-cell activation are secreted in vitro (Riechelmann et al. 2007; Ruf et al. 2004) and in vivo (Heiss et al. 2005). Especially, significant IL-2 production combined with increased expression of the IL-2R α-chain (CD25) exemplifies a kind of self-supporting system characterized by strong T-cell proliferation and most efficacious eradication of targeted tumor cells (Stanglmaier et al. 2008; Zeidler et al. 1999). Remarkably, Triomab® antibody-mediated tumor cell killing requires no additional co-stimulation of immune effector cells, as other bispecific antibodies do. Once bound, T cells are induced to secrete pore-forming perforin at

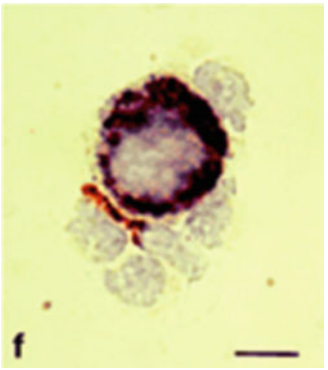

Fig. 16.5 Triomab® antibody-induced pore-forming perforin attack against a targeted tumor cell. PBMC (*blue*) partly expressing pore-forming perforin proteins (*brown*) concentrated toward the targeted tumor cell (LNCaP, *black*). Bar = 10 μm. Reproduced with permission from Riesenberg et al. (2001)

the membrane contact side to the targeted tumor cells, resulting in the osmotic lysis of the target (Riesenberg et al. 2001, Fig. 16.5). In vitro, tumor cells burst 9–17 h after incubation initiation, as monitored by computerized sequential video microscopy. Interestingly, increased granzyme B secretion by PBMCs indicates an additional contribution of apoptotic processes to Triomab®-mediated cytotoxic efficacy (Riechelmann et al. 2007). Thus, along with necrosis for tumor cell killing, the induction of apoptosis is likely another T-cell-mediated killer mechanism that occurs in the context of Triomab® antibody therapies.

16.3.2.2 Monocytes/Macrophages and DCs

As Zeidler et al. (1999) have shown, monocytes and macrophages clearly contribute to Triomab® antibody-mediated tumor cell elimination. Further studies have revealed enhanced phagocytosis of tumor cells by CD14-positive monocytes after addition of Triomab® antibodies (Zeidler et al. 2000). The profound activation of monocytes is indicated by high IL-6 secretion and by the upregulation of CD25 and CD40 (Stanglmaier et al. 2008). Thus, the interaction of monocytes with T cells is essential. Cross-talk of T cells and monocytes at the tumor cell results in mutual effector cell stimulation and enhanced tumor destruction. Accordingly, Triomab® antibody-induced tumor cell killing is much greater when the whole PBMC population is used instead of purified T cells (Zeidler et al. 1999). The preferential binding of the chimeric mouse IgG2a × rat IgG2b Fc region of Triomab® antibodies to activating FcγRI, IIa, and III on monocytes/macrophages and DCs may be the key to understanding this strong stimulation effect. As other groups have shown, selective blockade of the inhibitory FcγRIIb (Dhodapkar et al. 2007) or selective binding to the activating FcgRIIa by Fc engineering (Moore et al. 2010) leads to activation of DCs and monocytes and enhanced ADCP. The essential role of monocytes and macrophages in the destruction of tumor cells by Triomab® antibodies is further confirmed by experiments using multicellular tumor spheroids (MCTS) as a model for micrometastases. This work showed that co-cultures of

PBMC effector cells and Triomab® antibody-treated MCTS of about 0.9–1.8 mm diameter completely disintegrated into single cells and debris within 11 days (Hirschhaeuser et al. 2009). Further, at 6 days after therapy, clonogenic survival assays showed that around 98% of tumor cells were destroyed. Of importance, destruction of MCTS was accompanied by massive infiltration of CD45-positive leukocytes. So far, FcγRI-positive accessory immune cells have been identified as the major infiltrating cell population (Walz et al. 2004). Thus, reciprocal cross-talk and stimulation between T cells and monocytes/macrophages potentiates anti-tumor efficacy, resulting in the destruction of both the tumor cell monolayer and the three-dimensional tumor spheroids. Therefore, not only single disseminated tumor cells but also micrometastases or even solid tumors of patients might be attacked by Triomab® antibody-directed immune cells. Partial and complete remissions of metastases observed in clinical studies with Triomab® antibodies confirm this conclusion (Kiewe et al. 2006; Riechelmann et al. 2007).

16.3.2.3 NK Cells

Classical ADCC is mainly executed by NK cells that express the low affinity but activating FcγRIIIa. The binding of Triomab® antibodies to this receptor has been shown using $CD56^{+}CD3^{-}$ purified NK cells. Antibody binding was followed by increased expression of the NK cell-activation marker CD95 (APO-1, Fas) (Zeidler et al. 2000). Finally, high tumor cell lysis was observed, demonstrating that Triomab® antibodies can recruit cytotoxic NK cells. Thus, it is evident that NK cells can contribute to Triomab® antibody-mediated tumor cell killing. However, in consideration of their low frequency in regard to lymphocytes (2–13%), NK cells may add to a lesser extent to direct tumor cell killing in comparison to T cells and monocytes or macrophages.

16.4 The Tumor Cell Is a Smart Immunologist

Cancer cells can develop a number of resistance- and tolerance-inducing mechanisms during their evolution in the patient's body. These mechanisms are based on mutations in single tumor cells at different timepoints and are responsible for the heterogeneity of tumor cells or, for example, differences between the primary tumor and metastases. Thus, the patient's immune system must confront not only a single phenotype of tumor but a variety that can in total contribute different tumorigenic factors. Thus, tumor cells can, for example, secret immune modulators such as vascular endothelial growth factor (VEGF) (improving tumor vasculature) or IL-6 (a growth factor for lymphomas) and immunosuppressing factors such as IL-10 or TGF-β (Del Prete et al. 1993; de Souza and Bonorino 2009). Moreover, tumor cells obviously can raise peripheral tolerance against themselves

by inducing regulatory T cells (Onishi et al. 2010). Curiel et al. (2004) demonstrated that in MA, about 10–17% of T cells have a regulatory $CD4^+$ $CD25^+$ $FOXP3^+$ T cell phenotype that can inhibit cytotoxic T cells and the release of proinflammatory cytokines. In this context, it was of interest to determine whether Triomab® antibodies can overcome this highly immunosuppressive environment after locoregional application. Lindhofer et al. (2009) and Jäger et al. (2009a) showed that this is indeed possible: Analysis of ascites samples before, during, and after i.p. therapy with the therapeutic Triomab® antibody catumaxomab (anti-EpCAM × anti-CD3) demonstrated (a) elimination of $EpCAM^+$ tumor cells and $CD133^+/EpCAM^+$ double-positive putative cancer stem cells (CSCs) (Lindhofer et al. 2009) and (b) the in vivo activation of $CD4^+$ and $CD8^+$ T cells as well as a significant decrease in VEGF levels (Burges et al. 2007; Jäger et al. 2009a, 2010; Heiss et al. 2005).

16.5 From In Vitro to In Vivo

The important features of the Triomab® antibody treatment concept have already been demonstrated in preclinical and clinical settings using anti-EpCAM catumaxomab or anti-HER2/neu ertumaxomab or surrogate antibodies such as BiLu (Burges et al. 2007; Heiss et al. 2005, 2010; Kiewe et al. 2006; Ruf and Lindhofer 2001). Table 16.3 summarizes the important hallmarks for the clinical development program of these antibodies.

The efficient dose–response profile of the Triomab® antibody format [e.g., catumaxomab and ertumaxomab given intravenously (i.v.) up to a 7.5 μg per single dose (Sebastian et al. 2007) or at a triple dosing of 10–200–200 μg at weekly intervals, respectively (Kiewe et al. 2006), is characterized by its fine-tuned balance among (a) the binding affinity for the tumor target antigen (e.g., EpCAM or HER2/neu); (b) the FcγR binding capacity of the constant Fc region (e.g., on NK cells, monocytes/macrophages, DCs) without considerable engagement of the inhibitory FcγRIIb; (c) the additional polyclonal CD3-specific T cell recruitment and

Table 16.3 Clinical studies with Triomab® antibodies

Name	Target	Cancer type	Trial stage	References
Catumaxomab	EpCAM × CD3	Malignant ascites	Phase I/II	Burges et al. (2007)
Catumaxomab	EpCAM × CD3	Malignant ascites	Phase II/III	Heiss et al. (2010)
Catumaxomab	EpCAM × CD3	Peritoneal carcinomatosis	Phase I	Ströhlein et al. (2009)
Catumaxomab	EpCAM × CD3	Pleural effusion	Phase I/II	Sebastian et al. (2009a, b)
Catumaxomab	EpCAM × CD3	Non-small cell lung cancer	Phase I	Sebastian et al. (2007)
Ertumaxomab	HER2/neu × CD3	Metastatic breast cancer	Phase I	Kiewe et al. (2006)
FBTA05	CD20 × CD3	CLL and NHL	Proof-of-concept	Buhmann et al. (2009)

activation; (d) the IL-2-mediated self-supporting maintenance of the immune response without co- or restimulation (e.g., w/o additional administration of IL-2); (e) the scientifically tenable pharmacokinetic and toxicological features (e.g., slow renal clearance rate and avoidance of severe adverse events such as "cytokine storm"); and (f) a convenient treatment window prior to the occurrence of anti-antibody-based immune responses.

16.5.1 Clinical Studies with Triomab® Antibodies

16.5.1.1 Catumaxomab

Based on these structural and underlying therapeutic properties, the Triomab® family member catumaxomab (Removab®) represents the first bispecific anti-tumor antibody worldwide (Sebastian et al. 2009a, b; Shen and Zhu 2008) that has clearly met its expected clinical endpoints in a pivotal phase II/III study and was therefore approved for the treatment of MA in 2009 in European Union (Heiss et al. 2010). Malignant ascites is a manifestation of an advanced disease in a variety of indications including ovarian, breast, or gastric cancer, and is characterized by strong fluid influx and tumor growth into the peritoneal cavity. The onset and progression of MA is associated with significant morbidity and deterioration in quality of life. The prognosis of MA patients is generally poor, with a median survival following diagnosis of ascites that varies from 75 days (non-ovarian) up to 7–12 months (ovarian) (Parsons et al. 1996).

Briefly, 258 patients suffering from epithelial ovarian cancer or non-ovarian cancer with confirmed $EpCAM^+$ MA were enrolled and randomized to catumaxomab treatment (one paracentesis plus four subsequent intraperitoneal (i.p.) catumaxomab infusions of 10, 20, 50, and 150 μg within 11 days) or to the control intervention (paracentesis alone as the best supportive care). Here, it should be noted that the unusual i.p. route of antibody administration represents the best delivery mode for attacking tumor cells that reside within the peritoneum. Moreover, in a pharmacokinetic analysis with ten MA patients, it was determined that catumaxomab became increasingly concentrated in ascites during the course of treatment, attaining effective concentrations in the ng/ml range (Ruf et al. 2010). Median antibody concentrations in the ascites arose from 552 to over 1,721 and then to 6,121 pg/ml, in proportion to the dosing scheme of 10, 20, and 50 μg. However, due to the established peritoneal carcinomatosis, the catumaxomab transfer from the peritoneal cavity into the systemic circulation was low (<1%); a median plasma concentration of 403 pg/ml was achieved with an antibody half-life of 2.13 days using the i.p. infusion route for MA treatment. Interestingly, catumaxomab showed a high in vivo stability, thus retaining its cytotoxic potential even after several days in the systemic circulation (Ruf et al. 2010).

By means of i.p. application, catumaxomab therapy reached a statistically significant puncture-free survival of 46 compared to 11 days for control treatment ($P < 0.0001$), respectively, underlining the potency of the catumaxomab treatment

even in MA patients with such a poor prognosis. Moreover, median puncture-free time was 77 versus 13 days ($P < 0.0001$), indicating a clear gain in life quality. Although treatment benefits with regard to overall survival (OS) are unlikely in such advanced disease, the Kaplan–Meier curves indicate that catumaxomab prolonged OS in ~50% of patients (Heiss et al. 2010). In particular, a prospectively planned subgroup analysis of gastric cancer patients, demonstrated a statistically significant prolongation of OS ($n = 66$; 71 versus 44 days; $p = 0.0313$). One reason for this observation could be that nonovarian cancer patients (including gastric cancer) had fewer previous chemotherapies (median of one) compared to ovarian cancer patients (median of three) resulting in a less impaired immune system (see also Sect. 16.5.4).

Of greatest importance, tumor cells were almost completely eliminated from ascites fluid, suggesting the induction of prominent catumaxomab-mediated anti-tumor responses. Pyrexia, nausea, vomiting, and a transient elevation of liver enzymes as the most frequent adverse events were generally mild to moderate and reversible.

Interestingly, in a preliminary monitoring study, putative $CD133^+/EpCAM^+$ CSCs were present in peritoneal fluids of 62% of analyzed MA patients with different underlying primary tumor entities (Lindhofer et al. 2009) before therapeutic intervention. After four i.p. catumaxomab infusions, these presumed CSCs were completely eliminated from the peritoneal fluids of all tested MA patients. Thus, even under enhanced immunosuppressive conditions (Curiel et al. 2004, see also Sect. 16.4), catumaxomab efficiently destroyed $CD133^+/EpCAM^+$ CSCs within the peritoneal fluids of MA patients. Therefore, catumaxomab-based therapeutic measures may offer an additional treatment opportunity to eliminate CSCs in $EpCAM^+$ malignancies.

Taken together, results suggest that patients with $EpCAM^+$ MA do benefit from catumaxomab therapy by the prolongation of puncture-free survival and the reduction of ascites symptoms in comparison with MA patients receiving palliative paracentesis only. Of note, two further studies targeting treatment of peritoneal carcinomatosis (phase I) and pleural effusion (phase I/II) have been performed with acceptable safety and promising efficacy results (Sebastian et al. 2009a, b; Ströhlein and Heiss 2009). Moreover, because EpCAM is frequently over-expressed, as in prostate, breast, lung, colon, ovarian, or gastric carcinomas, catumaxomab may offer novel future treatment opportunities for a variety of cancers provided that the trifunctional antibody will be administered via the systemic route, which will be investigated in future trials.

16.5.1.2 Ertumaxomab

Ertumaxomab is a Triomab® antibody targeting CD3 and HER2/neu, the latter representing a well-characterized breast tumor marker that is also central to trastuzumab (Herceptin®) therapy. In vitro ertumaxomab has been compared to trastuzumab for its ability to kill tumor cells expressing various levels of HER2/neu. Exposure to ertumaxomab led to the efficient lysis of cells expressing

high or very low amounts of tumor antigen, whereas trastuzumab was completely ineffective even at high concentrations at low HER2/neu expression (Jäger et al. 2009b). As already outlined in Sect. 16.3.2, this difference can be probably explained by the mode of action of these antibodies; trastuzumab triggers NK-mediated ADCC, while ertumaxomab mainly relies on T-cell-mediated killing and the interaction between T cells and accessory cells, as demonstrated in a phase I trial by the release of proinflammatory Th1-biased cytokines such as IFN-γ. To summarize the study briefly, 15 metastatic breast cancer patients who completed the study received i.v. three ascending doses of ertumaxomab (10–200 μg) on days 1, 7, and 14. Adverse events (e.g., fever, rigors, headache, nausea, and vomiting) related to ertumaxomab were generally mild, transient, and fully reversible. An anti-tumor response was seen in 5 out of 15 evaluable patients (one with a complete response, two with a partial response, and two with stable disease) (Kiewe et al. 2006).

16.5.2 Autologous In Situ Immunization Mediated by Triomab® Antibodies

The world's first therapeutic cancer vaccine, which was recently approved by the U. S. Food and Drug Administration, represents a major breakthrough in harnessing the immune system to fight tumors. Moreover, this therapeutic vaccine approval symbolizes a "proof-of-concept" development in cancer immunotherapy in general. The novel vaccine, Provenge (sipuleucel-T) from Dendreon (USA), will be used to treat men for advanced prostate cancer in an individual-targeted manner. In view of this successful cancer vaccine development, it should be noted that trifunctional IgG-shaped antibodies with anti-CD3 recognition of T cells can mediate vaccine-like adaptive immune responses against tumors. As already outlined, Triomab® antibodies have the potential to target and activate DCs in situ while initiating phagocytosis of tumor cells via opsonization (see also Sect. 16.3.1). After the destruction of tumor cells phagocytosed by DCs, subsequent antigen trafficking along MHC class I and class II processing and presentation pathways leads to the stimulation of tumor-specific CD8 or CD4 T cells, respectively. Data taken from preclinical experiments and preliminary clinical observations have indicated the induction of long-lasting CD4 and CD8 T cell responses (Ruf and Lindhofer 2001). If comprehensive immunomonitoring programs confirm these interesting findings (e.g., long-term immune and memory responses against tumors) in parallel with a beneficial therapeutic outcome, Triomab® antibodies may give rise to the induction of adaptive immunity, bridging the gap between active and passive immunization. Chames and Baty (2009) have described this mode of action as the "Holy Grail" of antibody-based cancer therapy.

In fact, in the A20 mouse lymphoma model, it has been demonstrated that the trifunctional antibody BiLu (anti-human EpCAM × anti-mouse CD3) could be used as an immunizing agent for anti-tumor vaccination. The immunization

efficacy was dependent on the presence of the chimeric mouse/rat Fc-region, as the $F(ab')_2$ fragment of the same trifunctional antibody did not elicit long-term protection (Ruf and Lindhofer 2001). Furthermore, a strong correlation has been observed between the induction of a humoral immune response with tumor-reactive antibodies and mouse survival.

Moreover, case observations with patients suffering from progressive peritoneal carcinomatosis from gastric and ovarian cancer were immunomonitored for long-lasting cell-mediated immune responses as a consequence of Triomab® therapy (Ströhlein et al. 2009). Briefly, after chemotherapy, the patient received three escalating doses of catumaxomab (10 μg–20 μg–40 μg) given i.p. The already primed immune responses were restimulated 30 days later after the end of catumaxomab therapy by means of an intradermal antigenic boost immunization via PBMCs loaded with 100-Gy irradiated autologous tumor cells and Triomab® antibody. As demonstrated by IFN-γ secretion assays, activation of tumor-reactive T cells was measured from day 39 up to day 110 at least after restimulation (Ströhlein et al. 2009). For these reasons, we have initiated for the next 2 years a clinical immunomonitoring program addressing the detection of memory responses. We are confident that important immunological parameters will be identified that will clearly mark the beginning of a new era in antibody therapy when the Triomab® concept will become a part of vaccinology.

16.5.3 Avoiding Graft-Versus-Host Disease While Redirecting Graft Versus Leukemia After Allogeneic Transplantation by Means of Triomab® FBTA05

With an annual incidence of four new cases per 100,000 population, chronic lymphocytic leukemia (CLL) represents the most frequent leukemia in the western world. In addition, about 19 new non-Hodgkin's lymphoma (NHL) cases per 100,000 population annually are currently diagnosed in Europe or the USA. CLL is not curable by means of the usual chemotherapeutic regimes even in combination with antibody application (e.g., anti-CD52 alemtuzumab and/or anti-CD20 rituximab). Although evidence for a graft-versus-lymphoma (GVL) effect of allogeneic stem cell transplantation (SCT) in highly malignant lymphoma is limited and controversial, allogeneic SCT represents the only curative option for CLL patients (Grigg and Ritchie 2004). However, several studies and observations report a GVL effect by donor lymphocyte infusions (DLI) following allogeneic hematopoietic SCT (Porter et al. 2006). Nevertheless, the use of allogeneic cell therapy in clinical practice can be frequently accompanied by life-threatening graft-versus-host disease (GVHD), which is difficult to control effectively with current treatment protocols. Thus, more sophisticated treatment regimes are required that allow optimal GVL effects while reducing the frequency and severity of devastating GVHD. Monoclonal antibodies such as rituximab have been used

with increasing success in the treatment of patients with indolent and aggressive lymphoma as well as with CLL. The application of rituximab has improved anti-tumor response and survival of patients, but the mechanism of action is not entirely clear; ADCC, CDC, and induction of apoptosis have been demonstrated in in vitro experiments (Cartron et al. 2004).

Bispecific antibodies may represent a highly attractive therapeutic option for directing T cells efficiently toward tumor cells. In this context, the trifunctional anti-CD20 × anti-CD3 Triomab® antibody FBTA05 may improve the targeting of tumor cells by immune allogeneic effector cells while simultaneously reducing the risk of undesirable reactivity against normal host cells. In other words, FBTA05 may maximize GVL effects by simultaneously decreasing the incidence and severity of GVHD. Morecki and co-workers (2006, 2008) provided the first evidence that the hallmark of autologous vaccination also holds true for the concerted interaction of allogeneic lymphocytes and a trifunctional BiLu antibody (anti-EpCAM × anti-murine CD3) in an experimental B16-EpCAM tumor model. By means of this BiLu antibody-targeted DLI, long-lasting anti-tumor responses were induced that protected treated mice against a second B16-EpCAM tumor challenge (Morecki et al. 2008). Of importance, the use of this trifunctional antibody BiLu prevented mice from GVHD induced by the application of allogeneic haplo-identical lymphocytes. These preclinical findings represent the basic principle for the combination of DLI and trifunctional antibodies for CLL or NHL treatment. Based on the preclinical success described, a proof-of-concept clinical study was performed with a combination of the trifunctional anti-CD20 × anti-CD3 Triomab® antibody FBTA05 and DLI as therapeutic measures against B cell malignancies (Buhmann et al. 2009). This therapeutic setting showed FBTA05-induced anti-tumor responses in extensively pre-treated, p53-mutated, alemtuzumab- and rituximab-refractory patients, indicating the therapeutic capacity of this biopharmaceutical. It should be noted that FBTA05 antibodies can mediate effective killing of lymphoma cells even at very low surface expression levels of CD20 in vitro (Stanglmaier et al. 2008). Taken together, these findings suggest that the trifunctional antibody FBTA05 may offer novel treatment options, such as SCT followed by DLI plus FBTA05 antibodies, for therapy-resistant low-grade lymphoma (including CLL) and NHL patients in relapse.

16.5.4 HAMA Response as a Predictive Marker

Because Triomab® antibodies are considered "foreign" to the human immune system, it is not surprising that human anti-mouse (HAMA) and anti-rat antibodies (HARA) are induced in patients with preceding antibody applications. Nevertheless, results from clinical studies have demonstrated that Triomab® antibodies administered within a given treatment window, e.g., prior to the induction of neutralizing HAMA/HARA responses, may be sufficient for the therapeutic benefit in many cancer indications (Heiss et al. 2005, 2010; Kiewe et al. 2006; Ruf et al. 2010).

On the other hand, HAMA responses could be a predictive measure for the early onset of antigen-specific immune responses. In fact, these HAMA responses have been monitored through a pivotal phase II/III study with catumaxomab against MA and represented a predictive measure in that study. Briefly, 76% of the tested MA patients in the pivotal phase II/III trial were HAMA positive 8 days after completion of catumaxomab treatment (Ott et al. 2010). In contrast to HAMA-negative patients, they showed a significantly longer puncture-free survival, time to puncture, and especially overall survival. In fact, median overall survival in HAMA-positive patients was more than twofold increased compared to patients in the control group or HAMA-negative patients in the pivotal trial (129 days versus 64 days [HAMA neg.], $p = 0.0003$; Ott et al. 2010). Catumaxomab therapy was also beneficial for HAMA-negative patients in terms of time to first puncture; however, catumaxomab failed to improve overall survival in HAMA-negative patients. In summary, only early positive HAMA responses were strongly correlated with increased overall survival data with catumaxomab treatment. Thus, the beneficial clinical outcome may also be attributed to the contribution of acquired anti-tumor effector mechanisms mediated by catumaxomab, especially distinguished through its T-cell-recruiting capacity. Moreover, HAMA data may additionally reflect immunological fitness and antigen responsiveness with emphasis on acquired immunity. Therefore, the development of HAMA may represent an early biomarker and predictor for beneficial tumor therapy with Triomab® antibodies.

16.6 Future Perspectives

After the first approval in Europe for the treatment of MA, catumaxomab will be intensely developed against $EpCAM^+$ carcinomas including cancer stem cells and for alternative routes of administration. Moreover, ertumaxomab treatment of HER2/neu-expressing breast cancer patients, FBTA05 against B cell malignancies, and novel target antigens such as GD2-gangliosides will be further investigated and implemented into the Triomab® technology platform. Another interesting focus of research will be the aspect of active immunization. Therefore, immunomonitoring will be intensified to analyze possibly induced tumor-specific T cells as well as humoral anti-tumor responses.

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