1 General outline and aim of the thesis

Antibodies (Abs) are part of the adaptive humoral immune response, which provides long-lasting protection against pathogens such as viruses and bacteria. During this immune response naïve B-cells recognize an antigen through their B-cell receptor. This results in clonal expansion of the B-cells and differentiation into plasma cells, which secrete large amounts of Abs. These Abs can bind to pathogens thereby flagging the opsonized pathogen for destruction. Abs of the IgG isotype contain two binding arms, each containing a Fab (fragment antigen binding) region through which they recognize their cognate antigen (**Figure 1**). The Abs selectivity for the antigen is determined by the complementary determining region (CDRs) located at the top of the Fab-region. The population of B-cells in the human body may be able to respond to as many as 1 x 10^{11} different antigens. This huge diversity is determined by the different gene segments encoding variable, joining and diversity regions that recombine randomly allowing for nearly 2.5 x 10^{6} combinations. Nucleotide insertions and hypermutations further diversify the CDRs.

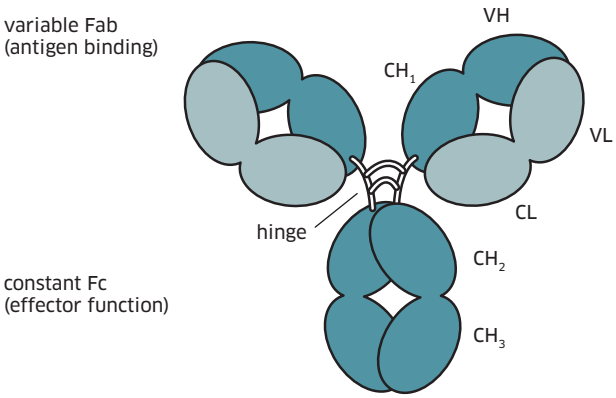

FIGURE 1 *Schematic representation of an IgG1 antibody structure.*

Abs are able to interact with the immune system through the constant Fc (fragment crystallizable) region. In humans, nine different antibody isotypes exist (IgA1 and 2, IgD, IgE, IgG1, 2, 3 and 4 and IgM), each having a unique Fc region. The most abundant class in circulation is IgG1 (~50%). This antibody class is able to eliminate pathogens through a number of Fc-mediated effector mechanisms. Each of these mechanisms requires that the pathogen is opsonized with antibodies resulting in a

high density of Fc regions on the pathogen surface. These Fc regions may interact to form a high-avidity binding scaffold for C1q, which initiates complement dependent cytotoxicity (CDC) [1]. Alternatively, the IgG Fc region can be recognized by Fcγ-receptors that are expressed on immune effector cells such as NK-cells, granulocytes and macrophages. Binding of Fcγ-receptors expressed on NK-cells and granulocytes triggers the release of cytotoxic granules that kill the pathogen through a mechanism called antibody-dependent cellular cytotoxicity (ADCC) [2]. Binding of Fcγ-receptors on macrophages leads to the engulfing of the opsonized pathogen, also known as antibody-dependent cellular phagocytosis (ADCP) [3].

The ability to engage the immune system to induce killing of opsonized cells via Fc-mediated mechanisms led to the notion that monoclonal Abs might have great potential for the treatment of cancer. Numerous monoclonal Abs for the treatment of cancer have been developed in the past two decades, some of which have revolutionized treatment of cancers such as non-Hodgkin lymphoma (Rituxan®) and breast cancer (Herceptin®). In addition to Fc-mediated effector functions, therapeutic antibodies can exert anti-tumor activity through a number of different mechanisms. For example, inhibition of growth factor receptor signaling and induction of receptor downmodulation (e.g. zalututumab). Furthermore, therapeutic antibodies may interact with the tumor microenvironment, for example by inhibiting regulatory interactions between tumor cells and the adaptive immune system (e.g. ipilumumab, PD-1, PD-L1). Although the generation of tumor-targeting antibodies has generally been very successful, only a limited number of antibodies have been clinically effective [4]. As of today, 17 monoclonal antibodies have been approved for the treatment of cancer by the Food and Drug Administration (FDA) and 15 by the European Medicines Agency (EMA) in addition to a comparable number of Abs for the treatment of inflammatory, cardiovascular, infectious and other diseases. The challenge in cancer treatment is that tumors often develop resistance to antibody therapy. Downstream signaling pathways can be mutated (KRAS/BRAF) which limits the antibody's capacity to inhibit growth factor receptor signaling [5]. Tumors can overexpress complement inhibitory receptors such as CD46, CD55 and CD59, thereby blocking CDC [6]. Overexpression of certain HLA molecules (i.e. HLA-E and -G) that inhibit NK-cell mediated ADCC has been described for various tumors [7,8] and the tumor microenvironment can be infiltrated by T-regulatory cells and myeloid-derived suppressor cells that serve to suppress the anti-tumor immune response [9].

One approach to overcome such limitations in efficacy is the conjugation of cytotoxic compounds to monoclonal antibodies. These antibody-drug conjugates (ADCs) combine the tumor specificity, pharmacokinetics and biodistribution properties of antibodies with the potent cell-killing activity of small molecules. This concept was already postulated in the early 20th century by Paul Ehrlich who reasoned that if a compound could be made that selectively targeted a disease-causing organism,

then a toxin for that organism could be delivered along with the agent of selectivity [10]. Hence, a "magic bullet" would be created that only killed the targeted organism. An antibody would be extremely suitable for this purpose as antibodies can selectively bind to tumor antigens while maintaining a long half-life in circulation (~21 days for IgG1). The first generation of ADCs were conjugated with clinically approved chemotherapeutic agents such as vinblastine, mitomycin, methotrexate and doxorubicin [11]. These ADCs showed limited clinical success which was mostly attributed to the low potency of the conjugated drug. The second generation of ADCs made use of more potent payloads, namely calicheamicin, auristatin and maytansin analogs. Besides, pharmacokinetics of the conjugation linker was optimized and fully human mAbs were used to solve immunogenicity problems observed with murine mAbs. In 2000 the first ADC, gemtuzumab ozogamicin (Mylotarg®), was clinically approved for use in refractory acute myeloid leukemia (AML) [12]. Here, a CD33 antibody was conjugated to calicheamicin, a drug that specifically binds to DNA and generates single and double strand DNA breaks. Gemtuzumab ozogamicin received accelerated approval for the treatment of patients with relapse AML. Unfortunately, ten years later, gemtuzumab ozogamicin was withdrawn from the US market due to lack of clinical benefit [13]. A confirmatory phase III trial showed no improvement in clinical benefit for patients who received standard chemotherapy plus gemtuzumab ozogamicin, but instead a greater number of deaths occurred in the group of patients who received gemtuzumab ozogamicin compared with those receiving chemotherapy alone [13]. Several factors have been identified that have limited the clinical efficacy of gemtuzumab ozogamicin, including poor stability of the acid-labile conjugation linker, heterogeneous drug loading (approximately 50% of the CD33 antibodies are unconjugated) and sensitivity to multidrug resistance pumps that are often overexpressed in AML.

More recently, two novel ADCs were approved by the FDA for the treatment of Hodgkin lymphoma and anaplastic large-cell lymphoma, brentuximab vedotin (Adcetris®) and HER2 positive breast cancer, trastuzumab emtansine (Kadcyla®) [14,15]. These ADCs showed improved liker stability and pharmacokinetics. Their clinical success has led to an impressive expansion of the clinical ADC pipeline (**Chapter 2, Table 1**). An overview of the recent developments in ADC based therapy is summarized in **Chapter 2**.

Although simple in concept, the success of a given ADC depends on careful selection of the tumor antigen, antibody, linker as well as the payload. The aim of this thesis was to better understand the antibody and antigen requirements that are essential for developing a therapeutically effective ADC. **Chapter 2** reviews the different cytotoxic compounds that are currently being used as payloads, and the type of tumor antigens that can be utilized for their intracellular delivery, as well as the interplay of ADCs with the immune system. In **Chapter 3** we explore tissue factor (TF) as a

novel target for an auristatin-based ADC. An effective ADC treatment requires that in circulation, the payload remains attached to the antibody. Following selective antigen binding, the ADC should be internalized and targeted to the lysosomes to be processed by lysosomal enzymes such as cathepsins. This leads to cleavage of the linker and/or degradation of the antibody moiety of the ADC, resulting in release of the payload. Once released, the payload can exert its cytotoxic effect through inhibition of microtubule formation (**Figure 2**).

To investigate the suitability of TF as a target for an ADC approach, we compared the distribution, internalization and lysosomal targeting of TF with that of the clinically validated ADC target HER2 as well as for EGFR, for which an ADC is currently in phase II clinical development. ADCs were generated by conjugating TF-, HER2- and EGFR- Abs with the microtubule inhibiting agent duostatin-3. These ADCs allowed us to compare efficacy of TF-, HER2- and EGFR-specific ADCs in different *in vitro* and *in vivo* tumor models. **Chapter 4** describes the selection of monoclonal antibody TF-011 as the optimal candidate for the development of a TF-specific ADC. A large panel of TF Abs was generated from which clone 011 was selected based on excellent target binding characteristics, rapid internalization and efficient lysososomal targeting and the capacity to inhibit TF-Factor VIIa (FVIIa)-dependent intracellular signaling, while having minimal impact on coagulation *in vitro*. The *in vivo* efficacy of the lead

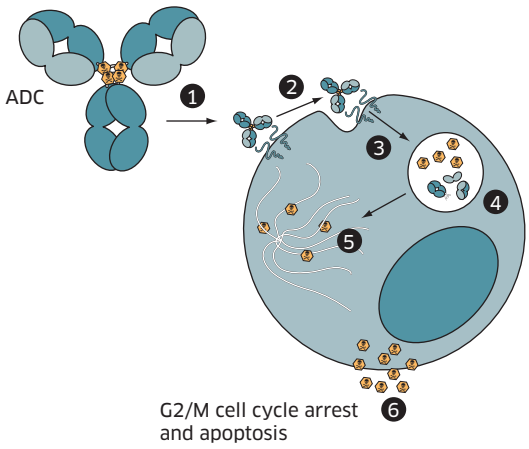

FIGURE 2 *Mechanism of action of auristatin-based ADCs.* The ADC should be stable in circulation (**1**) and bind (**2**) to its antigen when a tumor cell is encountered. The antigen/ADC complex has to be internalized and targeted to the lysosomes (**3**) where lysosomes enzymes can process the ADC and release the auristatin payload (**4**). The payload can then exert its cytotoxic effect by inhibition of tubulin formation (**5**), resulting in cell cycle arrest and apoptosis (**6**).

candidate HuMax-TF-ADC (TF-011-MMAE) is analyzed in detail. Different patient-derived xenograft (PDX) models with variable levels of TF expression were treated with TF-011-MMAE. In addition, tumor models that showed tumor recurrence after treatment with TF-011-MMAE and paclitaxel were retreated with TF-011-MMAE.

Chapter 5 describes the development of a high throughput assay that can be used to screen large antibody panels for their suitability to facilitate intracellular delivery of toxic payloads. A modified version of the Pseudomonas exotoxin-A was fused to a human kappa light chain binding antibody fragment. The resulting fusion protein (α-kappa-ETA') was tested for binding to Abs with a human kappa light chain and its ability to inhibit proliferation of EGFR expressing cells when non-covalently linked to an EGFR Ab. In **Chapter 6** we used the α-kappa-ETA' assay to screen a large and diverse panel of HER2 antibodies for their ability to deliver α-kappa-ETA' into tumor cells. **Chapter 7** describes the development of a Fab-arm that can be used to facilitate internalization and lysosomal delivery of poorly internalizing tumor antigens in a bispecific antibody approach. **Chapter 8** covers the general discussion of this thesis and addresses the key findings in comparison to the literature. General rules of thumb providing a road map for ADC development are presented and summarized.

To summarize, the clinical success of brentuximab vedotin and trastuzumab emtansine has led to an extensive expansion of the clinical ADC pipeline. Although the concept of an ADC seems simple, designing a successful ADC is complex and requires careful selection of the tumor antigen, antibody, linker and payload. In this thesis, different tumor antigens and targeting antibodies were compared for their capacity to deliver cytotoxic payloads to tumor cells, uncovering general mechanisms. In the course of this work, TF was identified as an excellent ADC target because of its rapid internalization and lysosomal targeting characteristics. Furthermore we have explored a novel Ab platform that improves the intracellular delivery of cytotoxic payloads. These findings provide a better insight in the Ab and antigen requirements needed for optimal payload delivery and support the development of novel and improved ADCs for the treatment of cancer.

REFERENCES

1 Diebolder CA, Beurskens FJ, de Jong RN, Koning RI, Strumane K, Lindorfer MA, Voorhorst M, Ugurlar D, Rosati S, Heck AJ *et al*: Complement is activated by IgG hexamers assembled at the cell surface. *Science* 2014, 343(6176):1260-1263.

2 Overdijk MB, Verploegen S, van den Brakel JH, Lammerts van Bueren JJ, Vink T, van de Winkel JG, Parren PW, Bleeker WK: Epidermal growth factor receptor (EGFR) antibody-induced antibody-dependent cellular cytotoxicity plays a prominent role in inhibiting tumorigenesis, even of tumor cells insensitive to EGFR signaling inhibition. *J Immunol* 2011, 187(6):3383-3390.

3 Overdijk MB, Verploegen S, Bogels M, van Egmond M, Lammerts van Bueren JJ, Mutis T, Groen RW, Breij E, Martens AC, Bleeker WK *et al*: Antibody-mediated phagocytosis contributes to the anti-tumor activity of the therapeutic antibody daratumumab in lymphoma and multiple myeloma. *MAbs* 2015, 7(2):311-321.

4 Nelson AL, Dhimolea E, Reichert JM: Development trends for human monoclonal antibody therapeutics. *Nat Rev Drug Discov* 2010, 9(10):767-774.

5 Van Cutsem E, Kohne CH, Lang I, Folprecht G, Nowacki MP, Cascinu S, Shchepotin I, Maurel J, Cunningham D, Tejpar S *et al*: Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. *J Clin Oncol* 2011, 29(15):2011-2019.

6 Jurianz K, Maslak S, Garcia-Schuler H, Fishelson Z, Kirschfink M: Neutralization of complement regulatory proteins augments lysis of breast carcinoma cells targeted with rhumAb anti-HER2. *Immunopharmacology* 1999, 42(1-3):209-218.

7 Lin A, Yan WH, Xu HH, Gan MF, Cai JF, Zhu M, Zhou MY: HLA-G expression in human ovarian carcinoma counteracts NK cell function. *Ann Oncol* 2007, 18(11):1804-1809.

8 Levy EM, Bianchini M, Von Euw EM, Barrio MM, Bravo AI, Furman D, Domenichini E, Macagno C, Pinsky V, Zucchini C *et al*: Human leukocyte antigen-E protein is overexpressed in primary human colorectal cancer. *Int J Oncol* 2008, 32(3):633-641.

9 Greten TF, Manns MP, Korangy F: Myeloid derived suppressor cells in human diseases. *Int Immunopharmacol* 2011, 11(7):802-807.

10 Ehrlich P: Address in Pathology, ON CHEMIOTHERAPY: Delivered before the Seventeenth International Congress of Medicine. *Br Med J* 1913, 2(2746):353-359.

11 Perez HL, Cardarelli PM, Deshpande S, Gangwar S, Schroeder GM, Vite GD, Borzilleri RM: Antibody-drug conjugates: current status and future directions. *Drug Discov Today* 2014, 19(7):869-881.

12 Sievers EL, Larson RA, Stadtmauer EA, Estey E, Lowenberg B, Dombret H, Karanes C, Theobald M, Bennett JM, Sherman ML *et al*: Efficacy and safety of gemtuzumab ozogamicin in patients with CD33-positive acute myeloid leukemia in first relapse. *J Clin Oncol* 2001, 19(13):3244-3254.

13 Kopecky K, Stuart RK, Larson RA, Nevill TJ, Stenke L, Slovak ML, Tallman MS, Willman CL, Erba H, Appelbaum FR: Preliminary Results of Southwest Oncology Group Study S0106: An International Intergroup Phase 3 Randomized Trial Comparing the Addition of Gemtuzumab Ozogamicin to Standard Induction Therapy Versus Standard Induction Therapy Followed by a Second Randomization to Post-Consolidation Gemtuzumab Ozogamicin Versus No Additional Therapy for Previously Untreated Acute Myeloid Leukemia *Annual Meeting of the American Society of Hematology; New Orleans; 2009; abstract 790*.

14 Younes A, Bartlett NL, Leonard JP, Kennedy DA, Lynch CM, Sievers EL, Forero-Torres A: Brentuximab vedotin (SGN-35) for relapsed CD30-positive lymphomas. *N Engl J Med* 2010, 363(19):1812-1821.

15 Burris HA, Rugo HS, Vukelja SJ, Vogel CL, Borson RA, Limentani S, Tan-Chiu E, Krop IE, Michaelson RA, Girish S *et al*: Phase II study of the antibody drug conjugate trastuzumab-DM1 for the treatment of human epidermal growth factor receptor 2 (HER2)-positive breast cancer after prior HER2-directed therapy. *J Clin Oncol* 2011, 29(4):398-405.