Cancer Immunotherapy Meets Oncology

In Honor of Christoph Huber

Cedrik Michael Britten
Sebastian Kreiter
Mustafa Diken
Hans-Georg Rammensee
Editors

Springer

Cancer Immunotherapy Meets Oncology

Cedrik Michael Britten
Sebastian Kreiter • Mustafa Diken
Hans-Georg Rammensee
Editors

Cancer Immunotherapy Meets Oncology

In Honor of Christoph Huber

Springer

Editors
Cedrik Michael Britten
Immunotherapy Development Center (IDC)
TRON-Translational Oncology
University Medical Center
Johannes Gutenberg University
Mainz
Germany

BioNTech RNA Pharmaceuticals
Mainz
Germany

Sebastian Kreiter
Department of Immunotherapy Development Center
TRON-Translational Oncology
University Medical Center
Johannes Gutenberg-University
Mainz
Germany

BioNTech AG
Mainz
Germany

Mustafa Diken
Department of Immunotherapy Development Center
TRON-Translational Oncology
University Medical Center
Johannes Gutenberg University
Mainz
Germany

Hans-Georg Rammensee
Department of Immunology
University of Tübingen
Tübingen
Germany

ISBN 978-3-319-05103-1 ISBN 978-3-319-05104-8 (eBook)
DOI 10.1007/978-3-319-05104-8
Springer Heidelberg Dordrecht London New York

Library of Congress Control Number: 2014938034

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

This book is a tribute to Professor Dr. Christoph Huber and his lifetime achievements. It is also a testimony to the scientific and medical progress in the growing field of immuno-oncology which is about to improve the standard of care for cancer patients.

In 2002, anticipating the possibilities of immunotherapy in the treatment of cancer, Christoph Huber, together with a core group of basic scientists and clinicians founded the Association for Cancer Immunotherapy (CIMT). Since then, CIMT has grown into the largest European platform and expert meeting with sole focus on cancer immunology.

Over the last 12 years, speakers and contributors of the CIMT faculty have taken us on a dynamic journey: The efforts of numerous scientists in the field have revealed novel mechanisms of how the immune system is able to control tumor growth, while at the same time increasing our general knowledge about the interdependencies of the human immune system. We have witnessed the translation of this knowledge into the first-time approval of vaccines and immune-modulatory antibodies and have seen the formation of the first dedicated regulatory frameworks in Europe and the USA that address the peculiar features of cancer immunotherapies. Recently, clinical trials with adoptively transferred *ex vivo* generated or immunoreceptor-engineered lymphocytes have shown unprecedented effects in patients. Novel combinations of immune-modulatory treatments with immunological and non-immunological treatments promise to lead to further breakthroughs in the near future. Increasing financial constraints in global health-care systems mandate the wise use of innovative drugs. This may be achieved by selecting patients who are most likely to respond to the use of novel immunological and molecular biomarkers. The advent of affordable whole genome sequencing has opened the door to a new discipline of immune-genomics that will lead to better diagnostics and personalized therapies.

Under the chairmanship of Christoph Huber, CIMT has developed into a thriving platform for disseminating the latest research findings among specialists working in academia, industry, and regulatory agencies. Throughout the years, CIMT has invited the most relevant experts in the world. In addition, CIMT has supported young scientists by giving them an opportunity to present their recent findings and awarding prizes for the best abstracts and posters. The CIMT working groups have been instrumental in harmonizing cellular immune assays and the generation of a reporting framework for T-cell assays as well as providing input on new regulatory documents and the

generation of a blueprint for personalized mutanome vaccines that was aligned with the European Medicines Agency. All these achievements would have been impossible without people who are willing to share their innovative ideas for the greater good. Christoph Huber is one of these visionaries who is interested not only in science but also in the people he has been working with, he has mentored, or he has treated as a medical doctor.

Therefore, this book is dedicated to Christoph Huber and all scientists and investigators who share his vision of immune-oncology and work passionately to develop better treatments for cancer patients.

Mainz, Germany — Cedrik Michael Britten
Mainz, Germany — Sebastian Kreiter
Mainz, Germany — Mustafa Diken
Tübingen, Germany — Hans-Georg Rammensee

Contents

Part I

Immunological and Regulatory Framework for Immuno-oncology

From Basic Immunology to New Therapies for Cancer Patients

Hans-Georg Rammensee

Origins

Paul Ehrlich obviously was fascinated by the then newly discovered adaptive immune receptor molecules able to distinguish between different infectious agents and by the plasticity of the immune system to select such receptors and to make many copies on demand. Constructing "ein Gedankengebäude" to explain the observations made by Emil von Behring and Shibasaburo Kitasato (1890), he not only created the term "Antikörper" (antibody) to describe such adaptive receptors but also considered the problems connected to their development within a mouse or human being, that is, the way how self-reactive antibodies are to be avoided. Presumably within this context, he hypothesized that antibodies, respectively, the immune system, should be able to somehow recognize and attack cancer cells, leading to his famous 1909 postulate of cancer immunosurveillance (Ehrlich 1909): We would have a much higher incidence of cancer without an immune system constantly chasing and destroying newly developing cancer cells. "… Würden diese (*die Schutzvorrichtungen des Organismus*) nicht bestehen, so könnte man vermuten, dass das Karzinom in einer geradezu ungeheuerlichen Frequenz auftreten würde." Independently of Paul Ehrlich, and earlier, two surgeons, Wilhelm Busch (1866) in Bonn (Hartmann 2008) and William B. Coley (1893) in New York (Coley 1991), reported a positive correlation between infection and tumor regression, early hints on TLR ligands and cytokines.

In the century thereafter, a tremendous amount of work searching for manifestations of such cancer immunity was performed, mostly leading to nothing or to discoveries seemingly unrelated to cancer. One such prominent case was the discovery of histocompatibility antigens (Klein 1986), following the observation that transplanted mouse tumors are readily rejected by recipient mice, but normal tissue from the other mouse as well, because the mice at that time were not inbred sufficiently (reviewed in (Klein 1986)).

Modern Cancer Immunology

It took almost 50 years until Richmond Prehn and Joan Main were able to show that at least methylcholanthrene-induced tumors could be rejected by an immune reaction in syngeneic mice (Prehn and Main 1957), and shortly thereafter, in 1960, George Klein and colleagues found tumor rejection to be also possible for an autologous tumor (Klein et al. 1960). The decades to follow brought a long row of ups and downs in the perception of the relevance of cancer immunity by the scientific community. A severe blow to the cancer immunosurveillance theory was the thymusless nude mouse, showing no higher incidence of spontaneous cancer than fully

H.-G. Rammensee
Department of Immunology, University of Tübingen, Auf der Morgenstelle 15, 72076 Tübingen, Germany
e-mail: rammensee@uni-tuebingen.de

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_1,

immunocompetent mice, as reported by Osiasis Stutman in 1974, again with a chemically induced tumor model (Stutman 1974). Another blow to the belief in cancer immunity was Prehn's experiment in 1972, demonstrating that, in opposite to Ehrlich's view, an immune reaction could also enhance rather that inhibit tumor growth (Prehn 1972). This experiment actually picked up an older observation of 1962 from the Old group (Boyse et al. 1962). (This collection of phenomena can now be put into the drawer of "tumor-promoting inflammation" (Hanahan and Weinberg 2011).) During all these years, a rather small number of scientists still were of the opinion that there must be something to it and continued to invest in experiments to discover mechanisms and target structures of cancer immunity, by studying both antibody and T-cell responses. Some of the leading figures were Lloyd Old et al. (2005), Robert North (1982), and Thierry Boon et al. (1988), to name only a few who influenced my own education. It took until the 1980s to molecularly identify in the mouse the first nonviral tumor antigen recognized by T cells, with a contribution from Mainz (Thomas Wölfel) (De Plaen et al. 1988). This actually turned out to be a mutated antigen, and in collaboration with the Boon group, we were able to identify and to quantify the mutated peptide presented on the MHC molecules of the tumor cells (Wallny et al. 1992). The first human T-cell epitope representing a tumor antigen again was reported by the Boon group in 1991 (van der Bruggen et al. 1991) and again with essential contribution from the University of Mainz (Alexander Knuth). Tumor-associated antigens spontaneously recognized by antibodies were analyzed early on by Lloyd Old and Edward Boyse in mice (Old and Boyse 1964), extended by Old's group to patients' sera (Pfreundschuh et al. 1978) and brought to high throughput in the 1990s by the SEREX approach, pioneered by Ugur Sahin, Özlem Türeci, and Michael Pfreundschuh (Sahin et al. 1997; Tureci et al. 1997).

Since the days of Paul Ehrlich, a full century was required to understand the basic molecules and mechanisms our immune system uses for its daily tasks in fighting infections. We still are far away from having gained complete knowledge but what we know to date is just sufficient to manipulate the immune system such that it can attack and destroy cancer cells. Currently, several of such attempts are proving to be successful. After getting to know the structures and functions of antibodies, T-cell receptors, MHC molecules and their ligands, cytokines and their receptors, cells of the innate immune system including their receptors and ligands, T-cell populations (chapter by T. Bopp et al.), and their co-receptors and inhibitory receptors, we now start to get insight into the complex interactions between immune mechanisms attacking tumors and the counteracting measures of tumors to defend themselves against this attack, formulated by Bob Schreiber into the "immunoediting" concept (Schreiber et al. 2011).

Modern Cancer Immunotherapy

The first hopes into cancer immunotherapy were raised by the discovery of the first cytokines, the interferons, in the 1950s by Alick Isaacs and Jean Lindenmann (1957) and later in the mid-1970s by the invention of making monoclonal antibodies on demand by Georges Köhler and Cesar Milstein (1975).

The first successful cancer immunotherapy, however, was a special kind of adoptive T-cell transfer, the donor lymphocyte infusion in the setting of bone marrow transplantation. This was a result from the development of bone marrow transplantation into irradiated recipients as a treatment of leukemias performed by the Edward Donnall Thomas lab with Rainer Storb in Seattle, who observed that the detrimental graft-versus-host reaction regularly occurring in human patients or outbred dogs, but not within inbred mice, was beneficial since it seemed to have an effect against leukemia (Weiden et al. 1979). This observation could be attributed to donor leukocytes in the late 1980s by Hans-Jochem Kolb (1990), who then systematically developed the use of DLI (donor leukocyte infusion) for the treatment of leukemia relapses after the original

bone marrow transplantation (Weiden et al. 1979). Such donor-derived T cells, including those already present in the bone marrow graft, induced not only graft-versus-host disease but also a graft-versus-leukemia effect. The recurrence of leukemia after transplantation could be successfully treated by additional transfer of a small number of leukocytes from the original donor, which in many cases led not only to an aggravation of GvHD but also to complete cure. Other early successes in antigen nonspecific cancer immunotherapy were the development of cytokines, in particular interferon alpha in hairy cell leukemia, where Christoph Huber was a pioneer (Gastl et al. 1985a, b; Huber et al. 1985; Aulitzky et al. 1985), and the use of a TLR ligand, BCG, for the treatment of bladder carcinoma (De Jager et al. 1991).

The first attempts of using monoclonal antibodies for passive immunotherapy of cancer were by the groups of Stuart Schlossman et al. (1980) and Ronald Levy and Miller (1981). It took, however, until the late 1990s to use monoclonal antibodies for passive immunotherapy of cancer on a routine basis, pioneered by Ralph Reisfeld et al. (1992), Gert Riethmüller et al. (1998), and others. In 1997, the first antibody was approved by the FDA for the treatment of cancer – rituximab (Grillo-Lopez et al. 2000) – directed not against a cancer antigen but rather against a cell type-specific antigen, CD20, expressed on normal cells dispensable for survival, the B cells.

Three principal problems in these developments were (1) the task to produce humanized antibodies in suitable formats to achieve sufficient production rates in cell cultures as well as to avoid anti-antibody reactions in the recipient, (2) achieving efficient effector function in the patient, and (3) finding the right antigen. The first problem has been largely solved by now, and the second is being solved at present by enhancing Fc-receptor interaction or by using bispecific antibodies capable of recruiting T cells with their superior proliferative potential, as pioneered by Uwe Staerz et al. (1985), Gundram Jung et al. (1986, 2001), and Gert Riethmüller (Topp et al. 2011). The third problem, finding suitable target structures on the surface of cancer cells that are not, or at least not much, expressed on normal cells, is still unsolved. Finding cancer cell surface antigens as target structures for therapeutic antibodies essentially follows three strategies:

1. Using information derived from cancer biology; epithelial carcinomas, for example, express epithelial markers, such as Epcam (Riethmuller et al. 1998). In growth factor receptor-driven cancers, in particular, this receptor or others of the EGFR family can be used as target, as pioneered by Axel Ullrich for HER2/neu in breast cancer (Hudziak et al. 1987; Fischer et al. 2003).
2. Looking at the antibody response produced spontaneously by cancer patients, as followed by the SEREX technology.
3. By systematically comparing cell surface antigens of tumor cells with that of normal cells, an approach that has been attempted surprisingly late in a systematic way, but then very successfully as shown by the work of Özlem Türeci and colleagues (Sahin et al. 2008).

The design of present and future cancer immunotherapies is drawing essential benefit from the revelations of cancer biology in the last 30 years. The insight that not only viral but also cellular oncogenes (Doolittle et al. 1983; Waterfield et al. 1983; Downward et al. 1984) are causative for cancer development, and the first indications that mutations in genes regulating cellular signaling or DNA repair such as K-Ras or p53 (Vogelstein et al. 1988; Hollstein et al. 1991) already hinted toward interesting targets for cancer immunotherapy. This is true in particular for T cells, since we know that HLA molecules present peptides from all cellular compartments, including nuclear proteins. Indeed, Thomas Wölfel showed that T cells specific for peptides representing mutated gene products can spontaneously develop in melanoma patients (Wolfel et al. 1995), and Gustav Gaudernack introduced peptide vaccination against K-ras mutations in a clinical trial followed over many years, with encouraging clinical results (Weden et al. 2011). The recent methodological improvements in genome sequencing have been used to systematically analyze the spectrum of mutations in many individual cancers, the result being an amazing heterogeneity of number and sites

of mutations, many of them drivers of cancer development but even more so just passenger mutations (Vogelstein et al. 2013). Since peptides derived from mutated gene products can principally be presented by HLA molecules (Falk et al. 1991) at the surface of the tumor cells, such mutated peptides have been recognized as ideal tumor-specific antigens, not shared by any normal cells (Rammensee and Singh-Jasuja 2013; Rammensee 2006; Castle et al. 2012; Segal et al. 2008).

Present cancer biology indeed is a field characterizable with Ehrlich's words "Auf dem Gebiete der Geschwulstforschung hat sich im letzten Dezennium eine durchgreifende Umwälzung vollzogen (Ehrlich 1909)." The foremost of the new insights comes from the genome sequence information we now have available for thousands of individual human cancers of all frequent entities, indicating hundreds to thousands of mutations in every human cancer (Vogelstein et al. 2013). Many of these mutations are drivers of cancer hallmarks, whereas others are inert passengers. Other new revelations come from the most striking new branch of cancer immunotherapies, inhibition of immunoregulatory checkpoints, pioneered by Jim Allison (1994). The exciting clinical benefit first of CTLA-4 (Wolchok et al. 2013a), and later PD1 antibodies (Wolchok et al. 2013b), convinced classical cancer biologists that after all the immune system can do something against cancer (compare the famous Hanahan and Weinberg reviews from 2000 and 2011 (Hanahan and Weinberg 2000, 2011)). What is recognized by the T cells supposedly released from suppression by these antibodies are most likely peptides representing cancer-specific mutations, as shown already in a few examples (van Rooij et al. 2013). A further solid demonstration of immunity at work against cancer comes from detailed analysis ("immunoscore") of tumor-infiltrating T cells (Fridman et al. 2012).

Perspectives

There are several interesting developments in cancer immunotherapy, as reviewed in detail by (Fox et al. 2011). The four most promising main strategies are as follows: (1) active vaccination with cancer antigens in various forms, e.g., peptides (Walter et al. 2012; Kenter et al. 2009) (see the chapters by C. Melief and H. Singh), mRNAs (Rittig et al. 2011; Kallen et al. 2013) (chapters by K-J Kallen and S. Kreiter et al.), proteins, viral constructs, or autologous tumor lysates, applied directly or on dendritic cells (Kreutz et al. 2013; Schierer et al. 2012) (chapters by H. Westdorp et al. and C.M. Britten et al.), an approach that can be validated by deep immunomonitoring (see the chapter by S.H. van der Burg et al. from the CIMT Immunoguiding Programme); (2) passive vaccination with function-improved antibodies directed against cancer antigens (Bargou et al. 2008; Hofmann et al. 2012) (chapters by M. Glennie et al. and G. Jung et al.); (3) adoptive transfer (Morgan et al. 2013; Meyer and Herr 2010) of T cells or T-cell receptors or chimeric antigen receptors (CAR) (Kalos and June 2013; Grupp et al. 2013; Riet et al. 2013; Chmielewski et al. 2013) (chapters by D. Schendel et al., U. Hartwig et al. and H. Abken et al.); and (4) manipulation of the patient's immune response by inhibition of immunoregulatory checkpoints (Page et al. 2014) (chapter by A. Hoos). Additional strategies are other antigen nonspecific interventions, such as the application of oncolytic or immuno-enhancing viruses (chapters by M.D. Mühlebach et al.), or innate immunity stimulators like toll-like receptor ligands or cytokines, or agents or measures inducing immunogenic cell death (Kroemer et al. 2013) (chapter by J.M. Pitt et al.), such as certain conventional or new drugs targeting cancer cells directly, or irradiation or local tumor ablation (chapters by T.M. Gorges et al., J.-P. Marschner et al. and S. Kasper et al.).

Chances and Pitfalls

Whereas active vaccination in general has been proven to be rather safe, but not as efficient as desired, the other three main strategies – function-improved antibodies, in particular, bispecific antibodies targeting T cells, adoptive transfer of effector cells, and checkpoint inhibition – can be extremely efficient but at the cost of toxicities. The contributions collected in this Festschrift

indicate the directions to go for improvement of these items. I see particular exciting potential in the development of active vaccination against the really tumor-specific antigens, the mutations (Britten et al. 2013), which, however, requires an individualized approach so that a new drug (e.g., peptides or RNAs) has to be manufactured for every patient, as we had suggested a while ago (Rammensee et al. 2002; Weinschenk et al. 2002), obviously a logistic and regulatory challenge (see the chapters by C.M. Britten et al. from the CIMT Regulatory Research Group and J.C. Castle et al.). In addition, combinations of two or more strategies – e.g., vaccination accompanied by checkpoint inhibition or immunotherapy together with any kind of conventional chemo- or radiotherapy – seem extremely promising. The limitations of antibody and CAR-mediated therapies are in the limited selection of suitable target antigens, which almost never will be entirely tumor specific. For therapies involving adoptive transfer of T cells or TCR gene transfer, care should be taken if the TCR has not been educated in the very patient's thymus. If the TCR comes from a mouse, or from a human individual with a different HLA restriction, or if it is affinity optimized, the danger of cross-reactivity with unpredictable target peptides certainly exists. I was the first to demonstrate allorestricted CTL (Rammensee and Bevan 1984) and at that time was convinced that such T cells should be great for cancer immunotherapy (Rammensee 1997), but later turned from Paulus to Saulus because we saw the unpredictable cross-reactivities (Obst et al. 1998, 2000). A problem with the mutation-directed vaccination approach lies in the difficulty we are presently experiencing with the verification of HLA presentation of peptides harboring a mutation, perhaps partially due to negative selection of tumor cells presenting an immunodominant mutated peptide. Exome or transcriptome sequencing cannot provide this information; the only way to prove the physical existence of peptides is mass spectrometry (Rammensee and Singh-Jasuja 2013) or, if possible, the recognition of tumor cells by T cells (van Rooij et al. 2013) as treated in this book by M.V. van Buuren et al.

Bright Future

After prophylactic vaccination against virus-induced cancer has proven to be successful and has entered clinical routine (Michels and zur Hausen 2009), it appears now that immunotherapy of clinically manifest cancer other than passive vaccination with antibodies has reached the bedside. Many scientists have contributed to this success; one eminent of these is Christoph Huber from Mainz, the "target" of this Festschrift. Christoph, we all thank you for your tremendous contributions to the field, by your own science, and by creating a surrounding fostering progress in cancer immunotherapy.

Acknowledgments The author thanks Gundram Jung and Cedrik M. Britten for improving the manuscript.

References

Allison JP (1994) CD28-B7 interactions in T-cell activation. Curr Opin Immunol 6(3):414–419

Aulitzky W, Gastl G, Troppmair J, Tilg H, Abbrederis K, Nerl C, Flener R, Rokos H, Huber C (1985) Results of a phase II study on the treatment of hairy cell leukemias with various doses of alpha-2-recombinant interferon. Acta Med Austriaca 12(5):115–121

Bargou R, Leo E, Zugmaier G, Klinger M, Goebeler M, Knop S, Noppeney R, Viardot A, Hess G, Schuler M, Einsele H, Brandl C, Wolf A, Kirchinger P, Klappers P, Schmidt M, Riethmuller G, Reinhardt C, Baeuerle PA, Kufer P (2008) Tumor regression in cancer patients by very low doses of a T cell-engaging antibody. Science 321(5891):974–977

Behring E, Kitasato S (1890) Ueber das Zustandekommen der Diphterie-Immunität und der Tetanus-Immunität bei Thieren. Dtsch Med Wochenschr 49:1113–1114

Boyse EA, Old LJ, Stockert E (1962) Immunological enhancement of a leukaemia. Nature 194:1142–1144

Britten CM, Singh-Jasuja H, Flamion B, Hoos A, Huber C, Kallen KJ, Khleif SN, Kreiter S, Nielsen M, Rammensee HG, Sahin U, Hinz T, Kalinke U (2013) The regulatory landscape for actively personalized cancer immunotherapies. Nat Biotechnol 31(10):880–882

Castle JC, Kreiter S, Diekmann J, Lower M, van de Roemer N, de Graaf J, Selmi A, Diken M, Boegel S, Paret C, Koslowski M, Kuhn AN, Britten CM, Huber C, Tureci O, Sahin U (2012) Exploiting the mutanome for tumor vaccination. Cancer Res 72(5):1081–1091

Chmielewski M, Hombach AA, Abken H (2013) Antigen-specific T-cell activation independently of the MHC: chimeric antigen receptor-redirected T cells. Front Immunol 4:371

Coley WB (1991) The treatment of malignant tumors by repeated inoculations of erysipelas. With a report of ten original cases. 1893. Clin Orthop Relat Res (262):3–11

De Jager R, Guinan P, Lamm D, Khanna O, Brosman S, De Kernion J, Williams R, Richardson C, Muenz L, Reitsma D et al (1991) Long-term complete remission in bladder carcinoma in situ with intravesical TICE bacillus Calmette Guerin. Overview analysis of six phase II clinical trials. Urology 38(6):507–513

De Plaen E, Lurquin C, Van Pel A, Mariame B, Szikora JP, Wolfel T, Sibille C, Chomez P, Boon T (1988) Immunogenic (tum-) variants of mouse tumor P815: cloning of the gene of tum- antigen P91A and identification of the tum- mutation. Proc Natl Acad Sci U S A 85(7):2274–2278

Doolittle RF, Hunkapiller MW, Hood LE, Devare SG, Robbins KC, Aaronson SA, Antoniades HN (1983) Simian sarcoma virus onc gene, v-sis, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science 221(4607):275–277

Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, Ullrich A, Schlessinger J, Waterfield MD (1984) Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature 307(5951):521–527

Ehrlich P (1909) Über den jetzigen Stand der Karzinomforschung. Ned Tijdschr Geneeskd 5:273–290

Falk K, Rotzschke O, Stevanovic S, Jung G, Rammensee HG (1991) Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature 351(6324):290–296

Fischer OM, Streit S, Hart S, Ullrich A (2003) Beyond Herceptin and Gleevec. Curr Opin Chem Biol 7(4):490–495

Fox BA, Schendel DJ, Butterfield LH, Aamdal S, Allison JP, Ascierto PA, Atkins MB, Bartunkova J, Bergmann L, Berinstein N, Bonorino CC, Borden E, Bramson JL, Britten CM, Cao X, Carson WE, Chang AE, Characiejus D, Choudhury AR, Coukos G, de Gruijl T, Dillman RO, Dolstra H, Dranoff G, Durrant LG, Finke JH, Galon J, Gollob JA, Gouttefangeas C, Grizzi F, Guida M, Hakansson L, Hege K, Herberman RB, Hodi FS, Hoos A, Huber C, Hwu P, Imai K, Jaffee EM, Janetzki S, June CH, Kalinski P, Kaufman HL, Kawakami K, Kawakami Y, Keilholtz U, Khleif SN, Kiessling R, Kotlan B, Kroemer G, Lapointe R, Levitsky HI, Lotze MT, Maccalli C, Maio M, Marschner JP, Mastrangelo MJ, Masucci G, Melero I, Melief C, Murphy WJ, Nelson B, Nicolini A, Nishimura MI, Odunsi K, Ohashi PS, O'Donnell-Tormey J, Old LJ, Ottensmeier C, Papamichail M, Parmiani G, Pawelec G, Proietti E, Qin S, Rees R, Ribas A, Ridolfi R, Ritter G, Rivoltini L, Romero PJ, Salem ML, Scheper RJ, Seliger B, Sharma P, Shiku H, Singh-Jasuja H, Song W, Straten PT, Tahara H, Tian Z, van Der Burg SH, von Hoegen P, Wang E, Welters MJ, Winter H, Withington T, Wolchok JD, Xiao W, Zitvogel L, Zwierzina H, Marincola FM, Gajewski TF, Wigginton JM, Disis ML (2011) Defining the critical hurdles in cancer immunotherapy. J Transl Med 9(1):214

Fridman WH, Pages F, Sautes-Fridman C, Galon J (2012) The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer 12(4):298–306

Gastl G, Aulitzky W, Margreiter R, Flener R, Van Camp B, Bememan Z, Peetermans M, Huber C (1985a) Recombinant alpha 2 interferon for induction and maintenance of remission in hairy cell leukaemia. Br J Haematol 61(3):581–582

Gastl G, Denz H, Abbrederis C, Huber H, Troppmair J, Wiegele J, Niederwieser D, Flener R, Huber C (1985b) Treatment with low dose human recombinant interferon-alpha-2-ARG induces complete remission in patients with hairy cell leukemia. Onkologie 8(3):143–144

Grillo-Lopez AJ, White CA, Dallaire BK, Varns CL, Shen CD, Wei A, Leonard JE, McClure A, Weaver R, Cairelli S, Rosenberg J (2000) Rituximab: the first monoclonal antibody approved for the treatment of lymphoma. Curr Pharm Biotechnol 1(1):1–9

Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR, Teachey DT, Chew A, Hauck B, Wright JF, Milone MC, Levine BL, June CH (2013) Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. N Engl J Med 368(16): 1509–1518

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100(1):57–70

Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144(5):646–674

Handgretinger R, Baader P, Dopfer R, Klingebiel T, Reuland P, Treuner J, Reisfeld RA, Niethammer D (1992) A phase I study of neuroblastoma with the anti-ganglioside GD2 antibody 14.G2a. Cancer Immunol Immunother 35(3):199–204

Hartmann G (2008) http://www3.uni-bonn.dePresse-mitteilungen/alles-rund-um-krebs?set_language=en

Hofmann M, Grosse-Hovest L, Nubling T, Pyz E, Bamberg ML, Aulwurm S, Buhring HJ, Schwartz K, Haen SP, Schilbach K, Rammensee HG, Salih HR, Jung G (2012) Generation, selection and preclinical characterization of an Fc-optimized FLT3 antibody for the treatment of myeloid leukemia. Leukemia 26(6): 1228–1237

Hollstein M, Sidransky D, Vogelstein B, Harris CC (1991) p53 mutations in human cancers. Science 253(5015): 49–53

Huber C, Flener R, Gastl G (1985) Interferon-alpha-2C in the treatment of advanced hairy cell leukaemia. Results of a phase II trial. Oncology 42(Suppl 1): 7–9

Hudziak RM, Schlessinger J, Ullrich A (1987) Increased expression of the putative growth factor receptor p185HER2 causes transformation and tumorigenesis of NIH 3T3 cells. Proc Natl Acad Sci U S A 84(20):7159–7163

Isaacs A, Lindenmann J (1957) Virus interference. I. The interferon. Proc R Soc Lond B Biol Sci 147(927): 258–267

Jung G, Honsik CJ, Reisfeld RA, Muller-Eberhard HJ (1986) Activation of human peripheral blood mononuclear cells by anti-T3: killing of tumor target cells coated with anti-target-anti-T3 conjugates. Proc Natl Acad Sci U S A 83(12):4479–4483

Jung G, Brandl M, Eisner W, Fraunberger P, Reifenberger G, Schlegel U, Wiestler OD, Reulen HJ, Wilmanns W (2001) Local immunotherapy of glioma patients with a combination of 2 bispecific antibody fragments and resting autologous lymphocytes: evidence for in situ t-cell activation and therapeutic efficacy. Int J Cancer 91(2):225–230

Kallen KJ, Heidenreich R, Schnee M, Petsch B, Schlake T, Thess A, Baumhof P, Scheel B, Koch SD, Fotin-Mleczek M (2013) A novel, disruptive vaccination technology: self-adjuvanted RNActive vaccines. Hum Vaccin Immunother 9(10):2363–2276

Kalos M, June CH (2013) Adoptive T cell transfer for cancer immunotherapy in the era of synthetic biology. Immunity 39(1):49–60

Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP, Essahsah F, Fathers LM, Offringa R, Drijfhout JW, Wafelman AR, Oostendorp J, Fleuren GJ, van der Burg SH, Melief CJ (2009) Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. N Engl J Med 361(19):1838–1847

Klein J (1986) Natural history of the major histocompatibility complex. Wiley, New York

Klein G, Sjogren HO, Klein E, Hellstrom KE (1960) Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary autochthonous host. Cancer Res 20:1561–1572

Kohler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256(5517):495–497

Kolb HJ, Mittermuller J, Clemm C, Holler E, Ledderose G, Brehm G, Heim M, Wilmanns W (1990) Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. Blood 76(12):2462–2465

Kreutz M, Tacken PJ, Figdor CG (2013) Targeting dendritic cells–why bother? Blood 121(15):2836–2844

Kroemer G, Galluzzi L, Kepp O, Zitvogel L (2013) Immunogenic cell death in cancer therapy. Annu Rev Immunol 31:51–72

Meyer RG, Herr W (2010) Addressing tumour tolerance to improve cancer immunotherapy. Eur J Immunol 40(12):3302–3305

Michels KB, zur Hausen H (2009) HPV vaccine for all. Lancet 374(9686):268–270

Miller RA, Levy R (1981) Response of cutaneous T cell lymphoma to therapy with hybridoma monoclonal antibody. Lancet 2(8240):226–230

Morgan RA, Chinnasamy N, Abate-Daga D, Gros A, Robbins PF, Zheng Z, Dudley ME, Feldman SA, Yang JC, Sherry RM, Phan GQ, Hughes MS, Kammula US, Miller AD, Hessman CJ, Stewart AA, Restifo NP, Quezado MM, Alimchandani M, Rosenberg AZ, Nath A, Wang T, Bielekova B, Wuest SC, Akula N, McMahon FJ, Wilde S, Mosetter B, Schendel DJ, Laurencot CM, Rosenberg SA (2013) Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy. J Immunother 36(2):133–151

Nadler LM, Stashenko P, Hardy R, Kaplan WD, Button LN, Kufe DW, Antman KH, Schlossman SF (1980) Serotherapy of a patient with a monoclonal antibody directed against a human lymphoma-associated antigen. Cancer Res 40(9):3147–3154

North RJ (1982) Cyclophosphamide-facilitated adoptive immunotherapy of an established tumor depends on elimination of tumor-induced suppressor T cells. J Exp Med 155(4):1063–1074

Obst R, Munz C, Stevanovic S, Rammensee HG (1998) Allo- and self-restricted cytotoxic T lymphocytes against a peptide library: evidence for a functionally diverse allorestricted T cell repertoire. Eur J Immunol 28(8): 2432–2443

Obst R, Netuschil N, Klopfer K, Stevanovic S, Rammensee HG (2000) The role of peptides in T cell alloreactivity is determined by self-major histocompatibility complex molecules. J Exp Med 191(5):805–812

Old LJ, Boyse EA (1964) Immunology of experimental tumors. Annu Rev Med 15:167–186

Page DB, Postow MA, Callahan MK, Allison JP, Wolchok JD (2014) Immune modulation in cancer with antibodies. Annu Rev Med 65:185–202

Pfreundschuh M, Shiku H, Takahashi T, Ueda R, Ransohoff J, Oettgen HF, Old LJ (1978) Serological analysis of cell surface antigens of malignant human brain tumors. Proc Natl Acad Sci U S A 75(10): 5122–5126

Prehn RT (1972) The immune reaction as a stimulator of tumor growth. Science 176(4031):170–171

Prehn RT, Main JM (1957) Immunity to methylcholanthrene-induced sarcomas. J Natl Cancer Inst 18(6):769–778

Rammensee H-G (1997) Tissue specific cytotoxic T cells produced by subtractive immunization in vitro (1984). In: Rammensee H-G, Bachmann J, Stevanovic S (eds) MHC ligands and peptide motifs. Springer/LANDES Bioscience, New York, pp 375–380

Rammensee HG (2006) Some considerations on the use of peptides and mRNA for therapeutic vaccination against cancer. Immunol Cell Biol 84(3):290–294

Rammensee HG, Bevan MJ (1984) Evidence from in vitro studies that tolerance to self antigens is MHC-restricted. Nature 308(5961):741–744

Rammensee HG, Singh-Jasuja H (2013) HLA ligandome tumor antigen discovery for personalized vaccine approach. Expert Rev Vaccines 12(10):1211–1217

Rammensee HG, Weinschenk T, Gouttefangeas C, Stevanovic S (2002) Towards patient-specific tumor antigen selection for vaccination. Immunol Rev 188:164–176

Riet T, Holzinger A, Dorrie J, Schaft N, Schuler G, Abken H (2013) Nonviral RNA transfection to transiently modify T cells with chimeric antigen receptors for adoptive therapy. Methods Mol Biol 969:187–201

Riethmuller G, Holz E, Schlimok G, Schmiegel W, Raab R, Hoffken K, Gruber R, Funke I, Pichlmaier H, Hirche H, Buggisch P, Witte J, Pichlmayr R (1998) Monoclonal antibody therapy for resected Dukes' C colorectal cancer: seven-year outcome of a multicenter randomized trial. J Clin Oncol 16(5):1788–1794

Rittig SM, Haentschel M, Weimer KJ, Heine A, Muller MR, Brugger W, Horger MS, Maksimovic O, Stenzl A, Hoerr I, Rammensee HG, Holderried TA, Kanz L, Pascolo S, Brossart P (2011) Intradermal vaccinations with RNA coding for TAA generate CD8+ and CD4+ immune responses and induce clinical benefit in vaccinated patients. Mol Ther 19(5):990–999

Sahin U, Tureci O, Pfreundschuh M (1997) Serological identification of human tumor antigens. Curr Opin Immunol 9(5):709–716

Sahin U, Koslowski M, Dhaene K, Usener D, Brandenburg G, Seitz G, Huber C, Tureci O (2008) Claudin-18 splice variant 2 is a pan-cancer target suitable for therapeutic antibody development. Clin Cancer Res 14(23):7624–7634

Schierer S, Hesse A, Knippertz I, Kaempgen E, Baur AS, Schuler G, Steinkasserer A, Nettelbeck DM (2012) Human dendritic cells efficiently phagocytose adenoviral oncolysate but require additional stimulation to mature. Int J Cancer 130(7):1682–1694

Schreiber RD, Old LJ, Smyth MJ (2011) Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science 331(6024):1565–1570

Segal NH, Parsons DW, Peggs KS, Velculescu V, Kinzler KW, Vogelstein B, Allison JP (2008) Epitope landscape in breast and colorectal cancer. Cancer Res 68(3):889–892

Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ (2005) Cancer/testis antigens, gametogenesis and cancer. Nat Rev Cancer 5(8):615–625

Staerz UD, Kanagawa O, Bevan MJ (1985) Hybrid antibodies can target sites for attack by T cells. Nature 314(6012):628–631

Stutman O (1974) Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. Science 183(4124):534–536

Topp MS, Kufer P, Gokbuget N, Goebeler M, Klinger M, Neumann S, Horst HA, Raff T, Viardot A, Schmid M, Stelljes M, Schaich M, Degenhard E, Kohne-Volland R, Bruggemann M, Ottmann O, Pfeifer H, Burmeister T, Nagorsen D, Schmidt M, Lutterbuese R, Reinhardt C, Baeuerle PA, Kneba M, Einsele H, Riethmuller G, Hoelzer D, Zugmaier G, Bargou RC (2011) Targeted therapy with the T-cell-engaging antibody blinatumomab of chemotherapy-refractory minimal residual disease in B-lineage acute lymphoblastic leukemia patients results in high response rate and prolonged leukemia-free survival. J Clin Oncol 29(18):2493–2498

Tureci O, Sahin U, Pfreundschuh M (1997) Serological analysis of human tumor antigens: molecular definition and implications. Mol Med Today 3(8):342–349

van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T (1991) A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 254(5038):1643–1647

van Rooij N, van Buuren MM, Philips D, Velds A, Toebes M, Heemskerk B, van Dijk LJ, Behjati S, Hilkmann H, El Atmioui D, Nieuwland M, Stratton MR, Kerkhoven RM, Kesmir C, Haanen JB, Kvistborg P, Schumacher TN (2013) Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. J Clin Oncol 31(32):e439–e442

Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL (1988) Genetic alterations during colorectal-tumor development. N Engl J Med 319(9):525–532

Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA Jr, Kinzler KW (2013) Cancer genome landscapes. Science 339(6127):1546–1558

Wallny HJ, Deres K, Faath S, Jung G, Van Pel A, Boon T, Rammensee HG (1992) Identification and quantification of a naturally presented peptide as recognized by cytotoxic T lymphocytes specific for an immunogenic tumor variant. Int Immunol 4(10):1085–1090

Walter S, Weinschenk T, Stenzl A, Zdrojowy R, Pluzanska A, Szczylik C, Staehler M, Brugger W, Dietrich PY, Mendrzyk R, Hilf N, Schoor O, Fritsche J, Mahr A, Maurer D, Vass V, Trautwein C, Lewandrowski P, Flohr C, Pohla H, Stanczak JJ, Bronte V, Mandruzzato S, Biedermann T, Pawelec G, Derhovanessian E, Yamagishi H, Miki T, Hongo F, Takaha N, Hirakawa K, Tanaka H, Stevanovic S, Frisch J, Mayer-Mokler A, Kirner A, Rammensee HG, Reinhardt C, Singh-Jasuja H (2012) Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. Nat Med 18:1254–1261

Waterfield MD, Scrace GT, Whittle N, Stroobant P, Johnsson A, Wasteson A, Westermark B, Heldin CH, Huang JS, Deuel TF (1983) Platelet-derived growth factor is structurally related to the putative transforming protein p28sis of simian sarcoma virus. Nature 304(5921):35–39

Weden S, Klemp M, Gladhaug IP, Moller M, Eriksen JA, Gaudernack G, Buanes T (2011) Long-term follow-up of patients with resected pancreatic cancer following vaccination against mutant K-ras. Int J Cancer 128(5):1120–1128

Weiden PL, Flournoy N, Thomas ED, Prentice R, Fefer A, Buckner CD, Storb R (1979) Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts. N Engl J Med 300(19):1068–1073

Weinschenk T, Gouttefangeas C, Schirle M, Obermayr F, Walter S, Schoor O, Kurek R, Loeser W, Bichler KH, Wernet D, Stevanovic S, Rammensee HG (2002) Integrated functional genomics approach for the design of patient-individual antitumor vaccines. Cancer Res 62(20):5818–5827

Wolchok JD, Hodi FS, Weber JS, Allison JP, Urba WJ, Robert C, O'Day SJ, Hoos A, Humphrey R, Berman

DM, Lonberg N, Korman AJ (2013a) Development of ipilimumab: a novel immunotherapeutic approach for the treatment of advanced melanoma. Ann N Y Acad Sci 1291:1–13

Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, Segal NH, Ariyan CE, Gordon RA, Reed K, Burke MM, Caldwell A, Kronenberg SA, Agunwamba BU, Zhang X, Lowy I, Inzunza HD, Feely W, Horak CE, Hong Q, Korman AJ, Wigginton JM, Gupta A, Sznol M (2013b) Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med 369:122–133

Wolfel T, Hauer M, Schneider J, Serrano M, Wolfel C, Klehmann-Hieb E, De Plaen E, Hankeln T, Meyer zum Buschenfelde KH, Beach D (1995) A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science 269(5228):1281–1284

How T Cells Single Out Tumor Cells: "And That Has Made All the Difference…"

Marit M. van Buuren, Pia Kvistborg, and Ton N.M. Schumacher

Introduction

Two roads diverged in a yellow wood,
And sorry I could not travel both.
…
I took the one less traveled by,
And that has made all the difference…

The final lines from this poem (Robert Frost, 1920) take on a double meaning in the context of this short review. First the targeting of neo-antigens in human cancer can without doubt be considered "the road less traveled," a road of which the tracks are only just now becoming somewhat visible. Second, the targeting of "the difference," those determinants that can be used by the immune system to distinguish healthy cells from cancer, forms the central goal of cancer immunotherapy, and – conceivably – neo-antigens make up a large, if not essential, part of this difference.

There is now solid proof that the immune system can recognize a variety of different human cancers. Early – and admittedly weak – evidence for this has been the occasional spontaneous regression of tumor lesions in cancers such as melanoma (Kalialis et al. 2009). More recently, direct evidence for tumor control by the human immune system has been provided by the clinical success of different forms of immunotherapy in melanoma (Rosenberg and Dudley 2009; Hodi et al. 2010), but also in other cancer types such as renal-cell carcinoma (RCC) and non-small-cell lung cancer (NSCLC) (Topalian et al. 2012).

In spite of the recent successes in other tumor types, the potential of cancer immunotherapy and the mechanisms underlying immune-mediated cancer regression are to date still most clearly established for melanoma. Tumor-infiltrating lymphocyte (TIL) therapy, in which patients are treated with ex vivo-expanded autologous tumor-infiltrating T cells, has shown objective responses in about 50 % of patients treated in multiple centers, with a good fraction of patients showing a complete response (range 6.5–22 %) (Rosenberg et al. 2011; Radvanyi et al. 2012; Besser et al. 2013). Furthermore, from studies that entailed the infusion of $CD8^+$-enriched T-cell products, it is now evident that cytotoxic T cells are responsible for at least part of the reactivity observed (Dudley et al. 2010, 2013). Further (indirect) support for the notion that $CD8^+$ T cells can control tumor growth is provided by a large number of studies that demonstrate that for several tumor types a strong infiltrate of $CD8^+$ T cells correlates with a good clinical prognosis (Fridman et al. 2012).

In parallel work, the clinical use of antibodies directed against T-cell checkpoint molecules has shown impressive results in a number of studies. In two phase III studies, treatment of patients

M.M. van Buuren • P. Kvistborg
T.N.M. Schumacher (✉)
Division of Immunology,
The Netherlands Cancer Institute,
Plesmanlaan 121, 1066 CX Amsterdam,
The Netherlands
e-mail: t.schumacher@nki.nl

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_2,

with advanced melanoma with the anti-CTLA4 antibody ipilimumab was shown to improve overall survival (Hodi et al. 2010). A remarkable observation in these clinical trials has been the long duration of these responses in 10–20 % of the patients treated (Ott et al. 2013). Furthermore, substantial clinical activity has now also been seen with anti-PD1 antibodies, inducing objective response rates in around 30 % of melanoma patients treated in phase I studies (Topalian et al. 2012; Hamid et al. 2013), and early evidence suggests substantial synergy between the two treatment strategies (Wolchok et al. 2013).

These studies provide clear evidence that human tumors must express determinants, "antigens," that can be recognized by the human immune system. However, it is currently unclear which antigenic determinants are the main targets in the observed tumor regression. Knowledge of such antigens could provide a way not only to increase the activation state of the immune system by checkpoint blockade but also to specifically alert this activated immune system to tumor determinants of interest.

The Roads Toward Tumor Recognition: The Antigens That Can Be Targeted by CD8$^+$ T Cells

There are two major classes of antigens that can be targeted by T cells. The first class is formed by the "self-antigens," non-mutated antigens that are only expressed in a restricted set of cell types and for which T-cell tolerance is (therefore) incomplete. An interesting subset of the class of self-antigens is formed by the group of cancer germline antigens (C/G antigens). The expression pattern of many of the C/G antigens is to a large part – if not fully – restricted to germline tissue (which forms an immune privileged site), and, because of this, C/G antigens can be considered an attractive target in immunotherapy (vide infra).

The second major class of antigens is formed by the so-called neo-antigens. During cancer development, human tumors acquire large numbers of mutations within their DNA. These mutations are not restricted to driver mutations that contribute to cellular transformation but also include "passenger mutations" that haphazardly occur during the process of tumor outgrowth. An interesting feature of those mutations is the fact that they can result in the presentation of novel peptides by MHC molecules on the tumor cell surface. Furthermore, as these newly formed antigens ("neo-antigens") are fully tumor restricted, the targeting of these antigens should result in an immune response that is most likely (see below) entirely specific for the tumor tissue.

The potential relevance of neo-antigens in cancer was recognized many years ago by a few pioneers in the field, and early work using cDNA library screening tools provided the first evidence for recognition of neo-antigens by (autologous) T cells in human cancer (Wolfel et al. 1995; Robbins et al. 1996; Lennerz et al. 2005). However, with tools lacking to study the recognition of neo-antigens in human cancer in a systematic way, attention shifted rapidly toward the class of tumor-associated self-antigens, and this has been the main road taken since.

With the developments in next-generation sequencing technology over the past years, analysis of the human cancer genome has suddenly become straightforward. The definition of the genomic alterations in cancers, such as single nucleotide variants (SNVs) and insertions and deletions (indels), has now been performed for over thousands of human tumors (Stratton 2011; Alexandrov et al. 2013; Kandoth et al. 2013). With the technology to rapidly determine the genomic alterations in human tumors established, a key next step has been to assess whether such information can be utilized to dissect neo-antigen-specific CD8$^+$ T-cell recognition. Sahin and colleagues were the first to show the feasibility of using whole-exome sequencing data in order to identify neo-antigens encoded by a cancer genome. In this seminal work, mutations within the B16F10 murine melanoma cell line – a workhorse of cancer immunologists for the past decades – were first identified, and this information was then used to assess the immunogenicity of predicted neo-antigens by peptide vaccination. Interestingly, these experiments showed that a

good part of the mutated epitopes was able to elicit an immune response, and vaccination with some of these could be shown to result in immune control of tumor growth (Castle et al. 2012). Parallel work from Schreiber and colleagues, using a distinct murine cancer model, demonstrated that cancer-exome sequencing could be utilized to reveal the identity of a neo-antigen that was recognized during in vivo tumor outgrowth. Furthermore, this work also showed how such a neo-antigen can be lost under immune pressure (Matsushita et al. 2012), an important aspect to take into account when considering the road toward personalized immunotherapy.

Following these initial studies in mouse models, recent efforts by the group of Rosenberg and by our group have shown how cancer-exome data can be used to reveal T-cell responses against neo-antigens in humans (van Rooij et al. 2013; Robbins et al. 2013) and also how these T-cell responses can be influenced by immunotherapy (van Rooij et al. 2013). In the first study, neo-antigen-specific T-cell reactivity within the TIL compartment of melanoma patients was detected in four out of five patients analyzed, with the remark that this analysis primarily focused on patients with a clinical response upon TIL therapy (Robbins et al. 2013). In our work, neo-antigen-specific T-cell reactivity was analyzed in a patient with metastatic melanoma that showed a partial response to ipilimumab treatment. First, analysis of the tumor exome revealed a striking number of mutations (>1,000 non-synonymous mutations), and with the use of computational algorithms to predict proteasomal cleavage (Kesmir et al. 2002) and MHC binding (Nielsen et al. 2003), a list of HLA-A- and HLA-B-restricted possible neo-antigens was generated. Second, using a combination of MHC-based monitoring tools that we established in prior work (Toebes et al. 2006; Hadrup et al. 2009), we measured reactivity against any of these potential epitopes in T-cell populations of this patient. This analysis revealed two neo-antigen-specific T-cell responses within the TIL: one low-level response, targeting the mutated product of ZNF462 (0.003 % of $CD8^+$ T cells) that was not pursued further, and one T-cell response directed against a nonameric peptide encoded by the mutated ATR gene product (3.3 % of $CD8^+$ T cells). Furthermore, this ATR-specific T-cell response was also present in the peripheral blood compartment of this patient and showed a marked increase after treatment with ipilimumab (van Rooij et al. 2013).

Following this initial work, we have utilized cancer-exome data to analyze neo-antigen-specific T-cell reactivity in an additional seven patients, revealing neo-antigen-specific T-cell responses in five of them (range 0.002–65 % of $CD8^+$ T cells, van Buuren et al, unpublished observations). Importantly, for a number of reasons, we consider it highly likely that we are still underestimating the number of neo-antigen-specific T-cell responses in these patients. Among other factors, exome capture is incomplete, alternative open reading frames are not taken into account, and the prediction of epitope presentation is far from optimal for many HLA class I alleles.

Taking into account that neo-antigen reactivity has been found in most of the melanoma patients thus far analyzed (10 out of 13 in the combined NIH/NKI studies), these data indicate that neo-antigen recognition in melanoma must be a common phenomenon. Furthermore, as the size of the pool of neo-antigens is expected to roughly correlate with the mutational load in different tumor types, these data can also be used as a benchmark to determine the potential for neo-antigen recognition in other human malignancies. Comparison of the number of mutations across a large set of different human tumor types has revealed that melanoma forms the tumor type with the highest average mutational load (Alexandrov et al. 2013; Kandoth et al. 2013). Importantly though, the variation in the number of mutations between different melanomas is very high, and the mutation load in many human melanomas is comparable to that of other common human cancers, such as lung cancer, esophageal cancer, and colorectal cancer. Because of this, we hypothesize that also in these human tumor types, a significant repertoire of neo-antigens should be present that may be targeted by T cells. As a side note, the observation that NSCLC patients treated with anti-PD1 also do

show objective responses (27 % OR) (Topalian et al. 2012) is in line with the idea that tumors with a high mutation load may be particularly attractive for the development of cancer immunotherapy.

The Road Not Taken

In previous work, we and others have set out to define which of the antigens expressed by tumor cells can be considered true "cancer-regression antigens," antigens that are critically involved in (immunotherapy-induced) cancer regression (Gilboa 1999; Kvistborg et al. 2013). Two characteristics are thought to be particularly important for this. First and foremost, it is likely that T-cell responses directed against antigens that show a fully tumor-restricted expression pattern are of superior value, as central tolerance toward these antigen is not an issue (Gilboa 1999; Kvistborg et al. 2013). Furthermore, T-cell responses against such antigens are also of interest from a safety perspective, as toxicity due to the recognition of healthy tissues is not likely to form a concern. In this regard it should be noted that it can at this point not be excluded that a strong T-cell response against a neo-antigen would result in cross-reactivity with the wild-type counterpart (e.g., in a case in which the WT counterpart has the same TCR-exposed surface but is only expressed at a lower level). However, experimental evidence for this has thus far not been obtained. Second, T-cell responses against antigens that are unlikely to be lost from tumor cells may be of particular value, as tumor escape due to antigen loss is less likely to occur. From a more pragmatic point of view, when a set of antigens would exist that displays these characteristics and that is shared among many patients, this would be of interest, as it would allow the use of off-the-shelf targeted immunotherapies to enhance reactivity against such antigens.

With respect to the tumor-restricted nature of antigen expression, two classes of antigens stand out, the neo-antigens and the C/G antigens. As mentioned previously, expression of C/G antigens is thought to be largely restricted to cancer cells and germline tissues, and the targeting of at least one C/G antigen with a high-magnitude T-cell response has been shown to be safe (Robbins et al. 2011). Nevertheless, for a substantial number of C/G antigens, expression within somatic tissues has also been observed (Hofmann et al. 2008), and because of this, the targeting of these antigens can lead to toxicity, as shown in a recent example where an affinity-enhanced TCR was used to target an epitope from the MAGE A3 gene product (Linette et al. 2013). By the same token, data from Kyewski and co-workers (Gotter et al. 2004) has revealed that at least some C/G antigens are expressed within the thymic epithelium. Because of this, the T-cell repertoire available for tumor recognition may be restricted to lower-affinity TCRs/epitopes that are presented inefficiently.

Importantly, at present the quality of T-cell responses directed against neo-antigens and C/G antigens has not been compared. As a first thought, one could compare the dissociation (K_{off}) rate of neo-antigen and C/G antigen-specific TCRs or T cells obtained from patients. As a more direct test, one could conceivably compare the ability of neo-antigen and C/G antigen-specific T cells isolated from patient material to recognize autologous tumor. Such experiments would be useful to establish whether T-cell responses directed against neo-antigens are commonly of a higher quality than T-cell responses directed against C/G antigens or whether the two classes are essentially indistinguishable.

As a second factor determining the relative role of T-cell responses against neo-antigens and C/G antigens, it will be important to quantify the size of the "antigenic space" of both antigen classes in different tumors, something that may be feasible on the basis of cancer-exome/RNAseq data. Without such data presently being available, perhaps the best argument in favor of a significant role of neo-antigens as cancer-regression antigens is the observation that tumors with a high mutation load are clearly responsive to T-cell checkpoint blockade. On the basis of this early evidence, and with the recently developed technology to describe the patient-specific neo-antigen repertoire available, it seems justified to take some first steps on the road of personalized immunotherapy.

A Roadmap to Personalized Immunotherapy

In the final part of this review, we will discuss three aspects of this road toward personalized cancer immunotherapy, in which T-cell responses against specific neo-antigens are induced or boosted by vaccination: (1) the approach that may be used to select the epitopes that should be contained within such vaccines, (2) the issue of tumor heterogeneity, and (3) the changes in the regulatory landscape that are required for the clinical development of patient-specific vaccines.

A vaccine that encodes a series of predicted neo-antigens can only be of value if at least some of the neo-antigens contained are truly present on the tumor cell surface. As such, our ability to select those neo-antigens that have a protective capacity will be key. A recent effort to predict the immunogenicity of antigenic determinants was performed by Singh-Jasuja and colleagues. Here a multipronged approach, including mass spectrometry, gene expression profiling, and literature-based functional assessment, was utilized to assemble a set of tumor-associated peptides to treat patients with RCC. Interestingly, evaluation of this vaccine revealed that a favorable clinical course could be associated with vaccine-induced immune responses (Walter et al. 2012). Importantly though, the selection process used here appears too complex to be used for individual patients, and profound improvements in mass spectrometry would also be required to allow the evaluation of the MHC-associated epitope repertoire on the basis of biopsy material.

A substantially more straightforward approach from a logistic point of view will be the use of computational algorithms such as NetChop and NetMHC (Kesmir et al. 2002; Nielsen et al. 2003) to predict neo-antigens with cancer exome (and RNAseq) data as sole input. While results are still limited to a few examples, potential neo-antigens that are predicted to bind tightly to MHC class I may be more likely to induce T-cell reactivity than neo-antigens with a lower predicted affinity, consistent with prior data (Harndahl et al. 2012). Nevertheless, even when focusing on predicted high-affinity ligands, the false-positive rate is still high, in particular for the less commonly studied HLA class I alleles for which epitope prediction algorithms are of a lower quality. For this reason, it would perhaps seem useful to focus early trials in this area to HLA alleles such as HLA-A*02:01, for which we have a solid understanding of ligand preference.

While being able to select those neo-antigens that are actually presented by tumor cells is a major challenge, tumor heterogeneity adds another layer of complexity to this. Within a given tumor, cells may be present which do not express a given neo-antigen, conferring a selective advantage to those cells at the moment immune pressure is imposed. Significant evidence for both intralesional and inter-lesional heterogeneity has accumulated over the past years. To provide some examples, a comparative study in patients with metastatic pancreatic cancer showed that there was only a partial overlap in the mutations encountered in different metastatic lesions or in the primary lesions (Campbell et al. 2010). Thus, the neo-antigens predicted on the basis of one tumor lesion will unlikely be present in all others. Furthermore even within the same lesion, the genetic landscape can differ between different tumor cells in that lesion, something that is particularly apparent when comparing the genetic landscape in different geographical areas of the tumor. In both breast and kidney cancer, it has already been shown how genomic lesions in tumors varied between adjacent areas (Navin et al. 2011; Gerlinger et al. 2012), and there is little reason to assume this will be different for other tumor types. As such, mutations encountered within a biopsy may not be present within the entire tumor mass, another challenge for vaccine design.

To increase the likelihood that a neo-antigen-directed vaccine will lead to the induction of T-cell responses against epitopes that are truly presented by tumor cells, and to at the same time reduce the likelihood of clonal escape, the inclusion of a series of predicted neo-antigens within personalized neo-antigen vaccines appears mandatory. How large the number of such neo-antigens needs to be is presently unclear, and this

is something that should be evaluated in preclinical patient-derived xenograft models and early phase clinical trials.

The final hurdle to take in the development of patient-specific neo-antigen vaccines is formed by the regulatory landscape. Contrary to conventional vaccines, extensive safety and efficacy testing of individual vaccines is not feasible. As such, safety/efficacy will need to be judged on the basis preclinical and clinical proof-of-principle studies in which (1) the intended immune response is induced, (2) an antitumor effect is conferred, and (3) the toxicity profile is acceptable. Additionally, a stepwise enrollment of patients should be done when clinical trials are started while implementing sensible strategies for managing risks and stopping rules (Britten et al. 2013). A recent paper by Kalinke and colleagues indicates that, conceivably, the regulatory landscape for personalized neo-antigen vaccines can be modeled after that used for autologous cell therapy, which would form an important step forward.

Here we have outlined the progress that has recently been made in cancer immunotherapy and in particular our ability to dissect melanoma neo-antigen recognition by T cells on the basis of cancer exome data. Based on analysis of the mutational load in different human tumors, we consider it likely that also in other major human tumor types, recognition of such antigens can occur. On the basis of the data available, there is some reason to assume that the targeting of this neo-antigen pool by vaccination will be worth our while. Development of high-quality neo-antigen-specific vaccines that do induce a broad tumor-reactive T-cell response will form a logistic and regulatory challenge, but the ultimate result may be significant.

Acknowledgments

We thank C. Linnemann for useful discussions.

This research was supported by KWF grant 2012-5463 and by a Stand Up To Cancer-Cancer Research Institute Cancer Immunology Translational Cancer Research grant. Stand Up To Cancer is a program of the Entertainment Industry Foundation administered by the American Association for Cancer Research.

References

Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, Bignell GR, Bolli N, Borg A, Borresen-Dale AL, Boyault S, Burkhardt B, Butler AP, Caldas C, Davies HR, Desmedt C, Eils R, Eyfjord JE, Foekens JA, Greaves M, Hosoda F, Hutter B, Ilicic T, Imbeaud S, Imielinsk M, Jager N, Jones DT, Jones D, Knappskog S, Kool M, Lakhani SR, Lopez-Otin C, Martin S, Munshi NC, Nakamura H, Northcott PA, Pajic M, Papaemmanuil E, Paradiso A, Pearson JV, Puente XS, Raine K, Ramakrishna M, Richardson AL, Richter J, Rosenstiel P, Schlesner M, Schumacher TN, Span PN, Teague JW, Totoki Y, Tutt AN, Valdes-Mas R, van Buuren MM, van't Veer L, Vincent-Salomon A, Waddell N, Yates LR, Zucman-Rossi J, Futreal PA, McDermott U, Lichter P, Meyerson M, Grimmond SM, Siebert R, Campo E, Shibata T, Pfister SM, Campbell PJ, Stratton MR (2013) Signatures of mutational processes in human cancer. Nature 500(7463):415–421. doi:10.1038/nature12477

Besser MJ, Shapira-Frommer R, Itzhaki O, Treves AJ, Zippel DB, Levy D, Kubi A, Shoshani N, Zikich D, Ohayon Y, Ohayon D, Shalmon B, Markel G, Yerushalmi R, Apter S, Ben-Nun A, Ben-Ami E, Shimoni A, Nagler A, Schachter J (2013) Adoptive transfer of tumor-infiltrating lymphocytes in patients with metastatic melanoma: intent-to-treat analysis and efficacy after failure to prior immunotherapies. Clin Cancer Res 19(17):4792–4800. doi:10.1158/1078-0432.CCR-13-0380

Britten CM, Singh-Jasuja H, Flamion B, Hoos A, Huber C, Kallen KJ, Khleif SN, Kreiter S, Nielsen M, Rammensee HG, Sahin U, Hinz T, Kalinke U (2013) The regulatory landscape for actively personalized cancer immunotherapies. Nat Biotechnol 31(10):880–882. doi:10.1038/nbt.2708

Campbell PJ, Yachida S, Mudie LJ, Stephens PJ, Pleasance ED, Stebbings LA, Morsberger LA, Latimer C, McLaren S, Lin ML, McBride DJ, Varela I, Nik-Zainal SA, Leroy C, Jia M, Menzies A, Butler AP, Teague JW, Griffin CA, Burton J, Swerdlow H, Quail MA, Stratton MR, Iacobuzio-Donahue C, Futreal PA (2010) The patterns and dynamics of genomic instability in metastatic pancreatic cancer. Nature 467(7319):1109–1113. doi:10.1038/nature09460

Castle JC, Kreiter S, Diekmann J, Lower M, van de Roemer N, de Graaf J, Selmi A, Diken M, Boegel S, Paret C, Koslowski M, Kuhn AN, Britten CM, Huber C, Tureci O, Sahin U (2012) Exploiting the mutanome for tumor vaccination. Cancer Res 72(5):1081–1091. doi:10.1158/0008-5472.CAN-11-3722

Dudley ME, Gross CA, Langhan MM, Garcia MR, Sherry RM, Yang JC, Phan GQ, Kammula US, Hughes MS, Citrin DE, Restifo NP, Wunderlich JR, Prieto PA, Hong JJ, Langan RC, Zlott DA, Morton KE, White DE, Laurencot CM, Rosenberg SA (2010) CD8+ enriched "young" tumor infiltrating lymphocytes can mediate regression of metastatic melanoma. Clin Cancer Res 16(24):6122–6131. doi:10.1158/1078-0432.CCR-10-1297

Dudley ME, Gross CA, Somerville RP, Hong Y, Schaub NP, Rosati SF, White DE, Nathan D, Restifo NP, Steinberg SM, Wunderlich JR, Kammula US, Sherry RM, Yang JC, Phan GQ, Hughes MS, Laurencot CM, Rosenberg SA (2013) Randomized selection design trial evaluating CD8+-enriched versus unselected tumor-infiltrating lymphocytes for adoptive cell therapy for patients with melanoma. J Clin Oncol 31(17):2152–2159. doi:10.1200/JCO.2012.46.6441

Fridman WH, Pages F, Sautes-Fridman C, Galon J (2012) The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer 12(4):298–306. doi:10.1038/nrc3245

Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, Martinez P, Matthews N, Stewart A, Tarpey P, Varela I, Phillimore B, Begum S, McDonald NQ, Butler A, Jones D, Raine K, Latimer C, Santos CR, Nohadani M, Eklund AC, Spencer-Dene B, Clark G, Pickering L, Stamp G, Gore M, Szallasi Z, Downward J, Futreal PA, Swanton C (2012) Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med 366(10):883–892. doi:10.1056/NEJMoa1113205

Gilboa E (1999) The makings of a tumor rejection antigen. Immunity 11(3):263–270

Gotter J, Brors B, Hergenhahn M, Kyewski B (2004) Medullary epithelial cells of the human thymus express a highly diverse selection of tissue-specific genes colocalized in chromosomal clusters. J Exp Med 199(2):155–166. doi:10.1084/jem.20031677

Hadrup SR, Bakker AH, Shu CJ, Andersen RS, van Veluw J, Hombrink P, Castermans E, Thor Straten P, Blank C, Haanen JB, Heemskerk MH, Schumacher TN (2009) Parallel detection of antigen-specific T-cell responses by multidimensional encoding of MHC multimers. Nat Methods 6(7):520–526. doi:10.1038/nmeth.1345

Hamid O, Robert C, Daud A, Hodi FS, Hwu WJ, Kefford R, Wolchok JD, Hersey P, Joseph RW, Weber JS, Dronca R, Gangadhar TC, Patnaik A, Zarour H, Joshua AM, Gergich K, Elassaiss-Schaap J, Algazi A, Mateus C, Boasberg P, Tumeh PC, Chmielowski B, Ebbinghaus SW, Li XN, Kang SP, Ribas A (2013) Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. N Engl J Med 369(2):134–144. doi:10.1056/NEJMoa1305133

Harndahl M, Rasmussen M, Roder G, Dalgaard Pedersen I, Sorensen M, Nielsen M, Buus S (2012) Peptide-MHC class I stability is a better predictor than peptide affinity of CTL immunogenicity. Eur J Immunol 42(6):1405–1416. doi:10.1002/eji.201141774

Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbe C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ (2010) Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 363(8):711–723. doi:10.1056/NEJMoa1003466

Hofmann O, Caballero OL, Stevenson BJ, Chen YT, Cohen T, Chua R, Maher CA, Panji S, Schaefer U, Kruger A, Lehvaslaiho M, Carninci P, Hayashizaki Y, Jongeneel CV, Simpson AJ, Old LJ, Hide W (2008) Genome-wide analysis of cancer/testis gene expression. Proc Natl Acad Sci U S A 105(51):20422–20427. doi:10.1073/pnas.0810777105

Kalialis LV, Drzewiecki KT, Klyver H (2009) Spontaneous regression of metastases from melanoma: review of the literature. Melanoma Res 19(5):275–282. doi:10.1097/CMR.0b013e32832eabd5

Kandoth C, McLellan MD, Vandin F, Ye K, Niu B, Lu C, Xie M, Zhang Q, McMichael JF, Wyczalkowski MA, Leiserson MD, Miller CA, Welch JS, Walter MJ, Wendl MC, Ley TJ, Wilson RK, Raphael BJ, Ding L (2013) Mutational landscape and significance across 12 major cancer types. Nature 502(7471):333–339. doi:10.1038/nature12634

Kesmir C, Nussbaum AK, Schild H, Detours V, Brunak S (2002) Prediction of proteasome cleavage motifs by neural networks. Protein Eng 15(4):287–296

Kvistborg P, van Buuren MM, Schumacher TN (2013) Human cancer regression antigens. Curr Opin Immunol 25(2):284–290. doi:10.1016/j.coi.2013.03.005

Lennerz V, Fatho M, Gentilini C, Frye RA, Lifke A, Ferel D, Wolfel C, Huber C, Wolfel T (2005) The response of autologous T cells to a human melanoma is dominated by mutated neoantigens. Proc Natl Acad Sci U S A 102(44):16013–16018. doi:10.1073/pnas.0500090102

Linette GP, Stadtmauer EA, Maus MV, Rapoport AP, Levine BL, Emery L, Litzky L, Bagg A, Carreno BM, Cimino PJ, Binder-Scholl GK, Smethurst DP, Gerry AB, Pumphrey NJ, Bennett AD, Brewer JE, Dukes J, Harper J, Tayton-Martin HK, Jakobsen BK, Hassan NJ, Kalos M, June CH (2013) Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. Blood 122(6):863–871. doi:10.1182/blood-2013-03-490565

Matsushita H, Vesely MD, Koboldt DC, Rickert CG, Uppaluri R, Magrini VJ, Arthur CD, White JM, Chen YS, Shea LK, Hundal J, Wendl MC, Demeter R, Wylie T, Allison JP, Smyth MJ, Old LJ, Mardis ER, Schreiber RD (2012) Cancer exome analysis reveals a T-cell-dependent mechanism of cancer immunoediting. Nature 482(7385):400–404. doi:10.1038/nature10755

Navin N, Kendall J, Troge J, Andrews P, Rodgers L, McIndoo J, Cook K, Stepansky A, Levy D, Esposito D, Muthuswamy L, Krasnitz A, McCombie WR, Hicks J, Wigler M (2011) Tumour evolution inferred by single-cell sequencing. Nature 472(7341):90–94. doi:10.1038/nature09807

Nielsen M, Lundegaard C, Worning P, Lauemoller SL, Lamberth K, Buus S, Brunak S, Lund O (2003) Reliable prediction of T-cell epitopes using neural networks with novel sequence representations. Protein Sci 12(5):1007–1017. doi:10.1110/ps.0239403

Ott PA, Hodi FS, Robert C (2013) CTLA-4 and PD-1/PD-L1 blockade: new immunotherapeutic modalities with durable clinical benefit in melanoma patients.

Clin Cancer Res 19(19):5300–5309. doi:10.1158/1078-0432.CCR-13-0143

Radvanyi LG, Bernatchez C, Zhang M, Fox PS, Miller P, Chacon J, Wu R, Lizee G, Mahoney S, Alvarado G, Glass M, Johnson VE, McMannis JD, Shpall E, Prieto V, Papadopoulos N, Kim K, Homsi J, Bedikian A, Hwu WJ, Patel S, Ross MI, Lee JE, Gershenwald JE, Lucci A, Royal R, Cormier JN, Davies MA, Mansaray R, Fulbright OJ, Toth C, Ramachandran R, Wardell S, Gonzalez A, Hwu P (2012) Specific lymphocyte subsets predict response to adoptive cell therapy using expanded autologous tumor-infiltrating lymphocytes in metastatic melanoma patients. Clin Cancer Res 18(24):6758–6770. doi:10.1158/1078-0432.CCR-12-1177

Robbins PF, El-Gamil M, Li YF, Kawakami Y, Loftus D, Appella E, Rosenberg SA (1996) A mutated beta-catenin gene encodes a melanoma-specific antigen recognized by tumor infiltrating lymphocytes. J Exp Med 183(3):1185–1192

Robbins PF, Morgan RA, Feldman SA, Yang JC, Sherry RM, Dudley ME, Wunderlich JR, Nahvi AV, Helman LJ, Mackall CL, Kammula US, Hughes MS, Restifo NP, Raffeld M, Lee CC, Levy CL, Li YF, El-Gamil M, Schwarz SL, Laurencot C, Rosenberg SA (2011) Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. J Clin Oncol 29(7):917–924. doi:10.1200/JCO.2010.32.2537

Robbins PF, Lu YC, El-Gamil M, Li YF, Gross C, Gartner J, Lin JC, Teer JK, Cliften P, Tycksen E, Samuels Y, Rosenberg SA (2013) Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. Nat Med. doi:10.1038/nm.3161

Rosenberg SA, Dudley ME (2009) Adoptive cell therapy for the treatment of patients with metastatic melanoma. Curr Opin Immunol 21(2):233–240. doi:10.1016/j.coi.2009.03.002

Rosenberg SA, Yang JC, Sherry RM, Kammula US, Hughes MS, Phan GQ, Citrin DE, Restifo NP, Robbins PF, Wunderlich JR, Morton KE, Laurencot CM, Steinberg SM, White DE, Dudley ME (2011) Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. Clin Cancer Res 17(13):4550–4557. doi:10.1158/1078-0432.CCR-11-0116

Stratton MR (2011) Exploring the genomes of cancer cells: progress and promise. Science 331(6024):1553–1558. doi:10.1126/science.1204040

Toebes M, Coccoris M, Bins A, Rodenko B, Gomez R, Nieuwkoop NJ, van de Kasteele W, Rimmelzwaan GF, Haanen JB, Ovaa H, Schumacher TN (2006) Design and use of conditional MHC class I ligands. Nat Med 12(2):246–251. doi:10.1038/nm1360

Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Horn L, Drake CG, Pardoll DM, Chen L, Sharfman WH, Anders RA, Taube JM, McMiller TL, Xu H, Korman AJ, Jure-Kunkel M, Agrawal S, McDonald D, Kollia GD, Gupta A, Wigginton JM, Sznol M (2012) Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 366(26):2443–2454. doi:10.1056/NEJMoa1200690

van Rooij N, van Buuren MM, Philips D, Velds A, Toebes M, Heemskerk B, van Dijk LJ, Behjati S, Hilkmann H, El Atmioui D, Nieuwland M, Stratton MR, Kerkhoven RM, Kesmir C, Haanen JB, Kvistborg P, Schumacher TN (2013) Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. J Clin Oncol. doi:10.1200/JCO.2012.47.7521

Walter S, Weinschenk T, Stenzl A, Zdrojowy R, Pluzanska A, Szczylik C, Staehler M, Brugger W, Dietrich PY, Mendrzyk R, Hilf N, Schoor O, Fritsche J, Mahr A, Maurer D, Vass V, Trautwein C, Lewandrowski P, Flohr C, Pohla H, Stanczak JJ, Bronte V, Mandruzzato S, Biedermann T, Pawelec G, Derhovanessian E, Yamagishi H, Miki T, Hongo F, Takaha N, Hirakawa K, Tanaka H, Stevanovic S, Frisch J, Mayer-Mokler A, Kirner A, Rammensee HG, Reinhardt C, Singh-Jasuja H (2012) Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. Nat Med 18(8):1254–1261. doi:10.1038/nm.2883

Wolchok JD, Hodi FS, Weber JS, Allison JP, Urba WJ, Robert C, O'Day SJ, Hoos A, Humphrey R, Berman DM, Lonberg N, Korman AJ (2013) Development of ipilimumab: a novel immunotherapeutic approach for the treatment of advanced melanoma. Ann N Y Acad Sci 1291:1–13. doi:10.1111/nyas.12180

Wolfel T, Hauer M, Schneider J, Serrano M, Wolfel C, Klehmann-Hieb E, De Plaen E, Hankeln T, Meyer zum Buschenfelde KH, Beach D (1995) A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science 269(5228):1281–1284

Effects of Regulatory T Cell–Dendritic Cell Interactions on Adaptive Immune Responses

Tobias Bopp, Hans Christian Probst, Markus Radsak, Edgar Schmitt, Michael Stassen, Hansjörg Schild, and Stefan Tenzer

Introduction

Organisms control the development of malignant transformation and infection with the help of efficient induction and maintenance of adaptive immune responses. Essential for this induction is the specific recognition of antigenic structures. T cell activation is initiated by the detection of major histocompatibility complex (MHC)-peptide complexes by T cell receptors delivering the so-called signal 1. The degradation of extracellular and intracellular proteins by different proteases in the cytosol, ER, phagolysosomes, or autophagolysosomes generates peptides, which are characteristic for their source proteins. After the peptides are loaded onto MHC molecules in distinct intracellular compartments, the MHC-peptide complexes are transported to the cell surface (Stoltze et al. 2000; Tenzer et al. 2009). Central tolerance controls the recognition of these MHC-peptide complexes resulting in the elimination of high-affinity self-reactive T cells in the thymus. Consequently, adaptive immune responses to self-antigens overexpressed in tumors are restricted to low-affinity T cells (Kuball et al. 2005; Stanislawski et al. 2001).

In addition, surface molecules tightly control adaptive immune responses (signal 2) comprising costimulatory and inhibitory molecules. An important costimulator is CD28, which is expressed on the surface of T cells and crucial for T cell proliferation and IL-2 production. The most prominent inhibitor is the CD28-relative CTLA-4, which is upregulated after T cell activation for up to 3 days, binds CD80 and CD86 molecules with a much higher affinity than CD28 and impairs T cell proliferation, IL-2 production, and IL-2 receptor expression (Walunas et al. 1996). In addition to CD28 and CTLA4, the CD28 superfamily consists of the inhibitory molecule PD1. PD1 interacts with PD-L1 and negatively regulates cytokine production and proliferation of T cells (Freeman et al. 2000). In animal models, the interference with PD1/PD-L1 interactions modulates peripheral tolerance mechanisms and has improved the control of tumor growth and revived exhausted T cells in chronic viral infections (Blank and Mackensen 2007; Probst et al. 2005). BTLA on T cells

All authors contributed equally to this work and are listed in alphabetical order

T. Bopp • H.C. Probst • E. Schmitt • M. Stassen
H. Schild (✉) • S. Tenzer
Institute for Immunology, University Medical Center of the Johannes Gutenberg-University Mainz, 55131 Mainz, Germany

Research Center for Immunology (FZI), University Medical Center of the Johannes Gutenberg-University Mainz, 55131 Mainz, Germany
e-mail: schild@uni-mainz.de

M. Radsak
Research Center for Immunology (FZI), University Medical Center of the Johannes Gutenberg-University Mainz, 55131 Mainz, Germany

III-Medical Clinic, University Medical Center of the Johannes Gutenberg-University Mainz, 55131 Mainz, Germany

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_3,

interacts with the herpesvirus entry mediator (HVEM) and affects important T cell functions like IL-2 production (Watanabe et al. 2003) and has been reported to control inflammatory immune responses (Steinberg et al. 2008). Its role in pathogen- or tumor-specific immune responses is to be studied further. Interestingly, recent findings suggest a different regulation of BTLA expression on tumor versus virus specific T cells (Derré et al. 2010). The MHC class II interacting molecule LAG-3 is upregulated during T cell activation and has been shown to interfere with this process. Thus, LAG-3 expression correlates with defective T cell function observed in chronic infections and inefficient control of tumor growth.

Furthermore and in addition to surface molecules, soluble mediators released by different regulatory cells contribute to the control of T cell activation and the maintenance of peripheral tolerance (signal 3). Regulatory T cells (Tregs) play an important role in this context. In addition to different cytokines (IL-10 and TGF-β), Tregs control the activation of T cells or dendritic cells (DC) in a cell contact-dependent manner by the transfer of the second messenger cAMP (Bopp et al. 2007; Fassbender et al. 2010).

The interplay between inhibitory and activating signals determines the fate of a T cell. Therefore, costimulatory signals prevent T cells recognizing MHC-peptide complexes from becoming anergic, and their nature controls the activation and differentiation of T cells into different subpopulations. Since various T cell populations communicate differently with B cells, signals 2 and 3 subsequently influence the activation of B cells and the nature of the humoral immune response substantially.

The different regulatory mechanisms are critically important and play an essential role for the control and the duration of adaptive immune responses while at the same time preserving peripheral tolerance. Hence, through concerted actions, these regulatory mechanisms allow for the efficient recognition and elimination of malignant or mutated self- and nonself-structures, yet preventing autoimmunity.

Tumor Immunity and Regulatory T Cells

The elimination of "CD4+CD25+ immunoregulatory cells" caused regression of transplanted tumors in a CD8+ T cell-dependent manner (Onizuka et al. 1999). While on the one hand being indispensable for the perpetuation of peripheral tolerance to self-antigens, the immune suppressive properties of Tregs obviously contribute to cancer pathogenesis and progression. Subsequent observations in experimental model systems showed the depletion or functional inactivation of Treg cells by anti-CD25 and/or anti-CD152 (anti-CTLA-4) mAb (Sutmuller et al. 2001) or chemotherapeutic agents (Ercolini et al. 2005) and did not only allow generation of effective tumor-specific cytotoxic T cells but also boosted tumor-specific T cell responses induced by vaccination leading to enhanced protection against tumors. In consecutive clinical work, the occurrence of elevated numbers of Tregs in the blood or tumor tissues of patients has been widely described and is associated with tumor progression at numerous occasions. In an extensive study, an inverse correlation of tumor-infiltrating Tregs and survival of patients with ovarian carcinoma was observed (Curiel et al. 2004). In the presence of Tregs, the tumor tissue produced high levels of CCL22 attracting CCR4-expressing Tregs able to inhibit the proliferation of tumor-specific effector T cells in vitro. Thus, the presence of Tregs at the tumor site might be a major reason why tumor-specific T cells are unable to eradicate their targets despite the fact that they are detectable and systemically functional (Yu et al. 2005).

Treg Mechanisms

There are different suppressive mechanisms known with regard to Tregs inhibiting the function of cells of the immune system (Josefowicz et al. 2012). Several of these mechanisms have been shown to target DC activation. Among them are the inhibitory cell surface molecules CTLA-4 and LAG-3 which suppress via direct interaction

with molecules on the DC surface. In addition to its role in cell autonomous tolerance, CTLA-4 is important for the suppressive function of Tregs (Walker and Sansom 2011). CTLA-4 expressed on Tregs mediates the downregulation or transendocytosis of its ligands, the costimulatory molecules CD80 and CD86, on DCs (Qureshi et al. 2011; Wing et al. 2008), and blocking CTLA-4 in vivo resulted in functional activation of steady-state DCs (Schildknecht et al. 2010). In addition, ligation of B7 molecules by CTLA-4 expressed on Tregs can contribute to the tolerogenic function of steady-state DCs by indoleamine 2,3-dioxygenase (IDO) expression leading to the deprivation of the essential amino acid tryptophan and thus limiting metabolism of immune cells (Fallarino et al. 2003).

LAG-3 expressed on Tregs has been shown to interact with MHC class II molecules on DCs and suppresses DC activation via an ITAM-mediated inhibitory signaling pathway (Liang et al. 2008). LAG-3-mediated suppression was found to depend on antigen-specific recognition, underpinning the necessity of cognate interactions between Tregs and DCs for peripheral tolerance.

In tumor-draining lymph nodes, another mechanism of immunosuppression by Tregs was observed where DCs are killed by Tregs through a perforin-dependent mechanism (Boissonnas et al. 2010). It remains to be proven whether this is a general mechanism of Treg-mediated suppression or a distinctive feature of immune responses to tumors. Coculture experiments suggest that cell contact-dependent suppression of DC by Tregs is a two-step process where the initial formation of Treg-DC aggregates involves the adhesion molecule LFA-1 (Onishi et al. 2008) before active suppression via effectors such as CTLA-4 can occur.

A central molecule for T cell homeostasis and peripheral tolerance is TGF-β (Li and Flavell 2008). In mice, the lack of TGF-β receptor 2 selectively on DCs leads to multiorgan autoimmunity, and DCs fail to induce certain Treg subtypes (Ramalingam et al. 2012). Also, mice in which DCs express a dominant negative TGF-β receptor show enhanced susceptibility to experimentally induced autoimmune encephalitis (Laouar et al. 2008). This indicates that DCs are indeed target of TGF-β-mediated suppression. In addition, deficiency of DCs for integrin αvβ8 which mediates the activation of latent TGF-β results in autoimmunity (Li et al. 2007).

Among many other cells, Tregs can produce TGF-β. Cell contact-dependent suppression by Tregs in vitro could be blocked by antibodies to TGF-β (Nakamura et al. 2001). Furthermore, TGF-β-deficient Tregs were unable to block colitis development upon cotransfer into *Rag1-deficient* mice (Li et al. 2007). Surprisingly, inactivation of TGF-β selectively in FoxP3$^+$ Tregs did not result in any autoimmune phenotype (Gutcher et al. 2011). Thus, while activation of TGF-β on DCs and TGF-β signaling into DCs appears to be critical for peripheral tolerance, it remains to be proven whether it is a mediator of DC suppression by Tregs.

cAMP: A Key Component of Treg Function

Initially, Thornton et al. were able to show in vitro that Treg-mediated suppression of cocultured conventional CD4+ T cells was not cytokine mediated but strictly dependent on cell contact between Tregs and the responder cells (Thornton and Shevach 1998). However, membrane-bound molecules exclusively expressed by Tregs and responsible for mediating this suppression could not be identified. Eventually, we were able to show that the suppression of conventional CD4+ T cells by Tregs is substantially based on a transfer of cAMP via gap junction intercellular communication (GJIC) (Bopp et al. 2007). Recently, this finding was further corroborated by Ring et al. showing that gap junction intercellular communication between Tregs and DC in vivo is essential for the sensitization of CD8+ T cells (Ring et al. 2010).

As an underlying mechanism for this observation, we found that the cAMP-cleaving enzyme phosphodiesterase 3b (PDE3b) is hardly expressed in Tregs when compared to conventional CD4+ T cells. As a consequence, a 20-fold higher intracellular cAMP concentration in Tregs

compared to conventional CD4+ T cells was observed. Interestingly, our further analyses revealed that not only nTregs harbor high amounts of cAMP in their cytosol but also CD4+ T cells contacted by Tregs show increased amounts of intracellular cAMP. The second messenger cAMP was reported to be a potent inhibitor of T cell proliferation and differentiation (Kammer 1988) as well as IL-2 production, a capability that was strictly attributed to Tregs (Thornton and Shevach 1998). This notion provides an explanation for a potential mechanism of Treg function.

Treg Control of DC Function

Dendritic cells (DCs) play a central role in the control and modulation of immune responses. They are the master regulators of T cell responses. During inflammation and cellular stress, DCs undergo a differentiation and maturation process that turns them into potent activators of naïve $CD4^+$ and $CD8^+$ T cells. In the steady state, DCs induce peripheral T cell tolerance. Tregs play a crucial role in controlling the maturation and activation of dendritic cells. While being indispensable for the perpetuation of tolerance to self-antigens, the immune suppressive properties of Tregs contribute to cancer pathogenesis and progression. Thus, understanding Treg interaction with DCs represents a promising therapeutic strategy to control adaptive immunity.

The suppression of conventional T cells by Tregs on a cell-to-cell basis in vitro is well documented. However, in vivo, 2-photon laser scanning microscopy showed an intimate interaction of Tregs with DCs (Tadokoro et al. 2006; Tang et al. 2006). Interestingly, both DCs and T cells are sensitive to cAMP-mediated inhibition. In this context, it was shown that cAMP-elevating agents or cAMP itself is able to considerably counteract TLR-mediated activation of DCs (Galgani et al. 2004). Moreover, by inducing IL-10 expression, cAMP induces a tolerogenic phenotype upon DC activation (Eigler et al. 1998; Hammad et al. 2007).

Elevation of the cytosolic cAMP levels in DCs by Tregs instantly abrogates the costimulatory potency of DCs by impairing the expression of CD80/86 costimulators and promoting the upregulation of B7H3/H4 co-suppressors. Moreover, this cAMP-dependent inhibition is subsequently strengthened and further intensified by Treg-derived IL-10, which inhibits cytokine production (IL-6, IL-12) by DCs (Fassbender et al. 2010). Therefore, Tregs use at least two immune suppressive molecules (cAMP, IL-10) in order to inhibit DCs at multiple levels ultimately leading to an inert and suppressive DC phenotype.

To overcome the suppressive features of Tregs, optimized stimulation of professional antigen-presenting cells might be a choice. This approach, successfully applied by Busch, Fehleisen, and Coley more than a century ago despite the complete lack of knowledge about adaptive immunity and immunosuppression, has been revisited by many groups since the identification of TLRs and their ligands.

As an example, it was demonstrated that co-administration of a TLR ligand bypassed Treg-mediated tolerance of tumor-specific T cells in vivo (Yang et al. 2004). This has been validated in a more systematic approach by Warger et al. showing that DC activation by TLR ligand combinations overruled the Treg-mediated suppression of CD4+ and CD8+ T cell proliferation in vitro (Warger et al. 2006). Thereafter, a detailed analysis of the immunosuppressive features of Tregs has been aided by the development of mice allowing the specific elimination of Tregs expressing the diphtheria toxin receptor under the control of the *Foxp3* promoter (Lahl et al. 2007). The depletion of Treg led to the development of autoimmune-like syndromes. So far, the exact mechanisms by which Tregs execute immunosuppression are still elusive. For the design of efficient T cell-based immunotherapies, a detailed understanding of mechanisms and regulatory networks Tregs are involved in will provide a major advancement.

Outlook

In the past years, numerous mechanisms which contribute or control signals 1–3 have been identified, and it became evident that in cancer and

also chronic infections many of the signals are deviated at comparable levels and add substantially to the manifestation of the disease. Consequently, dysfunctional T cells specific for tumors or viruses have been reported to express PD1, CTLA4, and LAG-3 molecules on their surface. Presently used immunotherapies in cancer patients mainly aim at the induction of specific immune responses and are based on vaccine formulations (e.g., antigens in combination with different adjuvants) and are found to be successful in naïve organisms without preexisting immune deviation. So far, they do not show clinical success. However, recently, the use of antigen-nonspecific strategies (anti-CTLA4 antibodies or IFN-α) in patients with tumors or chronic hepatitis has shown promising results for the first time. This indicates that immunotherapy of cancer and chronic infections is possible and that the correction/modulation/targeting of immune tolerance mechanisms might play a crucial role in the future treatment of these diseases. However, so far, it is unclear to which extent common or disease-specific mechanisms contribute to immune dysfunction in cancer or chronic infection and, as an important consequence, if common or disease-specific therapeutic strategies have to be applied or developed. Therefore, the future challenge is to develop novel therapeutic concepts based on the combination of antigen-specific and antigen-nonspecific strategies.

Acknowledgments The work discussed in this review was supported by the Deutsche Forschungsgemeinschaft and by the Forschungszentrum Immunologie of the government of Rhineland-Palatinate.

References

Blank C, Mackensen A (2007) Contribution of the PD-L1/PD-1 pathway to T-cell exhaustion: an update on implications for chronic infections and tumor evasion. Cancer Immunol Immunother CII 56(5):739–745. doi:10.1007/s00262-006-0272-1

Boissonnas A, Scholer-Dahirel A, Simon-Blancal V, Pace L, Valet F, Kissenpfennig A et al (2010) Foxp3+ T cells induce perforin-dependent dendritic cell death in tumor-draining lymph nodes. Immunity 32(2):266–278. doi:10.1016/j.immuni.2009.11.015

Bopp T, Becker C, Klein M, Klein-Hessling S, Palmetshofer A, Serfling E et al (2007) Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. J Exp Med 204(6):1303–1310. doi:10.1084/jem.20062129

Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P et al (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 10(9):942–949. doi:10.1038/nm1093

Derré L, Rivals J-P, Jandus C, Pastor S, Rimoldi D, Romero P et al (2010) BTLA mediates inhibition of human tumor-specific CD8+ T cells that can be partially reversed by vaccination. J Clin Invest 120(1):157–167. doi:10.1172/JCI40070

Eigler A, Siegmund B, Emmerich U, Baumann KH, Hartmann G, Endres S (1998) Anti-inflammatory activities of cAMP-elevating agents: enhancement of IL-10 synthesis and concurrent suppression of TNF production. J Leukoc Biol 63(1):101–107

Ercolini AM, Ladle BH, Manning EA, Pfannenstiel LW, Armstrong TD, Machiels J-PH et al (2005) Recruitment of latent pools of high-avidity CD8(+) T cells to the antitumor immune response. J Exp Med 201(10):1591–1602. doi:10.1084/jem.20042167

Fallarino F, Grohmann U, Hwang KW, Orabona C, Vacca C, Bianchi R et al (2003) Modulation of tryptophan catabolism by regulatory T cells. Nat Immunol 4(12):1206–1212. doi:10.1038/ni1003

Fassbender M, Gerlitzki B, Ullrich N, Lupp C, Klein M, Radsak MP et al (2010) Cyclic adenosine monophosphate and IL-10 coordinately contribute to nTreg cell-mediated suppression of dendritic cell activation. Cell Immunol 265(2):91–96. doi:10.1016/j.cellimm.2010.07.007

Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H et al (2000) Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 192(7):1027–1034

Galgani M, De Rosa V, De Simone S, Leonardi A, D'Oro U, Napolitani G et al (2004) Cyclic AMP modulates the functional plasticity of immature dendritic cells by inhibiting Src-like kinases through protein kinase A-mediated signaling. J Biol Chem 279(31):32507–32514. doi:10.1074/jbc.M403355200

Gutcher I, Donkor MK, Ma Q, Rudensky AY, Flavell RA, Li MO (2011) Autocrine transforming growth factor-β1 promotes in vivo Th17 cell differentiation. Immunity 34(3):396–408. doi:10.1016/j.immuni.2011.03.005

Hammad H, Kool M, Soullié T, Narumiya S, Trottein F, Hoogsteden HC, Lambrecht BN (2007) Activation of the D prostanoid 1 receptor suppresses asthma by modulation of lung dendritic cell function and induction of regulatory T cells. J Exp Med 204(2):357–367. doi:10.1084/jem.20061196

Josefowicz SZ, Lu L-F, Rudensky AY (2012) Regulatory T cells: mechanisms of differentiation and function. Annu Rev Immunol 30:531–564. doi:10.1146/annurev.immunol.25.022106.141623

Kammer GM (1988) The adenylate cyclase-cAMP-protein kinase A pathway and regulation of the immune response. Immunol Today 9(7–8):222–229. doi:10.1016/0167-5699(88)91220-0

Kuball J, Schmitz FW, Voss R-H, Ferreira EA, Engel R, Guillaume P et al (2005) Cooperation of human tumor-reactive CD4+ and CD8+ T cells after redirection of their specificity by a high-affinity p53A2.1-specific TCR. Immunity 22(1):117–129. doi:10.1016/j.immuni.2004.12.005

Lahl K, Loddenkemper C, Drouin C, Freyer J, Arnason J, Eberl G et al (2007) Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. J Exp Med 204(1):57–63. doi:10.1084/jem.20061852

Laouar Y, Town T, Jeng D, Tran E, Wan Y, Kuchroo VK, Flavell RA (2008) TGF-beta signaling in dendritic cells is a prerequisite for the control of autoimmune encephalomyelitis. Proc Natl Acad Sci U S A 105(31):10865–10870. doi:10.1073/pnas.0805058105

Li MO, Flavell RA (2008) TGF-beta: a master of all T cell trades. Cell 134(3):392–404. doi:10.1016/j.cell.2008.07.025

Li MO, Wan YY, Flavell RA (2007) T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. Immunity 26(5):579–591. doi:10.1016/j.immuni.2007.03.014

Liang B, Workman C, Lee J, Chew C, Dale BM, Colonna L et al (2008) Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. J Immunol 180(9):5916–5926

Nakamura K, Kitani A, Strober W (2001) Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. J Exp Med 194(5):629–644

Onishi Y, Fehervari Z, Yamaguchi T, Sakaguchi S (2008) Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. Proc Natl Acad Sci U S A 105(29):10113–10118. doi:10.1073/pnas.0711106105

Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T, Nakayama E (1999) Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. Cancer Res 59(13):3128–3133

Probst HC, McCoy K, Okazaki T, Honjo T, van den Broek M (2005) Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. Nat Immunol 6(3):280–286. doi:10.1038/ni1165

Qureshi OS, Zheng Y, Nakamura K, Attridge K, Manzotti C, Schmidt EM et al (2011) Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. Science 332(6029):600–603. doi:10.1126/science.1202947

Ramalingam R, Larmonier CB, Thurston RD, Midura-Kiela MT, Zheng SG, Ghishan FK, Kiela PR (2012) Dendritic cell-specific disruption of TGF-β receptor II leads to altered regulatory T cell phenotype and spontaneous multiorgan autoimmunity. J Immunol 189(8):3878–3893. doi:10.4049/jimmunol.1201029

Ring S, Karakhanova S, Johnson T, Enk AH, Mahnke K (2010) Gap junctions between regulatory T cells and dendritic cells prevent sensitization of CD8(+) T cells. J Allergy Clin Immunol 125(1):237–246.e1–7. doi:10.1016/j.jaci.2009.10.025

Schildknecht A, Brauer S, Brenner C, Lahl K, Schild H, Sparwasser T et al (2010) FoxP3+ regulatory T cells essentially contribute to peripheral CD8+ T-cell tolerance induced by steady-state dendritic cells. Proc Natl Acad Sci U S A 107(1):199–203. doi:10.1073/pnas.0910620107

Stanislawski T, Voss RH, Lotz C, Sadovnikova E, Willemsen RA, Kuball J et al (2001) Circumventing tolerance to a human MDM2-derived tumor antigen by TCR gene transfer. Nat Immunol 2(10):962–970. doi:10.1038/ni1001-962

Steinberg MW, Turovskaya O, Shaikh RB, Kim G, McCole DF, Pfeffer K et al (2008) A crucial role for HVEM and BTLA in preventing intestinal inflammation. J Exp Med 205(6):1463–1476. doi:10.1084/jem.20071160

Stoltze L, Schirle M, Schwarz G, Schröter C, Thompson MW, Hersh LB et al (2000) Two new proteases in the MHC class I processing pathway. Nat Immunol 1(5):413–418. doi:10.1038/80852

Sutmuller RP, van Duivenvoorde LM, van Elsas A, Schumacher TN, Wildenberg ME, Allison JP et al (2001) Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. J Exp Med 194(6):823–832

Tadokoro CE, Shakhar G, Shen S, Ding Y, Lino AC, Maraver A et al (2006) Regulatory T cells inhibit stable contacts between CD4+ T cells and dendritic cells in vivo. J Exp Med 203(3):505–511. doi:10.1084/jem.20050783

Tang Q, Adams JY, Tooley AJ, Bi M, Fife BT, Serra P et al (2006) Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. Nat Immunol 7(1):83–92. doi:10.1038/ni1289

Tenzer S, Wee E, Burgevin A, Stewart-Jones G, Friis L, Lamberth K et al (2009) Antigen processing influences HIV-specific cytotoxic T lymphocyte immunodominance. Nat Immunol 10(6):636–646. doi:10.1038/ni.1728

Thornton AM, Shevach EM (1998) CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med 188(2):287–296

Walker LSK, Sansom DM (2011) The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses. Nat Rev Immunol 11(12):852–863. doi:10.1038/nri3108

Walunas TL, Bakker CY, Bluestone JA (1996) CTLA-4 ligation blocks CD28-dependent T cell activation. J Exp Med 183(6):2541–2550

Warger T, Osterloh P, Rechtsteiner G, Fassbender M, Heib V, Schmid B et al (2006) Synergistic activation of dendritic cells by combined Toll-like receptor ligation

induces superior CTL responses in vivo. Blood 108(2):544–550. doi:10.1182/blood-2005-10-4015

Watanabe N, Gavrieli M, Sedy JR, Yang J, Fallarino F, Loftin SK et al (2003) BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. Nat Immunol 4(7):670–679. doi:10.1038/ni944

Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z et al (2008) CTLA-4 control over Foxp3+ regulatory T cell function. Science 322(5899):271–275. doi:10.1126/science.1160062

Yang Y, Huang C-T, Huang X, Pardoll DM (2004) Persistent Toll-like receptor signals are required for reversal of regulatory T cell-mediated CD8 tolerance. Nat Immunol 5(5):508–515. doi:10.1038/ni1059

Yu P, Lee Y, Liu W, Krausz T, Chong A, Schreiber H, Fu Y-X (2005) Intratumor depletion of CD4+ cells unmasks tumor immunogenicity leading to the rejection of late-stage tumors. J Exp Med 201(5):779–791. doi:10.1084/jem.20041684

Evolution of the Regulatory Landscape for Immunomodulatory Compounds and Personalized Therapeutic Cancer Vaccines

Cedrik Michael Britten, Thomas Hinz, Harpreet Singh-Jasuja, and Ulrich Kalinke

Diversity of Cancer Immunotherapies

So far no consistent and unambiguous definition of cancer immunotherapies exists. A plethora of different immunological approaches have been proposed for the treatment of cancer that involves noncellular as well as cellular components of the immune system (Finn 2008). They all have in common therapeutic effects conferred by components of the innate or adaptive immune system. Cancer immunotherapy approaches may range from the use of cytokines, immunoglobulins, monoclonal antibodies and various adjuvants up to vaccines that come in countless formats and are intended to induce antigen-specific B- and T-cell responses. Even *ex vivo* cultured or gene-modified immune cells are being tested in the context of adoptive cell transfer approaches. Although this is certainly not a complete listing of all therapeutic approaches that a given reader may associate with the term cancer immunotherapy, it becomes clear that the sheer number of widely divergent approaches mandate product-specific considerations for their clinical development. Such product-specific aspects should (in the optimal case) be appropriately covered by the existing regulatory landscape in this constantly changing field of medicine.

Active Versus Passive Immunizations

From the regulatory perspective, active immunization is an intervention aiming at the induction of a pathogen-specific immune response that is appropriate to protect against the development of the corresponding infectious disease. This view is reflected in the vaccine definition as presented in the European Community Code, Directive 2001/83/EC where vaccines are defined as agents used to produce active immunity, such as cholera vaccine, BCG, polio vaccines, and smallpox vaccine. Passive immunization strategies substitute active effectors of the immune system, such as immunoglobulins, antibodies, or immune cells. When translating the active versus passive immunization principle that was historically established for infectious diseases to the emerging

C.M. Britten (✉)
Immunotherapy Development Center (IDC), TRON-Translational Oncology, University Medical Center, Johannes Gutenberg University, Mainz, Germany

BioNTech RNA Pharmaceuticals, Mainz, Germany
e-mail: cedrik.britten@tron-mainz.de

T. Hinz
Paul-Ehrlich-Institut (PEI), Langen, Germany

H. Singh-Jasuja
Immatics Biotechnologies GmbH, Tuebingen, Germany

U. Kalinke
Twincore, Centre for Experimental and Clinical Infection Research, A Joint Venture Between the Hannover Medical School and the Helmholtz Centre for Infection Research, Hannover, Germany

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_4,

field of onco-immunology, it seems straightforward that the induction of tumor-specific immune responses after immunization against tumor-associated antigens (= cancer vaccines) should be considered an active immunization strategy. Accordingly, cancer immunotherapies based on the adoptive transfer of immune cells (e.g., T or NK cells) would fall under the passive approach.

Monoclonal Antibodies

The group of monoclonal antibodies (mAbs) need specific considerations because they are classified in three different categories. First, there are antibodies that target antigens expressed on the surface of tumor cells. Herceptin and rituximab are probably the two most commonly known antibodies that more than 10 years ago received marketing authorization both from the FDA and EMA for the treatment of Her2-positive breast cancer or CD20-positive B-cell malignancies, respectively. Although a great part of their therapeutic effects are certainly mediated by immune-effector mechanisms, such as complement-dependent cytotoxicity (CDC) and antibody-dependent cellular-cytotoxicity (ADCC), they probably may not count as "true" cancer immunotherapies, as they do not primarily trigger the immune system but hit tumor cells directly. In contrast the compounds of the second and third mAb categories do not bind directly to tumor cells but "hit" immune cells. Consequently, all effects on the tumor lesion will occur subsequently as an indirect effect of the treatment-induced modulation of the function, phenotype, and number of immune cells. The most prominent examples of the second category are mAbs targeting co-inhibitory molecules on T cells like CTLA-4 or PD-1, thereby "releasing the breaks" on T cells (Korman et al. 2006; Wolchok et al. 2013). The third category of mAbs that modulate the activity of immune cell subsets are antibodies directed against co-stimulatory molecules such as CD40, 4-1BB, or OX-40 (Melero et al. 2007). There is broad consensus that mAbs targeting checkpoint inhibitors or immune-activating receptors should be considered to be "true" cancer immunotherapies. To make things even more complex there is still no consensus which mode of action contributes to what extent to the well-documented therapeutic activity of ipilimumab, which is the most intensively studied immune-modulating mAb. Releasing the breaks of T cells as a rather general nonspecific mode of action, depletion of CTLA-4 positive Tregs, and active induction of mutation-specific T cells are being intensively discussed (Wolchok et al. 2013; van Rooij et al. 2013). Therefore, it might still be a matter of debate whether some mAb approaches can also be referred to as an active immunotherapy. By no means, however, they should be termed cancer vaccines.

Cytokines

Also cytokines require a specific consideration as they play multiple roles in the area of cancer immunotherapy. First, they are often used during the manufacture of cell-based cancer vaccines such as antigen-loaded DCs. In this context, cytokines have to be classified as a raw material which is used during manufacture of a medicinal product. They are normally removed from the cells by several washing steps and thus might only be present in the final product at trace amounts. Secondly, in some cases cytokines are administered concomitantly with cancer vaccines to enhance the desired immune response. For example, the simultaneous administration of GM-CSF and of a therapeutic vaccine helps to attract dendritic cells to the site of vaccine administration. In such a case, the cytokine can be envisaged as an immunomodulator rather than the medicinal product (EMEA/CHMP/VEG/134716/2004). Thirdly, cytokines are also used as a medicinal product in cancer immunotherapy. For example, interferon-alfa-2b (Interferon A) is approved in the EU for the treatment of hairy cell leukemia, chronic myelogenous leukemia, multiple myeloma, follicular lymphoma, carcinoid rumor, and malignant melanoma. Again, as in case of the mAb-based approaches, cytokine treatments obviously do not resemble active cancer immunotherapy.

In summary, the term cancer immunotherapy covers a huge range of different therapeutic interventions. The underlying modes of action can be rather divergent, and they cannot always be determined for one given product. Consequently, regulation of cancer immunotherapies needs to acknowledge the constantly changing state of the art of this broad area of medicine.

Terminology and Some Regulatory Consequences

From the regulatory point of view, it is important to note that available vaccine guidance is almost exclusively dedicated to vaccines against infectious diseases. Notably, the legally binding EU definitions for vaccines also make reference to infectious diseases. For example, the definition provided in the European Pharmacopoeia is as follows: "…Vaccines for human use are preparations containing antigens capable of inducing a specific and active immunity in man against an infecting agent or the toxin or antigen elaborated by it. Immune responses include the induction of the innate and the adaptive (cellular, humoral) parts of the immune system…" (*European Pharmacopoeia* 7th Edition 2012/2013, General Chapter 8, General Monographs – Vaccines for human use (0153)). A less specific definition is given in the "European Drug Law," Directive 2001/83/EC where in Article 1 vaccines, toxins, and serums are defined as "agents used to produce active immunity, such as cholera vaccine, BCG, polio vaccines, smallpox vaccine." In both EU documents, however, a vaccine is clearly defined as a medicinal product against infectious diseases. It is interesting to compare these definitions to the one given in the German Drug Law in Section 4: "Vaccines are […] containing antigens or recombinant nucleic acids and are intended for use in human beings or animals for the production of specific antitoxins and protective agents and, in so far as they contain recombinant nucleic acids, intended exclusively for the prevention or treatment of infectious diseases" (nonofficial translation). Thus, the German vaccine definition is not restricted to infectious diseases. It is remarkable that in case of using recombinant nucleic acids for the prevention of infectious disease, these products are defined as vaccines, not as gene therapy. This definition was included into the German Drug Law as a consequence of EU Directive 2001/83/EC, where in Part IV it is laid down that "Gene therapy medicinal products shall not include vaccines against infectious diseases." In summary, while with regard to the regulatory context in the EU legislation the term "vaccine" is restricted to the treatment or prophylaxis of infectious diseases, the German definition in principle also includes cancer vaccines.

Classifying a drug product as a vaccine will have very practical regulatory consequences. For example, in order to release an authorized vaccine to the German market, official batch release by the competent higher federal authority (Paul-Ehrlich-Institut) is required according to Section 32 of the German Drug Law. Classification also impacts on the expertise required for the so-called Qualified Person (QP) who needs to be installed in companies manufacturing medicinal products. The QP is responsible to certify that each batch of product has been manufactured according to GMP and that each batch will only be released if predefined quality attributes and associated specifications are fulfilled. The QP duties are described in both the German Drug Law and in Annex 16 of the Good Manufacturing Practices (GMP). The QP's expertise required in Germany for blood preparations, sera of human or animal origin, vaccines, allergens, test sera, and test antigens is identical. Of note, a different expertise is required for Advanced Therapy Medicinal Products (ATMP) and remaining medicinal products (Regulation (EC) No. 1394/2007).

Specific Guidelines Available for Cancer Vaccines

Since available definitions for vaccines in the EU restrict the term vaccine to infectious diseases, most, if not all, available vaccine guidelines are not suitable for the development of therapeutic

cancer vaccines. The most obvious difference between therapeutic cancer vaccines and prophylactic vaccines is related to their clinical development. The target population for prophylaxis normally is healthy individuals, in many cases children. In contrast, therapeutic cancer vaccines are intended to treat patients suffering from severe and life-threatening malignancies. This important difference impacts on how the anticipated benefits and risks are balanced. It is evident that during clinical development and at the time of marketing authorization, more risks are in principle acceptable in case of a therapeutic anticancer product. Vice versa, the benefit-risk ratio changes substantially in case of prophylactic vaccines which are characterized by a high benefit expectation and acceptance of only minimal risks.

Owing to these facts, the development of therapeutic cancer vaccines needs to comply with the guidelines dedicated to anticancer medicinal products. The EMA "parent guideline" addressing anticancer medicinal products originally was dedicated to cytotoxic agents, but noncytotoxic agents are meanwhile also included following a revision of the document in 2013 (EMA/CHMP/205/95/Rev.4). In the chapter on immune modulating compounds, the tumor vaccines and immune-activating mAbs are dealt with. An interesting aspect of the guideline is the nonclinical development of cancer vaccines, acknowledging that relevant animal models are often not available. In such cases homologous models, i.e., animal-derived or specific products tested in respective animals, can be used for nonclinical testing. Also, in vitro studies can be conducted to show preclinical proof of concept. As regards the clinical development of cancer vaccines, the guideline discusses several important topics such as the importance of immune monitoring, suitable indications, and the possibility for continued treatment after disease progression.

A further EMA guideline addressing therapeutic cancer vaccines discusses potency testing (EMEA/CHMP/BWP/271475/2006). The guideline indicates that, for example, in vitro lysis of target cells by tumor-specific cytotoxic T cells, in vitro cytokine production, and co-stimulatory capacity of dendritic cells can be considered as potency assays. In cases where the direct measure of potency is not possible or too laborious for routine analyses, surrogates for potency can be used, provided that a correlation between the surrogate and the real biological activity has been demonstrated. Surrogates may comprise expression of cell surface markers or secretion of factors. The reason why such specific guidance for cancer vaccines was published by EMA is that some cancer vaccines belong to the class of somatic cell therapies. The latter, together with gene therapy and tissue engineered products comprise the group of the so-called Advanced Therapy Medicinal Products (ATMP). For ATMPs a dedicated regulatory system has been established during the last years. Among others, the Committee for Advanced Therapies (CAT) has been set up at EMA which is responsible for the scientific review of marketing authorization applications for ATMPs. Based on the result of their review, the CAT will provide a recommendation on whether or not a product can be marketed in the EU. The final decision on that issue has to be taken by the European Commission. Besides a central marketing authorization which is valid for all EU member states, an ATMP in principle can be marketed nationally if the following conditions are fulfilled (Directive 2001/83/EC; Article 3, Nr. 7):

> Any advanced therapy medicinal product […] which is prepared on a nonroutine basis according to specific quality standards, and used within the same Member State in a hospital under the exclusive professional responsibility of a medical practitioner, in order to comply with an individual medical prescription for a custom-made product for an individual patient.

Under these circumstances national marketing of ATMPs is also called the hospital exemption. In Germany the competent authority licensing ATMPs via the hospital exemption is the Paul-Ehrlich-Institute. As soon as the abovementioned conditions are not fulfilled, the respective ATMP needs to be licensed by EMA/European Commission for marketing in the EU.

Overarching guidelines (EMA/CHMP/410869/2006; CPMP/BWP/3088/99) and more

specific ones related to the manufacturing and quality control as well as the nonclinical and clinical development of somatic cell therapy and gene therapy products have been published by EMA. In case a cancer vaccine falls in one of these product classes, the respective guidelines need to be considered.

Toward a Novel Development Framework for onco-immunology

Available "classical" therapies in oncology, such as surgery, chemotherapy, irradiation, or monoclonal antibodies targeting surface-expressed tumor antigens, invariantly exert their effects directly on tumor cells (Fig. 1). In contrast, vaccines and immunomodulatory compounds affect the immune system and induce changes in the frequency, phenotype, and number of immune subsets which are meant to subsequently confer control of tumor growth and thus provide survival benefit for patients. These unique biological features of cancer immunotherapies need to be acknowledged to enable successful clinical development of cancer immunotherapies. Clinical developers of cancer immunotherapies thus need to gain deep understanding of the subtle interplay between the immune system and the tumor. They also should anticipate the impact that stepwise evolving tumor immunity can have on study designs, clinical endpoints, and biomarker assessments. Studies performed by clinical investigators, basic scientists, and drug developers allowed deduction of a new development paradigm for cancer immunotherapies which includes several elements such as harmonized use of methods for measuring immune response as a foundation for immune biomarker development (Britten et al. 2007; van der Burg et al. 2011), improved study

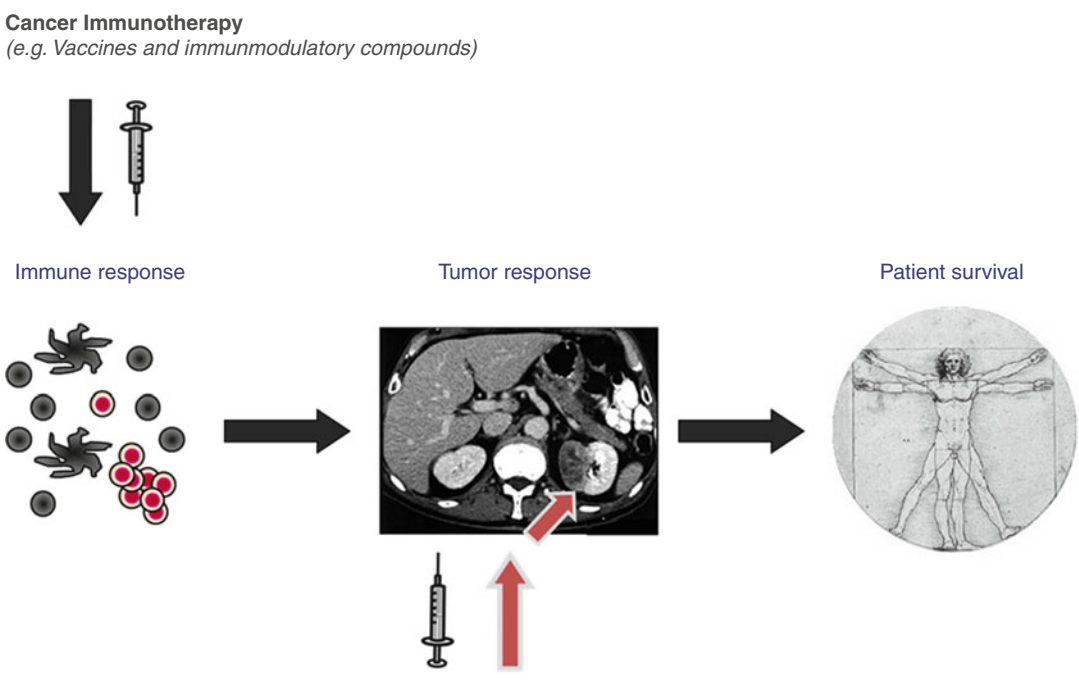

Fig. 1 Classical treatments in oncology versus vaccines and immunomodulatory compounds. Whereas all "classical" therapies in oncology hit the tumor, cancer immunotherapies hit the immune system. This different biological features need to be acknowledged during clinical development

designs (Britten et al. 2012) and clinical endpoints (Hoos et al. 2010; Wolchok et al. 2009), a publication framework for immune monitoring resulting from clinical trials (Britten et al. 2012), and scientific exchange and regulatory interactions to inform guidance document development by regulatory authorities. The described elements constituting a development paradigm for cancer immunotherapy evolved over several years with the participation of all major stakeholders from academia, biotechnology and pharmaceutical industries, and regulatory agencies in the USA and Europe. Both, the US-based Cancer Immunotherapy Consortium as well as the Association for Cancer Immunotherapy were strongly involved in driving this process (Hoos et al. 2011). In 2007, the FDA hosted a workshop where the peculiar features of cancer immunotherapies were reviewed. Subsequently, the agency drafted a guidance document on "Clinical Considerations for Therapeutic Cancer Vaccines" that for the first time constituted a dedicated regulatory document for immunotherapies. The final version of the FDA guidance has become available in late 2011 (FDA 2011). In 2012, the European Medicines Agency (EMA) published a concept paper to stimulate public feedback on a proposed revision of the guidance on "evaluation of anticancer medicinal products in man" (EMA 2010). The revised guidance document including a chapter dedicated to immunomodulatory compounds was finally published in 2012 (EMA 2011).

The gain of knowledge due to recent successes in the field together with a novel and dedicated regulatory framework now supports reproducible and likely more successful development of cancer immunotherapies and lays the foundation for the new clinical subspecialty of immune-oncology.

Individualized Approaches and APVACs and RRG

Genetic heterogeneity is a hallmark of cancer. Distinct genotypes are found for individual tumors and even within single lesions. This heterogeneity and the high diversity of the overall constitution of individual patients demand tailored approaches in tumor therapy. The promise of new approaches categorized as personalized medicine is that such drugs show increased efficacy and reduced adverse effects. Recent studies identified the therapeutic potential of immunogenic tumor mutations in mouse models. For the first time, it has become technologically feasible to integrate data from high-throughput genome sequencing to identify immunogenic mutations and to design therapies tailored to the mutational composition of individual tumor genomes (mutanomes). Translation of genome-based vaccine approaches into human clinical trials is imminent.

This is the introduction of a paper recently published in *Nature Biotechnology* (Britten et al. 2013) by the CIMT Regulatory Research Group (RRG). This publication is the first one describing principles of enhancing personalization of cancer immunotherapies finally leading to the ultimate objective, the development of *actively personalized vaccines* (APVACs). APVACs go far beyond current concepts of personalized medicine, also termed precision medicine. The latter is usually referring to the application of an approved drug to certain patient subgroups who are more likely to profit from the treatment with a given drug. A typical example is Her2/neu, which is expressed in 25 % of breast cancer patients and who are eligible to receive the monoclonal antibody Herceptin®. APVACs are also beyond previous concepts of passive personalization, where an individual cancer vaccine was manufactured for an individual patient based on the processing typically of autologous material, e.g., irradiated tumor lysates being reinjected into patients as a therapeutic vaccine. Though it is known that such tumor lysates do contain tumor-associated antigens (TAA), these TAAs are highly diluted among the vast majority of antigens not relevant for cancer therapy. Additionally, such *passively personalized* immunotherapies have difficulties to be manufactured in a fully reproducible and scalable fashion, making manufacturing and logistics highly expensive. So far only one product of this type has been approved in the EU and the USA (sipuleucel-T) relying on PAP (prostatic acid phosphatase)-loaded autologous dendritic cells. Like *passively personalized* immunotherapies, *actively personalized* immunotherapies including APVACs are also fully personalized,

but the components of APVACs are molecularly defined and can be manufactured fully synthetically, and their design and selection is based on a rational, biomarker-guided approach. While classical personalized medicine seeks patient subgroups fitting to the drug, APVACs take the inverse approach: the drug is fitted or tailored to the patient. APVACs can be based on antigens found highly overexpressed in many different tumors, but not being broadly expressed in a patient population, which would qualify such antigens for an off-the-shelf therapeutic vaccine. More excitingly, APVACs may be based on antigens which were newly created in a given tumor by somatic mutations. Two recent examples for projects translating APVACs to the clinical stage are the European Commission-funded consortia GAPVAC and MERIT.

The Glioma Actively Personalized Vaccines Consortium's (GAPVAC; http://gapvac.eu) goal is to develop highly personalized vaccines for glioblastoma patients. The Melanoma RNA Immuno-Therapy (MERIT; http://merit-consortium.eu) consortium's goal is to develop personalized vaccines for patients with triple-negative breast cancer.

In both consortia, patient that will enter a clinical phase I trial scheduled to start in 2014 will undergo a series of biomarker analyses. The personalized vaccines will be applied in two steps: Step 1 comprises the composition of a set of peptides or RNAs chosen from a pre-manufactured warehouse of tumor-associated lead structures. The goal is to match an antigen with the patient's tumor and immune system and to initiate vaccination treatment as early as possible. Step 2 comprises determination of tumor-specific somatic non-synonymous mutations by next-generation sequencing (NGS) and validation of identified mutations by different methods including Sanger sequencing or mass spectrometry-based analysis. Genomic analysis and mutation selection is followed by on-demand GMP manufacturing of the tailored mutanome vaccine that will become available for treatment following release testing.

Challenges in both highly innovative projects are manifold. The CIMT RRG has specifically looked at regulatory challenges associated with the development of APVACs. CIMT RRG concluded that existing regulatory principles applying to the manufacture and quality control of APVACs overlap in many aspects with those relevant for autologous cell therapies, and thus the development of APVACs may be pursued within the existing regulatory framework of the European Union. To validate this position, the CIMT RRG met with experts of the Innovation Task Force (ITF) of the European Medicines Agency (EMA). Experts from EMA acknowledged RRG's thoughts on several instants as described in the recent RRG publication:

> First, the current regulatory framework of the EU does not specifically address the development of APVACs; second, several aspects of APVAC development differ from the development of existing cancer vaccines, and thus some flexibility in the interpretation of the existing guidance would be needed; and third and most importantly, several regulatory principles already in place for the development of passively personalized vaccines, such as cellular/autologous therapeutics, might also be applicable to APVACs.

In summary, although most of the challenges are still ahead of us, laying out the principles of the regulatory framework for developing such complex personalized therapies is the first groundwork and will now be put into practice by the first consortia engaged in this space. These seem to be exciting times for the field of personalized medicine.

Concluding Remarks

The currently available regulatory landscape for cancer vaccines and immunomodulatory compounds has undergone substantial changes over the last years by constantly incorporating novel findings reflecting progress in science and medicine. With novel technologies enabling even newer concepts of personalized immune interventions, additional challenges have arisen that will surely fuel further evolution of new regulatory documents. The CIMT RRG is committed to continue the discussion on regulatory principles for novel personalized immune interventions. In summary, it is clear that on one hand medical

progress has influenced the development and evolution of dedicated regulatory frameworks. On the other hand, changes in regulation influence the way of how novel compounds will need to be clinically developed. It is the goal of the CIMT RRG to encourage stakeholders in immune-oncology not only to study the immunological mechanisms of tumor control but also to embark into regulatory research to facilitate and enhance the development of novel and highly innovative therapies for patients in a fashion that the medical need is addressed not only marginally but also substantially.

Acknowledgments The CIMT Regulatory Research Group is financially supported by a research grant from the German Ministry for Education and Research (BMBF; Förderkennzeichen 031A018). The authors would like to thank Christoph Huber for his invaluable contributions to the field of cancer immunotherapy and more specifically for being the Spiritus Rector and founder of the Regulatory Research Group of the Association for Cancer Immunotherapy (CIMT).

References

Britten CM, Janetzki S, van der Burg SH, Gouttefangeas C, Hoos A (2007) Toward the harmonization of immune monitoring in clinical trials: Quo vadis? Cancer Immunol Immunother 57:285–288

Britten CM, Janetzki S, Butterfield LH, Ferrari G, Gouttefangeas C, Huber C, Kalos M, Levitsky HI, Maecker HT, Melief CJ, O'Donnell-Tormey J, Odunsi K, Old LJ, Ottenhoff TH, Ottensmeier C, Pawelec G, Roederer M, Roep BO, Romero P, van der Burg SH, Walter S, Hoos A, Davis MM (2012) T-cell assays and MIATA: the essential minimum for maximum impact. Immunity 37:1–2

Britten CM, Singh-Jasuja H, Flamion B, Hoos A, Huber C, Kallen KJ, Khleif SN, Kreiter S, Nielsen M, Rammensee HG, Sahin U, Hinz T, Kalinke U (2013) The regulatory landscape for actively personalized cancer immunotherapies. Nat Biotechnol 31:880–882

European Medicines Agency (EMA) (2010) Concept paper on the need to revise the guideline on the evaluation of anticancer medicinal products in man. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/09/WC500096730.pdf

European Medicines Agency (EMA) (2011) Guideline on the evaluation of anticancer medicinal products in man. EMA/CHMP/205/95/rev.4. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/12/WC500119966.pdf

Finn OJ (2008) Cancer immunology. N Engl J Med 358:2704–2715

Food and Drug Administration (FDA) (2011) Guidance for industry: clinical considerations for therapeutic cancer vaccines. http://www.fda.gov/ucm/groups/fdagov-public/@fdagov-bio-gen/documents/document/ucm278673.pdf

Hoos A, Eggermont AM, Janetzki S, Hodi FS, Ibrahim R, Anderson A, Humphrey R, Blumenstein B, Old L, Wolchok J (2010) Improved endpoints for cancer immunotherapy trials. J Natl Cancer Inst 102:1388–1397

Hoos A, Britten CM, Huber C, O'Donnell-Tormey J (2011) A methodological framework to enhance the clinical success of cancer immunotherapy. Nat Biotechnol 29:867–870

Korman AJ, Peggs KS, Allison JP (2006) Checkpoint blockade in cancer immunotherapy. Adv Immunol 90:297–339

Melero I, Hervas-Stubbs S, Glennie M, Pardoll DM, Chen L (2007) Immunostimulatory monoclonal antibodies for cancer therapy. Nat Rev Cancer 7:95–106

van der Burg SH, Kalos M, Gouttefangeas C, Janetzki S, Ottensmeier C, Welters MJ, Romero P, Britten CM, Hoos A (2011) Harmonization of immune biomarker assays for clinical studies. Sci Transl Med 3:108ps44

van Rooij N, van Buuren MM, Philips D, Velds A, Toebes M, Heemskerk B, van Dijk LJ, Behjati S, Hilkmann H, El AD, Nieuwland M, Stratton MR, Kerkhoven RM, Kesmir C, Haanen JB, Kvistborg P, Schumacher TN (2013) Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. J Clin Oncol 31:e439–e442

Wolchok JD, Hoos A, O'Day S, Weber JS, Hamid O, Lebbe C, Maio M, Binder M, Bohnsack O, Nichol G, Humphrey R, Hodi FS (2009) Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. Clin Cancer Res 15:7412–7420

Wolchok JD, Hodi FS, Weber JS, Allison JP, Urba WJ, Robert C, O'Day SJ, Hoos A, Humphrey R, Berman DM, Lonberg N, Korman AJ (2013) Development of ipilimumab: a novel immunotherapeutic approach for the treatment of advanced melanoma. Ann N Y Acad Sci 1291:1–13. doi:10.1111/nyas.12180. Epub; 2013 Jun 17. 1-13

Immunoguiding, the Final Frontier in the Immunotherapy of Cancer

Sjoerd H. van der Burg, Steffen Walter, Sylvia Janetzki, Cedrik Michael Britten, Jérôme Galon, Marij J.P. Welters, and Cécile Gouttefangeas

Successes in Immunotherapy and Challenges Ahead

Effective immunotherapy of cancer has been practiced for many years. A multitude of therapeutic antibodies targeting tumor cell-surface molecules, growth receptors, or cytokines sustaining tumor growth (Sliwkowski and Mellman 2013) have found their way into current clinical practice. The clinical effects mediated by therapeutic antibodies sometimes require the presence of cells of the innate immune system (e.g., macrophages or NK cells), but essentially not that of the adaptive immunity. For long and despite a constantly growing pile of evidence on the protective role of T cells against cancer (Fridman et al. 2012; Rosenberg 2011), attempts to reinforce this arm have been dismissed by many within the clinical arena. In the last 5 years, however, a series of studies have consequently shown that therapeutic interventions which are based on transfer, activation and expansion, or de-blocking of tumor-specific T cells have met with clinical success (Kenter et al. 2009; Kantoff et al. 2010; Rosenberg 2011; Brahmer et al. 2012; Topalian et al. 2012; Hamid et al. 2013; Wolchok et al. 2013). The fraction of patients responding to these therapies varies widely, but successful responses can be impressive and durable. These outcomes suddenly position reinforcement of the systemic and local tumor-specific T-cell response at the forefront of cancer therapy. In the near future, the road to successfully treat tumors will be paved by different therapies that are currently being developed, many of which need to be combined to achieve the highest response rate. In a nutshell these therapies comprise methods to increase the number of tumor-specific T cells by (1) vaccines (Mocellin et al. 2004; Melief and van der Burg 2008) or by the transfer of ex vivo expanded (engineered) T cells (Restifo et al. 2012), (2) antibodies antagonizing early and late co-inhibitory molecules expressed on T cells and/or agonistic antibodies to amplify T-cell responses via engagement of co-stimulatory molecules (Melero et al. 2013), (3) compounds to

S.H. van der Burg (✉) • M.J.P. Welters
Department of Clinical Oncology, Leiden University Medical Center, Leiden, The Netherlands
e-mail: s.h.van_der_burg@lumc.nl

S. Walter
Immatics Biotechnologies GmbH, Tuebingen, Germany

S. Janetzki
ZellNet Consulting, Inc., Fort Lee, NJ, USA

C.M. Britten
Immunotherapy Development Center (IDC), TRON-Translational Oncology, University Medical Center, Johannes Gutenberg University, Mainz, Germany

BioNTech RNA Pharmaceuticals, Mainz, Germany
e-mail: cedrik.britten@tron-mainz.de

J. Galon
Laboratory of Integrative Cancer Immunology, INSERM, U872, Paris, France

C. Gouttefangeas
Department of Immunology, Institute for Cell Biology, Eberhard-Karls University, Tuebingen, Germany

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_5, © Springer International Publishing Switzerland 2014

relieve T cells from immunosuppressive elements (regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), M2 macrophages) and/or to stimulate tumor-rejecting innate immune cells (M1 macrophages, dendritic cells (DC)) (Gabrilovich et al. 2012; Rakhmilevich et al. 2012; Galluzzi et al. 2012) in order to change the tumor microenvironment. In addition, these immune-activating strategies may be combined with standard chemo(radio)therapy to reduce the tumor load.

Success comes with a downside. Clearly, it will be impossible to combine all therapies. It will be virtually impossible to test each and every possible combination of compounds for potential synergistic clinical effects for one single tumor type, let alone for the many different tumor types and stages which potentially could benefit from immunotherapy. There is simply not enough money nor are sufficient patients available for the high number of clinical trials required to undertake such a huge effort. Furthermore, one may question whether such an approach would lead to success as it becomes increasingly clear that the immune microenvironmental landscape differs between patients even having a tumor from the same cell origin (DeNardo et al. 2011; Sautes-Fridman et al. 2011; de Vos van Steenwijk et al. 2013; Gajewski et al. 2013). These differences are more likely to dictate the type of immunotherapeutic treatment that will be successful. As example, patients with dense T-cell infiltrated tumors may only require co-inhibitory molecule blocking, patients of whom the tumors are infiltrated with high numbers of MDSCs, M2 macrophages, or Tregs may need to be treated with depleting compounds, whereas in other cases, several weapons should be combined. The future will tell, but it is highly likely that treatment options will be determined not only by the tumor type but also by the patient's tumor immune microenvironment.

With this in mind and all the new choices on the horizon, there is an increased pressure to come up with appropriate biomarkers, which (1) provide a rationale for the choice of immunotherapeutic agents and combinations thereof, (2) reflect the in vivo activity of the used compound(s) based on the mechanism of action, and (3) correlate with clinical outcome after treatment so that for each and every patient, the right choice for a reasonable price is made. This process, for which we coined the term immunoguiding (van der Burg 2008), requires the use of immunological assays to identify and validate such biomarkers. Most of these are likely to reflect immunological processes and many will be related to T cells and myeloid cells since the immune-based cancer therapies primarily target these components of the immune system. As will be pointed out below, immunoguiding already started to leave some marks of success, especially in the field of vaccine development where the magnitude and reactivity of antigen-specific T cells are measured. However, for it to fully blossom necessitates solutions to at least two big challenges, the latter of which will be discussed in more detail.

The first challenge concerns the immunological landscape of tumors and comprises the determination of target antigens expressed, HLA expression, magnitude and orientation of the infiltrating T lymphocytes, as well as the presence of further supportive or suppressive immune cells. In order to make a rational choice from the toolbox of immune therapeutics, we need to define robust markers for the different immune cells or for those markers known to bear impact on clinical outcome. Furthermore, there is a requirement for objective scoring methods that are fast and can routinely be used in diagnostic laboratories not only for the immune cells but also for the antigens expressed, as they can form targets for therapeutic vaccines.

The second challenge concerns immunomonitoring of immune cells in a manner not only depending on antigen-specific assays. In particular, in treatments using T-cell immunostimulatory antibodies but also in the setting of certain adoptive T-cell therapies, the specificity of the responding T cells will not be fully known and potentially is unique to each individual patient. Hence, a completely different monitoring strategy is required than currently followed in vaccination studies. These problems will also play a role in the measurement of

other immune cells suppressing or enhancing immunity. A number of them (e.g., Tregs, myeloid cells) are already assessed, but due to the different methodologies and markers currently used in the field, it is difficult to get a clear picture of their impact.

Fundamental to defying these challenges is harmonization. The measurement, identification, and validation of (sets of) markers are not a simple task. There is a great need for a fast track to reliable markers and this requires many laboratories to work together and reach consensus. A relatively quick way to success is the currently ongoing assay harmonization effort. Harmonization is a rational, efficient, and effective mechanism that demonstrably improved the quality of immunological assays and the interpretation of the data in clinical trials (van der Burg et al. 2011).

Here we will discuss the role, success, and challenges in monitoring the immune system in patients with cancer and on immunotherapy, the impact of assay harmonization, and how to boost these in new areas.

Correlating Immune Responses and Clinical Outcome in Immunotherapy Trials

Immune reactivity, in particular the adaptive immune response and tumor immune microenvironment, are two recently recognized hallmarks of cancer (Hanahan and Weinberg 2011). Hence, the reinforcement of immune effector cells to fight cancer has been the focus of immunotherapeutic interventions. The efficacy of such interventions is generally assessed by parameters defining the clinical outcome such as overall or progression-free survival. Clearly, immune responses can be assessed much earlier than these clinical parameters and may be used for immunoguiding purposes during therapy and even before therapy starts in order to stratify patients for maximum treatment benefit (van der Burg 2008; Galon et al. 2013a; Gajewski et al. 2013). Importantly, effective immunoguiding requires reliable sets of immune measurements for each immunotherapeutic strategy. While numerous immune-monitoring assays exist and many are generally well established within the field, various factors can hamper the comparability of results obtained by different laboratories, as recently demonstrated (Janetzki et al. 2008; Britten et al. 2008a). Such factors include, but are not limited to, (1) the actual standard operating procedure (SOP) used; (2) materials, reagents, and equipment applied for testing; (3) sample handling techniques; (4) training and experience of operators; (5) data analysis; and (6) final reporting of study results. Therefore, mechanisms to control variability in biomarker assays are warranted. A tool to do this is standardization of assay protocols across all laboratories but this would be a doomed mission. Alternatively, one may harmonize the protocols between laboratories, an approach that has been well embraced within the field. Harmonization guidelines for MHC-multimer staining, ELISPOT, and gating in flow cytometry were introduced to the field. This has led to improved assay performance, decreased variability across labs (Britten et al. 2008a; Janetzki et al. 2008; Britten et al. 2009; Attig et al. 2011; McNeil et al. 2013; van der Burg et al. 2011), and recommendations for data analysis, as well as web-based analysis tools (Moodie et al. 2010). This ongoing and all steps encompassing harmonization process provides effective tools to immune-monitoring laboratories to adapt their protocol to highest possible efficiency and comparability, fostering the biomarker development and data interpretation within clinical trials. While assay harmonization is an effective mechanism to allow data comparability across laboratories, an assay can only be as good as the sample that is being monitored. To this end, first initiatives are evolving to ensure top quality samples. For example, the Duke Brain Tumor Immunotherapy Program collaborated with multiple SPORE (Specialized Program of Research Excellence) sites to harmonize and standardize PBMC collection, processing, and shipping procedures. Similar efforts were successfully undertaken across Europe as a prelude to a large multicenter vaccine trial in renal cancer patients (Walter et al. 2012).

Whereas the measurement of immune reactivity reflecting the proposed mechanism of action of an immune therapeutic agent is a first step for product development, it is absolutely essential for biomarker identification and immunoguiding to demonstrate a correlation between immune biomarkers and clinical outcome. Recently, various clinically successful immunotherapeutic approaches were reported, which has already led to Food and Drug Administration (FDA) approval for selected treatments, as sipuleucel-T for prostate cancer (Kantoff et al. 2010) and ipilimumab for melanoma (Hodi et al. 2010), based on their effects on patient survival. In contrast to many standard therapies (e.g., chemotherapy or radiation), objective clinical responses are typically delayed under immunotherapy due to the time required for activation and amplification of immune effector mechanisms (Thoren et al. 2013). Hence, the measurement of the early effects of immunotherapy on the immune system has become a recommendation as an early and additional endpoint (Hoos et al. 2010).

An early report of such successful assessment evolved from vaccine trials for high-grade premalignant lesions of the vulva (VIN3) induced by the human papillomavirus type 16 (HPV16). Upon treatment with a therapeutic vaccine consisting of long overlapping peptides covering the complete oncoproteins E6 and E7 of HPV16, a high number of patients with complete or partial regression were observed (Kenter et al. 2009). Clinical success was associated with a significantly larger magnitude and breadth of response by vaccine-induced IFNγ-producing T cells as well as higher IFNγ and IL-5 cytokine production levels, revealing that not only the frequency but also the polarization of T cells were related to clinical efficacy (Welters et al. 2010). Importantly, for the applied immune-monitoring assays, harmonization guidelines were implemented when relevant.

A second example is sipuleucel-T, a vaccine consisting of autologous PBMCs loaded with a fusion protein of prostatic acid phosphatase and GM-CSF for treatment of patients with castration-resistant prostate cancer. Immune cell activation as well as specific T-cell and B-cell responses could be demonstrated in a significant group of patients receiving the vaccine and correlated with overall survival (Sheikh et al. 2013). Again, immunomonitoring was based on internally validated and externally harmonized T-cell assays.

A third example is a report on two trials in which renal cell cancer patients were treated with IMA901, a therapeutic vaccine consisting of multiple tumor-associated peptides (TUMAPs) (Walter et al. 2012). In a phase 1 trial, immune responses to multiple TUMAPs measured by assays following established harmonization guidelines correlated with improved disease control, and this notion was confirmed in the subsequent phase 2 study by a longer survival of patients with such immune responses.

While most immune response assessments are performed using peripheral blood samples, some studies employ other cell sources. The presence of tumor-specific skin-infiltrating T cells, responding to intradermal injection with tumor antigen-pulsed DC and able to recognize naturally processed antigen after their isolation, were associated with a strongly improved overall survival of patients with melanoma who had been vaccinated with tumor antigen-loaded DC (Aarntzen et al. 2012; de Vries et al. 2005). The skin test was suggested to integrate multiple aspects of cellular functions required for effective immunity in melanoma.

But also out of the context of immunotherapy, there are studies employing immunomonitoring assays for which harmonization guidelines exist and of which the results show correlations with clinical benefit. In patients with advanced melanoma, endogenous T-cell responses against melan-A and NY-ESO-1, as assessed by intracellular cytokine staining in blood samples, had a strong prognostic impact on survival. Impressively, the median survival of patients with a response against one or both peptide antigens was 21 months, compared to 6 months for those without a response (Weide et al. 2012). Furthermore, in NY-ESO-1-positive metastatic melanoma patients, the integrated immune responses to NY-ESO-1 provided a predictive value for ipilimumab treatment. Patients with existing antibody and CD8+ T-cell responses

against NY-ESO-1 experienced a clinical benefit and significant survival advantage as compared to those without such responses (Yuan et al. 2011). In untreated breast cancer patients, measurement of tumor-specific T-cell responses in the bone marrow and intratumoral multiplex analysis were performed. Preexisting T-cell responses correlated with more favorable pathologic tumor characteristics and reduced cancer mortality risk (Domschke et al. 2009).

Many of the studies that successfully demonstrate a correlation between vaccine- or spontaneously induced immune responses and clinical benefits are based on well-established immuneassays, which underwent external validation and followed harmonization guidelines for optimal performance. However, we are still in need for larger data sets in order to establish reliable biomarkers that can be used for broad application of immunoguiding.

Beyond Monitoring of Vaccine-Specific T Cells

It is relatively straightforward to monitor T cells specific for antigen(s) used in vaccines. In other situations, there may be still a strong rationale to monitor T cells, as they are part of the known or expected mechanism of action. In the following, we will discuss four examples and the associated methods that may be available to address this challenge.

First, there is accumulating evidence that the immune system has a major impact on the prognosis of cancer patients (Galon et al. 2006; Fridman et al. 2012). The patients in most of these studies were treated with current standard of care, which were mostly chemotherapy and/or a targeted therapy. Based on these data, the view was expressed that "most, if not all, chemotherapies are immunotherapies." Although the densities, phenotype, and location of tumor-infiltrated lymphocytes (TILs) could be determined systematically, the antigens recognized by in situ prognostic T cells remain largely unknown. It could be hypothesized that the prognostic value of these naturally occurring immune responses in cancer patients would be even higher if the relevance of the recognized antigens would be known. One option is to test if the T cells respond to known tumor antigens. In one publication, high levels of tumor-specific T cells in the peripheral blood were reported to be prognostic (Weide et al. 2012). Such approaches will be facilitated in the future by novel tools that are available to monitor many T-cell specificities at the same time such as multidimensionally encoded MHC-multimers (Hadrup et al. 2009; Newell et al. 2009), but the main difficulty remains that such trial-and-error approaches are dwarfed by the number of potentially relevant epitopes which is much larger than the number of epitopes that can be experimentally tested. An alternative, more systematic approach employs screening of tumor-cDNA libraries with patient-derived T-cell clones (Lennerz et al. 2005), which is difficult to scale to large studies.

Second, the last years have witnessed a great interest in non-vaccine cancer immunotherapies, some of which have already reached the market or are in late-stage clinical development. These included the before-mentioned immunostimulatory antibodies and compounds to relieve T cells from immunosuppressive elements and alter the hostile local milieu for T cells. In these cases, a central role of activated T cells is clear from both theory and supportive animal models. The specificity of the T cells in patients is unfortunately largely unknown, which limits the rational biomarker-guided development of these drugs. In a few cases, however, potentially relevant epitopes for these types of immunotherapies have been identified by trial-and-error testing of known targets (Yuan et al. 2011) or by exploiting the cancer mutanome via next-generation sequencing, prediction of epitopes spanning those mutations, and confirmation by immunoassays (van Rooij et al. 2013).

Third, the therapeutic potential of autologous T cells, by means of adoptive cellular therapy of ex vivo expanded naturally occurring tumor-specific T cells in tumors or PBMCs, has been demonstrated over the last decade by several research groups (Dudley et al. 2002; Radvanyi et al. 2012; Verdegaal et al. 2011). Here too, the

nature of the antigen(s) recognized by reinfused T cells remains unknown in most cases, making it difficult to derive predictive markers for the success of adoptive T-cell therapy in a given patient. Approaches that aimed to decipher the TIL repertoire based on trial-and-error testing of known antigens could only account for a small fraction of TILs (Andersen et al. 2012). A more recent study reported the specificity for large fractions of cells within TIL samples, notably again by using next-generation sequencing of the cancer mutanome, prediction, and subsequent immunoassay confirmation of epitopes spanning those mutations (Robbins et al. 2013).

Fourth, even in the case of active immunotherapy using defined cancer vaccines, there is potential for epitope spreading of the immune response toward antigens not present in the vaccine. It is not yet clear whether this is an epiphenomenon or may be causally related to efficacy of immunotherapy. The relatively low magnitude of vaccine-specific T-cell responses described in many studies, which can nevertheless be associated with clinical benefit (Walter et al. 2012), may indirectly point to a role for epitope spreading. There is even direct evidence (Lurquin et al. 2005; Corbiere et al. 2011) that epitope spreading may be causally related to the efficacy of active immunotherapy. In the latter two studies, the relevant spreading antigens (which were again derived from somatic tumor-specific mutations) were identified by screening large cDNA libraries with T-cell clones.

Finally, a new and exciting methodological prospect to characterize the immune repertoire in cancer patients is the use of highly parallel next-generation sequencing of T-cell receptor (TCR) CDR3 regions (Freeman et al. 2009; Warren et al. 2011). With this method, it is possible to quantitate TCR clonotypes from inexpensive total blood DNA or RNA and even from archived formaldehyde-fixed paraffin tissues. Combined with enrichment of clonotypes of interest (Klinger et al. 2013), for example, by mixed lymphocyte-tumor culture, this method may complement existing antigen-specific approaches as it provides a comprehensive insight into the overall immune repertoire over time and at different sites.

Immunoscoring the Intratumoral T-Cell Response Predicts Patient Survival

The most common system for classifying the extent of spread of cancer is the TNM classification. This classification has been used for over 80 years and has been shown to be valuable in estimating the outcome of patients for a variety of cancers (Locker et al. 2006; Weitz et al. 2005) as well as to select patients for inclusion in clinical trials (Nagtegaal et al. 2012). While this approach is powerful, TNM classification provides incomplete prognostic information as the clinical outcome can dramatically vary among patients within the same histological tumor stage (Nagtegaal et al. 2012). The predictive accuracy of this traditional staging system still relies on the assumption that disease progression is largely a tumor cell-autonomous process and fails to incorporate the effects of the host immune response (Bindea et al. 2010). Still, albeit imperfect, the TNM classification was never surpassed in multivariate analysis by alternative methods such as immunohistochemistry for tumor biomarkers, flow cytometry for DNA content, molecular signatures, or genetic features. Until recently, when it became clear that the analysis of a specific type of intratumoral immune response, by a test called "Immunoscore," outperformed TNM classification in multivariate analysis (Galon et al. 2006; Mlecnik et al. 2011). As a result, tumor progression is now considered to be an imbalance between an invasive tumor process and a defense system whose major component is constituted by the host immune response (Galon et al. 2013a).

The immune contexture of a tumor, defined by the number, type, functional orientation, and location of immune infiltrates in primary tumors that are prognostic for the disease-free survival (DFS) and the overall survival (OS) (Galon et al. 2006; Mlecnik et al. 2011; Pages et al. 2005; Fridman et al. 2012; Galon et al. 2007; Bindea et al. 2013), was particularly described in patients with colorectal cancer, but the beneficial impact of the immune infiltrate comprising cytotoxic and memory T cells has been demonstrated also for cancers from diverse anatomical sites, including

melanoma; lung, gastric, esophageal, head and neck, breast, bladder, urothelial, ovarian, cervical, prostatic, and pancreatic cancer; hepatocellular carcinoma; medulloblastoma; and Merkel cell carcinoma (Fridman et al. 2012). Notably, the impact of immune infiltration extends to different cancer cell types (adenocarcinoma, squamous cell carcinoma, large cell cancer, and melanoma). An important aspect precluding the use of immune infiltration data in the clinical setting was the method used to determine the impact of the infiltrate, predominantly based on a statistical grouping of patients in low and high responders within the group analyzed. Broad application requires a scoring method that can objectively be applied in any cancer center. This hurdle has potentially been solved by the introduction of a scoring system designated "Immunoscore" (Angell and Galon 2013), which is based on the numeration of two lymphocyte populations both in the core of the tumor (CT) and in the invasive margin (IM) of tumors, as a clinically useful prognostic marker in colorectal cancer (Pages et al. 2009). The Immunoscore provides a score ranging from Immunoscore 0 (I0), when low densities of both cell types are found in both regions, to Immunoscore 4 (I4), when high densities are found in both regions. Current immunohistochemical technologies allow the application of such analyses in routine diagnostic pathology. Thus, considering the probable universal character of the immune control of tumors, it is essential for the patients to take into account the immune parameter as a prognostic factor and to introduce the Immunoscore as a component of cancer classification (Bindea et al. 2010; Angell and Galon 2013).

Clinical validation of the Immunoscore with standardized/harmonized procedures is necessary to reach clinical applicability for individual patients. To this end, multiple Immunoscore quality controls were performed to test the accuracy and repeatability of the method. Currently, a prospective multicenter national study to assess the use of the Immunoscore in clinical practice is tested with 600 colorectal cancer patients from 7 hospitals. Moreover, to promote the utilization of the Immunoscore in routine clinical settings, a worldwide Immunoscore consortium was erected (Galon et al. 2012a, b). This consortium, composed of international expert pathologists and immunologists, identified a strategy for the organization of worldwide retrospective study for the validation of the Immunoscore in colon cancer by scoring several thousands of tumors. Twenty-three international pathology expert centers, representing countries in Asia, Europe, North America, Australia, and the Middle East, are participating with the aim to implement the Immunoscore – when successful – as a new component for the classification of cancer TNM-I (Immune)(Galon et al. 2013b). This test has a dual advantage: firstly, it serves as a prognostic factor and secondly because of its biological meaning it will provide tools and targets for novel (immuno)therapeutic approaches.

Monitoring More Than Just Effector T Cells in the Tumor Microenvironment

Within the tumor microenvironment, the magnitude and functional capacity of effector T-cell responses are shaped by the cancer cells themselves and by a variety of infiltrating immune cell subsets belonging to the innate or adaptive immunity. Of particular significance are immune suppressive cells which may impair T-cell recruitment, differentiation, and function, as highlighted by many reports published in the recent years (Gabrilovich et al. 2012; Nishikawa and Sakaguchi 2010). In short, the rationale to monitor suppressive cells in cancer patients is based on two fundamental observations: (1) Immunosuppressive cells (Foxp3+ Tregs, MDSCs, M2 macrophages) are found at increased proportions in the blood and/or tumors of patients with various cancers as compared to healthy donors (Woo et al. 2002; Nishikawa and Sakaguchi 2010; Attig et al. 2009; Walter et al. 2012; Piersma et al. 2007) and (2) the number of intratumoral Tregs, M2 macrophages, and blood MDSCs often inversely correlates with patient survival (Curiel et al. 2004; Fridman et al. 2012; Jordanova et al. 2008; Solito et al. 2011; Heusinkveld and van der Burg 2011).

In the context of immunotherapy, measuring of suppressive cells (Tregs, MDSCs, possibly M2 macrophages) may be therefore relevant both prior to (as a predictive biomarker in order to assess the level of immunosuppression and to assist therapy decision) and during treatment (as a pharmacodynamic biomarker). This step forward has already been taken (Olson and McNeel 2013). In the phase I study of multipeptide vaccination for mRCC mentioned above, it was observed that low pretreatment levels of blood Foxp3+ Tregs were associated with broader anti-vaccine T-cell responses and enhanced disease control. In the subsequent phase II trial, overall survival of patients was improved if they received the immune modulator cyclophosphamide for Treg elimination before vaccination and developed vaccine-specific T cells. At the same time, overall survival was negatively correlated with the numbers of several MDSC subsets before treatment, suggesting that elimination of both Tregs and MDSCs may further improve vaccine efficacy (Walter et al. 2012). In patients with castrate-resistant metastatic prostate cancer (mPCa) treated with a poxvirus-based tumor vaccine, enhanced suppressive function, but not the mere number of peripheral Tregs posttreatment, was associated with shortened overall survival (Gulley et al. 2010). Last but not least, the group of patients who failed to display a complete response after HPV16 long peptide vaccination displayed a higher HPV-specific Treg frequency and a lower frequency of HPV16-specific effector cells after vaccination than patients having a complete regression of their lesion (Welters et al. 2010).

As our knowledge progresses new hurdles accumulate on the horizon. Notably, the populations of suppressive T cells, tumor-associated macrophages, and MDSCs are heterogeneous. Regulatory CD4+ T cells with suppressive properties can be natural or adaptive Foxp3-positive cells, Foxp3-negative type 1 Tregs (Tr1), or T-helper type 3 (Th3) cells, with different differentiation pathways and suppression mechanisms (Curotto de Lafaille and Lafaille 2009; Miyara et al. 2009). MDSCs may be of monocytic (CD14-positive) or granulocytic (CD15-positive) origin and come with multiple described phenotypes (Montero et al. 2012) similarly as was earlier reported for macrophages (Gabrilovich et al. 2012; Heusinkveld and van der Burg 2011). Depending on the local milieu, these immune cells can change their functional properties (Zhou et al. 2009; Sakaguchi et al. 2013; Gordon and Taylor 2005; Gabrilovich et al. 2012), a plasticity which may greatly complicate monitoring of these immune cells. Moreover, no consensus exists so far on surface or intracellular markers that should be used for identifying these various suppressive immune cells. For example, Foxp3 has been described in certain effector T cells while not all suppressive T cells do express this transcription factor (Wang et al. 2007; Curotto de Lafaille and Lafaille 2009; van der Burg et al. 2007). Although antibody panels have been proposed for staining Tregs (Murdoch et al. 2012), no standard phenotypes have been yet defined, and many investigators use a combination of between 3 and 7 antibodies. The picture is even more complex when it comes to the identification and measurement of MDSCs and macrophages. At the same time, discussions are ongoing whether an in vitro inhibition assay is necessary and sufficient to define regulatory subsets and which assay(s) should be recommended. At present, various assays are employed within the community. This heterogeneity in the techniques applied certainly impedes progression in the field. Once harmonization has been reached for the detection of suppressive cells by rationally choosing a set of minimal and necessary markers and assays, this will enable a better understanding of the relevance of each suppressive subset for immunotherapy. The Association for Cancer Immunotherapy's (CIMT) Immunoguiding Program (CIP; http://www.cimt.eu/workgroups/cip) is currently undertaking these endeavors. It can be predicted that the list of immune cell subsets which needs to be included as relevant biomarkers for cancer immunotherapy alone or in combination, will expand in the coming years.

Boosting Harmonization Efforts

In 2005, large-scale proficiency panel programs for the most commonly applied T-cell immunoassays were initiated by the CIP and the Cancer

Immunotherapy Consortium of the Cancer Research Institute (Britten et al. 2008b). The results generated in more than 25 independent panels in which so far more than 150 labs worldwide participated impressively showed that results generated across institutions varied and even variation within one lab tended to be higher as known for analytical tests of soluble analytes (Janetzki et al. 2009) . In subsequent panels, the critical assay steps having an impact on the assay performance were defined, and harmonization guidelines for ELISPOT, ICS, and MHC-multimers were derived (Janetzki et al. 2008; Britten et al. 2008a, 2009; Mander et al. 2010; Attig et al. 2011). Notably, variation was not only driven by the assay itself but also by the way how data was analyzed and processed (Moodie et al. 2012). In particular, results from flow-based assays were shown to critically depend on the gating strategy (Welters et al. 2012; McNeil et al. 2013). CIP has recently initiated a project to develop an algorithm for automated detection of rare antigen-specific T cells (Cron et al. 2013), in order to boost the harmonized analysis of flow data sets using computer software.

In addition to providing harmonization guidelines, the two networks characterized the assay performance (e.g., background spot production in ELISPOT or replicate variation) typically observed in heterogeneous groups of labs that can now be used as benchmarks (Moodie et al. 2010). Finally, the CIP developed cellular reference samples based on TCR-engineered lymphocytes that deliver predefined and reproducible signals and can be employed to control immune assay performance over time (Singh et al. 2013). Using a kit-based approach, CIP is now in the process of developing a robust and scalable RNA TCR technology that may enable the broad community to manufacture reference samples on demand as assay controls (Bidmon et al. 2014).

The benefits from assay harmonization derive from offering a complementary framework consisting of (1) external control of performance in proficiency panels, (2) harmonization guidelines, (3) benchmarks of assay performance, and (4) reference sample technology. The joint harmonization efforts of both CIP and CIC efficiently tackled major issues in the field of cellular immune monitoring that impede their broad adoption in clinical immunology. In summary, harmonization provides tools that support the development and use of immune assay in biomarker programs and thus guide the clinical development of new immunotherapies (van der Burg 2008; van der Burg et al. 2011). It needs to be emphasized that assay harmonization efforts cannot replace the need to develop, standardize, performance-control, qualify, and (if applicable) validate immunoassays prior to use in every single test facility (Kalos 2010).

As alluded to earlier, the results generated in the proficiency panels allowed defining assay variables impacting on the test results. Similar conclusions were made by independent efforts from the human immunophenotyping (HIP) consortium (Maecker et al. 2010). Despite the awareness of the most critical assay steps, most material and method parts published in peer-reviewed scientific journals still lack basic information on how the results from T-cell assays were generated (Janetzki et al. 2009). As a reaction, a field-wide consensus effort to identify the minimal information that should be provided about T-cell assays was initiated in 2008 (Janetzki et al. 2009; Britten et al. 2011). The outcome of the Minimal Information About T-cell Assays (MIATA) project was a consolidated and well-vetted guideline that is now available for use and adoption (Britten et al. 2012). Recently, the project website was relaunched and now offers templates for easy reporting that will not only help authors to speedup writing materials and method parts but in parallel introduce MIATA-compliant structure (http://www.miataproject.org). MIATA has already been endorsed by various journals.

Conclusion

The current clinical successes in immune-based cancer treatments demand biomarkers to guide their application and for new developments. This has resulted in a rapid growth of immunomonitoring activities, with increasing numbers of assays and immune cells to analyze. Furthermore, experts in the field are

continuously professionalizing their activities via large-scale proficiency panels, workshops, and by research and development activities, including setting up and establishing automated algorithms for analysis of flow data as well as the generation of reference samples. CIP as a working group of the Association for Cancer Immunotherapy will continue to support the development of robust immunological biomarkers to guide the development of new therapeutic compounds. There are still many challenges ahead, but based on current successes, there is no doubt that ongoing collaborative efforts struggling to solve these problems, in the end, will pay off.

Acknowledgments The CIP has been financially supported by a research grant from the Wallace Coulter Foundation (Florida, USA).

References

Aarntzen EH, Bol K, Schreibelt G, Jacobs JF, Lesterhuis WJ, Van Rossum MM, Adema GJ, Figdor CG, Punt CJ, De Vries IJ (2012) Skin-test infiltrating lymphocytes early predict clinical outcome of dendritic cell-based vaccination in metastatic melanoma. Cancer Res 72(23):6102–6110. doi:10.1158/0008-5472.CAN-12-2479

Andersen RS, Thrue CA, Junker N, Lyngaa R, Donia M, Ellebaek E, Svane IM, Schumacher TN, Thor Straten P, Hadrup SR (2012) Dissection of T-cell antigen specificity in human melanoma. Cancer Res 72(7):1642–1650. doi:10.1158/0008-5472.CAN-11-2614

Angell H, Galon J (2013) From the immune contexture to the Immunoscore: the role of prognostic and predictive immune markers in cancer. Curr Opin Immunol 25(2):261–267. doi:10.1016/j.coi.2013.03.004

Attig S, Hennenlotter J, Pawelec G, Klein G, Koch SD, Pircher H, Feyerabend S, Wernet D, Stenzl A, Rammensee HG, Gouttefangeas C (2009) Simultaneous infiltration of polyfunctional effector and suppressor T cells into renal cell carcinomas. Cancer Res 69(21):8412–8419. doi:10.1158/0008-5472.CAN-09-0852

Attig S, Price L, Janetzki S, Kalos M, Pride M, McNeil L, Clay T, Yuan J, Odunsi K, Hoos A, Romero P, Britten CM, Group C-CAW (2011) A critical assessment for the value of markers to gate-out undesired events in HLA-peptide multimer staining protocols. J Transl Med 9:108. doi:10.1186/1479-5876-9-108

Bidmon N, Attig S, Schroeder H, Rae R, Omoko T, Simon P, Kuhn A, Kreiter S, Sahin U, Gouttefangeas C, van der Burg SH, Britten CM (2014) Destigmatizing T-cell assays by continuous and tight control of performance (in press)

Bindea G, Mlecnik B, Fridman WH, Pages F, Galon J (2010) Natural immunity to cancer in humans. Curr Opin Immunol 22(2):215–222. doi:10.1016/j.coi.2010.02.006

Bindea G, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M, Obenauf AC, Angell H, Fredriksen T, Lafontaine L, Berger A, Bruneval P, Fridman WH, Becker C, Pages F, Speicher MR, Trajanoski Z, Galon J (2013) Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. Immunity 39(4):782–795. doi:10.1016/j.immuni.2013.10.003

Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsi K, Pitot HC, Hamid O, Bhatia S, Martins R, Eaton K, Chen S, Salay TM, Alaparthy S, Grosso JF, Korman AJ, Parker SM, Agrawal S, Goldberg SM, Pardoll DM, Gupta A, Wigginton JM (2012) Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med 366(26):2455–2465. doi:10.1056/NEJMoa1200694

Britten CM, Gouttefangeas C, Welters MJ, Pawelec G, Koch S, Ottensmeier C, Mander A, Walter S, Paschen A, Muller-Berghaus J, Haas I, Mackensen A, Kollgaard T, Thor Straten P, Schmitt M, Giannopoulos K, Maier R, Veelken H, Bertinetti C, Konur A, Huber C, Stevanovic S, Wolfel T, van der Burg SH (2008a) The CIMT-monitoring panel: a two-step approach to harmonize the enumeration of antigen-specific CD8+ T lymphocytes by structural and functional assays. Cancer Immunol Immunother 57(3):289–302

Britten CM, Janetzki S, van der Burg SH, Gouttefangeas C, Hoos A (2008b) Toward the harmonization of immune monitoring in clinical trials: Quo vadis? Cancer Immunol Immunother 57(3):285–288

Britten CM, Janetzki S, Ben-Porat L, Clay TM, Kalos M, Maecker H, Odunsi K, Pride M, Old L, Hoos A, Romero P, Group HL-pMPPotC-CIAW (2009) Harmonization guidelines for HLA-peptide multimer assays derived from results of a large scale international proficiency panel of the Cancer Vaccine Consortium. Cancer Immunol Immunother 58(10):1701–1713. doi:10.1007/s00262-009-0681-z

Britten CM, Janetzki S, van der Burg SH, Huber C, Kalos M, Levitsky HI, Maecker HT, Melief CJ, O'Donnell-Tormey J, Odunsi K, Old LJ, Pawelec G, Roep BO, Romero P, Hoos A, Davis MM (2011) Minimal information about T cell assays: the process of reaching the community of T cell immunologists in cancer and beyond. Cancer Immunol Immunother 60(1):15–22. doi:10.1007/s00262-010-0940-z

Britten CM, Janetzki S, Butterfield LH, Ferrari G, Gouttefangeas C, Huber C, Kalos M, Levitsky HI, Maecker HT, Melief CJ, O'Donnell-Tormey J, Odunsi K, Old LJ, Ottenhoff TH, Ottensmeier C, Pawelec G, Roederer M, Roep BO, Romero P, van der Burg SH, Walter S, Hoos A, Davis MM (2012) T cell assays and MIATA: the essential minimum for maximum impact. Immunity 37(1):1–2. doi:10.1016/j.immuni.2012.07.010, S1074-7613(12)00291-9 [pii]

Corbiere V, Chapiro J, Stroobant V, Ma W, Lurquin C, Lethe B, van Baren N, Van den Eynde BJ, Boon T, Coulie PG (2011) Antigen spreading contributes to MAGE vaccination-induced regression of melanoma metastases. Cancer Res 71(4):1253–1262. doi:10.1158/0008-5472.CAN-10-2693

Cron A, Gouttefangeas C, Frelinger J, Lin L, Singh SK, Britten CM, Welters MJ, van der Burg SH, West M, Chan C (2013) Hierarchical modeling for rare event detection and cell subset alignment across flow cytometry samples. PLoS Comput Biol 9(7):e1003130. doi:10.1371/journal.pcbi.1003130

Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, Zou W (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 10(9):942–949

Curotto de Lafaille MA, Lafaille JJ (2009) Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? Immunity 30(5):626–635. doi:10.1016/j.immuni.2009.05.002

de Vos van Steenwijk PJ, Ramwadhdoebe TH, Goedemans R, Doorduijn EM, van Ham JJ, Gorter A, van Hall T, Kuijjer ML, van Poelgeest MI, van der Burg SH, Jordanova ES (2013) Tumor-infiltrating CD14-positive myeloid cells and CD8-positive T-cells prolong survival in patients with cervical carcinoma. Int J Cancer 133(12):2884–2894. doi:10.1002/ijc.28309

de Vries IJ, Bernsen MR, Lesterhuis WJ, Scharenborg NM, Strijk SP, Gerritsen MJ, Ruiter DJ, Figdor CG, Punt CJ, Adema GJ (2005) Immunomonitoring tumor-specific T cells in delayed-type hypersensitivity skin biopsies after dendritic cell vaccination correlates with clinical outcome. J Clin Oncol 23(24):5779–5787

DeNardo DG, Brennan DJ, Rexhepaj E, Ruffel B, Shiao SL, Madden SF, Gallagher WM, Wadhwani N, Junaid SA, Rugo HS, Hwang ES, Jirstrom K, West BL, Coussens LM (2011) Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. Cancer Discover 1:54–67. doi:10.1158/2159-8274

Domschke C, Schuetz F, Ge Y, Seibel T, Falk C, Brors B, Vlodavsky I, Sommerfeldt N, Sinn HP, Kühnle MC, Schneeweiss A, Scharf A, Sohn C, Schirrmacher V, Moldenhauer G, Momburg F, Beckhove P (2009) Intratumoral cytokines and tumor cell biology determine spontaneous breast cancer-specific immune responses and their correlation to prognosis. Cancer Res 69(21):8420–8. doi:10.1158/0008-5472.CAN-09-1627

Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, Topalian SL, Sherry R, Restifo NP, Hubicki AM, Robinson MR, Raffeld M, Duray P, Seipp CA, Rogers-Freezer L, Morton KE, Mavroukakis SA, White DE, Rosenberg SA (2002) Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science 298(5594):850–854. doi:10.1126/science.1076514

Freeman JD, Warren RL, Webb JR, Nelson BH, Holt RA (2009) Profiling the T-cell receptor beta-chain repertoire by massively parallel sequencing. Genome Res 19(10):1817–1824. doi:10.1101/gr.092924.109

Fridman WH, Pages F, Sautes-Fridman C, Galon J (2012) The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer 12(4):298–306. doi:10.1038/nrc3245

Gabrilovich DI, Ostrand-Rosenberg S, Bronte V (2012) Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol 12(4):253–268. doi:10.1038/nri3175, nri3175 [pii]

Gajewski TF, Schreiber H, Fu YX (2013) Innate and adaptive immune cells in the tumor microenvironment. Nat Immunol 14(10):1014–1022. doi:10.1038/ni.2703

Galluzzi L, Senovilla L, Zitvogel L, Kroemer G (2012) The secret ally: immunostimulation by anticancer drugs. Nat Rev Drug Discov 11(3):215–233. doi:10.1038/nrd3626

Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoue F, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Pages F (2006) Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 313(5795):1960–1964

Galon J, Fridman WH, Pages F (2007) The adaptive immunologic microenvironment in colorectal cancer: a novel perspective. Cancer Res 67(5):1883–1886

Galon J, Pages F, Marincola FM, Angell HK, Thurin M, Lugli A, Zlobec I, Berger A, Bifulco C, Botti G, Tatangelo F, Britten CM, Kreiter S, Chouchane L, Delrio P, Arndt H, Asslaber M, Maio M, Masucci GV, Mihm M, Vidal-Vanaclocha F, Allison JP, Gnjatic S, Hakansson L, Huber C, Singh-Jasuja H, Ottensmeier C, Zwierzina H, Laghi L, Grizzi F, Ohashi PS, Shaw PA, Clarke BA, Wouters BG, Kawakami Y, Hazama S, Okuno K, Wang E, O'Donnell-Tormey J, Lagorce C, Pawelec G, Nishimura MI, Hawkins R, Lapointe R, Lundqvist A, Khleif SN, Ogino S, Gibbs P, Waring P, Sato N, Torigoe T, Itoh K, Patel PS, Shukla SN, Palmqvist R, Nagtegaal ID, Wang Y, D'Arrigo C, Kopetz S, Sinicrope FA, Trinchieri G, Gajewski TF, Ascierto PA, Fox BA (2012a) Cancer classification using the Immunoscore: a worldwide task force. J Transl Med 10:205. doi:10.1186/1479-5876-10-205

Galon J, Pages F, Marincola FM, Thurin M, Trinchieri G, Fox BA, Gajewski TF, Ascierto PA (2012b) The immune score as a new possible approach for the classification of cancer. J Transl Med 10:1. doi:10.1186/1479-5876-10-1

Galon J, Angell HK, Bedognetti D, Marincola FM (2013a) The continuum of cancer immunosurveillance: prognostic, predictive, and mechanistic signatures. Immunity 39(1):11–26. doi:10.1016/j.immuni.2013.07.008

Galon J, Mlecnik B, Bindea G, Angell HK, Berger A, Lagorce C, Lugli A, Zlobec I, Hartmann A, Bifulco C, Nagtegaal ID, Palmqvist R, Masucci GV, Botti G, Tatangelo F, Delrio P, Maio M, Laghi L, Grizzi F,

Asslaber M, D'Arrigo C, Vidal-Vanaclocha F, Zavadova E, Chouchane L, Ohashi PS, Hafezi-Bakhtiari S, Wouters BG, Roehrl M, Nguyen L, Kawakami Y, Hazama S, Okuno K, Ogino S, Gibbs P, Waring P, Sato N, Torigoe T, Itoh K, Patel PS, Shukla SN, Wang Y, Kopetz S, Sinicrope FA, Scripcariu V, Ascierto PA, Marincola FM, Fox BA, Pages F (2013b) Towards the introduction of the Immunoscore in the classification of malignant tumors. J Pathol. doi:10.1002/path.4287

Gordon S, Taylor PR (2005) Monocyte and macrophage heterogeneity. Nat Rev Immunol 5(12):953–964. doi:10.1038/nri1733

Gulley JL, Arlen PM, Madan RA, Tsang KY, Pazdur MP, Skarupa L, Jones JL, Poole DJ, Higgins JP, Hodge JW, Cereda V, Vergati M, Steinberg SM, Halabi S, Jones E, Chen C, Parnes H, Wright JJ, Dahut WL, Schlom J (2010) Immunologic and prognostic factors associated with overall survival employing a poxviral-based PSA vaccine in metastatic castrate-resistant prostate cancer. Cancer Immunol Immunother 59(5):663–674. doi:10.1007/s00262-009-0782-8

Hadrup SR, Bakker AH, Shu CJ, Andersen RS, van Veluw J, Hombrink P, Castermans E, Thor Straten P, Blank C, Haanen JB, Heemskerk MH, Schumacher TN (2009) Parallel detection of antigen-specific T-cell responses by multidimensional encoding of MHC multimers. Nat Methods 6(7):520–526. doi:10.1038/nmeth.1345

Hamid O, Robert C, Daud A, Hodi FS, Hwu WJ, Kefford R, Wolchok JD, Hersey P, Joseph RW, Weber JS, Dronca R, Gangadhar TC, Patnaik A, Zarour H, Joshua AM, Gergich K, Elassaiss-Schaap J, Algazi A, Mateus C, Boasberg P, Tumeh PC, Chmielowski B, Ebbinghaus SW, Li XN, Kang SP, Ribas A (2013) Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. N Engl J Med 369(2):134–144. doi:10.1056/NEJMoa1305133

Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144(5):646–674. doi:10.1016/j.cell.2011.02.013, S0092-8674(11)00127-9 [pii]

Heusinkveld M, van der Burg SH (2011) Identification and manipulation of tumor associated macrophages in human cancers. J Transl Med 9(1):216. doi:10.1186/1479-5876-9-216, 1479-5876-9-216 [pii]

Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbe C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ (2010) Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 363(8):711–723. doi:10.1056/NEJMoa1003466, NEJMoa1003466 [pii]

Hoos A, Eggermont AM, Janetzki S, Hodi FS, Ibrahim R, Anderson A, Humphrey R, Blumenstein B, Old L, Wolchok J (2010) Improved endpoints for cancer immunotherapy trials. J Natl Cancer Inst 102(18):1388–1397. doi:10.1093/jnci/djq310, djq310 [pii]

Janetzki S, Panageas KS, Ben-Porat L, Boyer J, Britten CM, Clay TM, Kalos M, Maecker HT, Romero P, Yuan J, Kast WM, Hoos A, Elispot Proficiency Panel of the CVCIAWG (2008) Results and harmonization guidelines from two large-scale international Elispot proficiency panels conducted by the Cancer Vaccine Consortium (CVC/SVI). Cancer Immunol Immunother 57(3):303–315

Janetzki S, Britten CM, Kalos M, Levitsky HI, Maecker HT, Melief CJ, Old LJ, Romero P, Hoos A, Davis MM (2009) "MIATA"-minimal information about T cell assays. Immunity 31(4):527–528. doi:10.1016/j.immuni.2009.09.007

Jordanova ES, Gorter A, Ayachi O, Prins F, Durrant LG, Kenter GG, van der Burg SH, Fleuren GJ (2008) Human leukocyte antigen class I, MHC class I chain-related molecule A, and CD8+/regulatory T-Cell ratio: which variable determines survival of cervical cancer patients? Clin Cancer Res 14(7):2028–2035

Kalos M (2010) An integrative paradigm to impart quality to correlative science. J Transl Med 8:26. doi:10.1186/1479-5876-8-26

Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, Redfern CH, Ferrari AC, Dreicer R, Sims RB, Xu Y, Frohlich MW, Schellhammer PF, Investigators IS (2010) Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med 363(5):411–422. doi:10.1056/NEJMoa1001294

Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP, Essahsah F, Fathers LM, Offringa R, Drijfhout JW, Wafelman AR, Oostendorp J, Fleuren GJ, van der Burg SH, Melief CJ (2009) Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. N Engl J Med 361(19):1838–1847. doi:10.1056/NEJMoa0810097, 361/19/1838 [pii]

Klinger M, Kong K, Moorhead M, Weng L, Zheng J, Faham M (2013) Combining next-generation sequencing and immune assays: a novel method for identification of antigen-specific T cells. PLoS One 8(9):e74231. doi:10.1371/journal.pone.0074231

Lennerz V, Fatho M, Gentilini C, Frye RA, Lifke A, Ferel D, Wolfel C, Huber C, Wolfel T (2005) The response of autologous T cells to a human melanoma is dominated by mutated neoantigens. Proc Natl Acad Sci U S A 102(44):16013–16018. doi:10.1073/pnas.0500090102

Locker GY, Hamilton S, Harris J, Jessup JM, Kemeny N, Macdonald JS, Somerfield MR, Hayes DF, Bast RC Jr, ASCO (2006) ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. J Clin Oncol 24(33):5313–5327. doi:10.1200/JCO.2006.08.2644

Lurquin C, Lethe B, De Plaen E, Corbiere V, Theate I, van Baren N, Coulie PG, Boon T (2005) Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen. J Exp Med 201(2):249–257. doi:10.1084/jem.20041378

Maecker HT, McCoy JP Jr, Consortium FHI, Amos M, Elliott J, Gaigalas A, Wang L, Aranda R, Banchereau

J, Boshoff C, Braun J, Korin Y, Reed E, Cho J, Hafler D, Davis M, Fathman CG, Robinson W, Denny T, Weinhold K, Desai B, Diamond B, Gregersen P, Di Meglio P, Nestle FO, Peakman M, Villanova F, Ferbas J, Field E, Kantor A, Kawabata T, Komocsar W, Lotze M, Nepom J, Ochs H, O'Lone R, Phippard D, Plevy S, Rich S, Roederer M, Rotrosen D, Yeh JH (2010) A model for harmonizing flow cytometry in clinical trials. Nat Immunol 11(11):975–978. doi:10.1038/ni1110-975

Mander A, Gouttefangeas C, Ottensmeier C, Welters MJ, Low L, van der Burg SH, Britten CM (2010) Serum is not required for ex vivo IFN-gamma ELISPOT: a collaborative study of different protocols from the European CIMT Immunoguiding Program. Cancer Immunol Immunother 59(4):619–627. doi:10.1007/s00262-009-0814-4

McNeil LK, Price L, Britten CM, Jaimes M, Maecker H, Odunsi K, Matsuzaki J, Staats JS, Thorpe J, Yuan J, Janetzki S (2013) A harmonized approach to intracellular cytokine staining gating: results from an international multiconsortia proficiency panel conducted by the Cancer Immunotherapy Consortium (CIC/CRI). Cytometry Part A: J Int Soc Anal Cytol 83(8):728–738. doi:10.1002/cyto.a.22319

Melero I, Grimaldi AM, Perez-Gracia JL, Ascierto PA (2013) Clinical development of immunostimulatory monoclonal antibodies and opportunities for combination. Clin Cancer Res 19(5):997–1008. doi:10.1158/1078-0432.CCR-12-2214

Melief CJ, van der Burg SH (2008) Immunotherapy of established (pre)malignant disease by synthetic long peptide vaccines. Nat Rev Cancer 8(5):351–360. doi:10.1038/nrc2373, nrc2373 [pii]

Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, Parizot C, Taflin C, Heike T, Valeyre D, Mathian A, Nakahata T, Yamaguchi T, Nomura T, Ono M, Amoura Z, Gorochov G, Sakaguchi S (2009) Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity 30(6):899–911. doi:10.1016/j.immuni.2009.03.019

Mlecnik B, Tosolini M, Kirilovsky A, Berger A, Bindea G, Meatchi T, Bruneval P, Trajanoski Z, Fridman WH, Pages F, Galon J (2011) Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. J Clin Oncol 29(6):610–618. doi:10.1200/JCO.2010.30.5425

Mocellin S, Mandruzzato S, Bronte V, Lise M, Nitti D (2004) Part I: Vaccines for solid tumours. Lancet Oncol 5(11):681–689

Montero AJ, Diaz-Montero CM, Kyriakopoulos CE, Bronte V, Mandruzzato S (2012) Myeloid-derived suppressor cells in cancer patients: a clinical perspective. J Immunother 35(2):107–115. doi:10.1097/CJI.0b013e318242169f

Moodie Z, Price L, Gouttefangeas C, Mander A, Janetzki S, Lower M, Welters MJ, Ottensmeier C, van der Burg SH, Britten CM (2010) Response definition criteria for ELISPOT assays revisited. Cancer Immunol Immunother 59(10):1489–1501. doi:10.1007/s00262-010-0875-4

Moodie Z, Price L, Janetzki S, Britten CM (2012) Response determination criteria for ELISPOT: toward a standard that can be applied across laboratories. Methods Mol Biol 792:185–196. doi:10.1007/978-1-61779-325-7_15

Murdoch DM, Staats JS, Weinhold KJ (2012) OMIP-006: phenotypic subset analysis of human T regulatory cells via polychromatic flow cytometry. Cytometry Part A: J Int Soc Anal Cytol 81(4):281–283. doi:10.1002/cyto.a.22024

Nagtegaal ID, Quirke P, Schmoll HJ (2012) Has the new TNM classification for colorectal cancer improved care? Nat Rev Clin Oncol 9(2):119–123. doi:10.1038/nrclinonc.2011.157

Newell EW, Klein LO, Yu W, Davis MM (2009) Simultaneous detection of many T-cell specificities using combinatorial tetramer staining. Nat Methods 6(7):497–499. doi:10.1038/nmeth.1344

Nishikawa H, Sakaguchi S (2010) Regulatory T cells in tumor immunity. Int J Cancer 127(4):759–767. doi:10.1002/ijc.25429

Olson BM, McNeel DG (2013) Monitoring regulatory immune responses in tumor immunotherapy clinical trials. Front Oncol 3:109. doi:10.3389/fonc.2013.00109

Pages F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R, Mlecnik B, Kirilovsky A, Nilsson M, Damotte D, Meatchi T, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Galon J (2005) Effector memory T cells, early metastasis, and survival in colorectal cancer. N Engl J Med 353(25):2654–2666. doi:10.1056/NEJMoa051424

Pages F, Kirilovsky A, Mlecnik B, Asslaber M, Tosolini M, Bindea G, Lagorce C, Wind P, Marliot F, Bruneval P, Zatloukal K, Trajanoski Z, Berger A, Fridman WH, Galon J (2009) In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. J Clin Oncol 27(35):5944–5951. doi:10.1200/JCO.2008.19.6147

Piersma SJ, Jordanova ES, van Poelgeest MI, Kwappenberg KM, van der Hulst JM, Drijfhout JW, Melief CJ, Kenter GG, Fleuren GJ, Offringa R, van der Burg SH (2007) High number of intraepithelial CD8+ tumor-infiltrating lymphocytes is associated with the absence of lymph node metastases in patients with large early-stage cervical cancer. Cancer Res 67(1):354–361

Radvanyi LG, Bernatchez C, Zhang M, Fox PS, Miller P, Chacon J, Wu R, Lizee G, Mahoney S, Alvarado G, Glass M, Johnson VE, McMannis JD, Shpall E, Prieto V, Papadopoulos N, Kim K, Homsi J, Bedikian A, Hwu WJ, Patel S, Ross MI, Lee JE, Gershenwald JE, Lucci A, Royal R, Cormier JN, Davies MA, Mansaray R, Fulbright OJ, Toth C, Ramachandran R, Wardell S, Gonzalez A, Hwu P (2012) Specific lymphocyte subsets predict response to adoptive cell therapy using expanded autologous tumor-infiltrating lymphocytes in metastatic melanoma patients. Clin Cancer Res 18(24):6758–6770. doi:10.1158/1078-0432.CCR-12-1177

Rakhmilevich AL, Alderson KL, Sondel PM (2012) T-cell-independent antitumor effects of CD40 ligation.

Int Rev Immunol 31(4):267–278. doi:10.3109/08830185.2012.698337

Restifo NP, Dudley ME, Rosenberg SA (2012) Adoptive immunotherapy for cancer: harnessing the T cell response. Nat Rev Immunol 12(4):269–281. doi:10.1038/nri3191

Robbins PF, Lu YC, El-Gamil M, Li YF, Gross C, Gartner J, Lin JC, Teer JK, Cliften P, Tycksen E, Samuels Y, Rosenberg SA (2013) Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. Nat Med 19(6):747–752. doi:10.1038/nm.3161

Rosenberg SA (2011) Cell transfer immunotherapy for metastatic solid cancer-what clinicians need to know. Nat Rev Clin Oncol 8(10):577–585. doi:10.1038/nrclinonc.2011.116, nrclinonc.2011.116 [pii]

Sakaguchi S, Vignali DA, Rudensky AY, Niec RE, Waldmann H (2013) The plasticity and stability of regulatory T cells. Nat Rev Immunol 13(6):461–467. doi:10.1038/nri3464

Sautes-Fridman C, Cherfils-Vicini J, Damotte D, Fisson S, Fridman WH, Cremer I, Dieu-Nosjean MC (2011) Tumor microenvironment is multifaceted. Cancer Metastasis Rev 30(1):13–25. doi:10.1007/s10555-011-9279-y

Sheikh NA, Petrylak D, Kantoff PW, Dela Rosa C, Stewart FP, Kuan LY, Whitmore JB, Trager JB, Poehlein CH, Frohlich MW, Urdal DL (2013) Sipuleucel-T immune parameters correlate with survival: an analysis of the randomized phase 3 clinical trials in men with castration-resistant prostate cancer. Cancer Immunol Immunother 62(1):137–147. doi:10.1007/s00262-012-1317-2

Singh SK, Tummers B, Schumacher TN, Gomez R, Franken KL, Verdegaal EM, Laske K, Gouttefangeas C, Ottensmeier C, Welters MJ, Britten CM, van der Burg SH (2013) The development of standard samples with a defined number of antigen-specific T cells to harmonize T cell assays: a proof-of-principle study. Cancer Immunol Immunother 62(3):489–501. doi:10.1007/s00262-012-1351-0

Sliwkowski MX, Mellman I (2013) Antibody therapeutics in cancer. Science 341(6151):1192–1198. doi:10.1126/science.1241145

Solito S, Falisi E, Diaz-Montero CM, Doni A, Pinton L, Rosato A, Francescato S, Basso G, Zanovello P, Onicescu G, Garrett-Mayer E, Montero AJ, Bronte V, Mandruzzato S (2011) A human promyelocytic-like population is responsible for the immune suppression mediated by myeloid-derived suppressor cells. Blood 118(8):2254–2265. doi:10.1182/blood-2010-12-325753

Thoren FB, Anderson H, Strannegard O (2013) Late divergence of survival curves in cancer immunotherapy trials: interpretation and implications. Cancer Immunol Immunother 62(10):1547–1551. doi:10.1007/s00262-013-1458-y

Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Horn L, Drake CG, Pardoll DM, Chen L, Sharfman WH, Anders RA, Taube JM, McMiller TL, Xu H, Korman AJ, Jure-Kunkel M, Agrawal S, McDonald D, Kollia GD, Gupta A, Wigginton JM, Sznol M (2012) Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 366(26):2443–2454. doi:10.1056/NEJMoa1200690

van der Burg SH (2008) Therapeutic vaccines in cancer: moving from immunomonitoring to immunoguiding. Expert Rev Vaccines 7(1):1–5

van der Burg SH, Piersma SJ, de Jong A, van der Hulst JM, Kwappenberg KM, van den Hende M, Welters MJ, Van Rood JJ, Fleuren GJ, Melief CJ, Kenter GG, Offringa R (2007) Association of cervical cancer with the presence of CD4+ regulatory T cells specific for human papillomavirus antigens. Proc Natl Acad Sci U S A 104(29):12087–12092

van der Burg SH, Kalos M, Gouttefangeas C, Janetzki S, Ottensmeier C, Welters MJ, Romero P, Britten CM, Hoos A (2011) Harmonization of immune biomarker assays for clinical studies. Sci Transl Med 3(108):108ps44. doi:10.1126/scitranslmed.3002785, 3/108/108ps44 [pii]

van Rooij N, van Buuren MM, Philips D, Velds A, Toebes M, Heemskerk B, van Dijk LJ, Behjati S, Hilkmann H, El Atmioui D, Nieuwland M, Stratton MR, Kerkhoven RM, Kesmir C, Haanen JB, Kvistborg P, Schumacher TN (2013) Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. J Clin Oncol. doi:10.1200/JCO.2012.47.7521

Verdegaal EM, Visser M, Ramwadhdoebe TH, van der Minne CE, van Steijn JA, Kapiteijn E, Haanen JB, van der Burg SH, Nortier JW, Osanto S (2011) Successful treatment of metastatic melanoma by adoptive transfer of blood-derived polyclonal tumor-specific CD4+ and CD8+ T cells in combination with low-dose interferon-alpha. Cancer Immunol Immunother 60(7):953–963. doi:10.1007/s00262-011-1004-8

Walter S, Weinschenk T, Stenzl A, Zdrojowy R, Pluzanska A, Szczylik C, Staehler M, Brugger W, Dietrich PY, Mendrzyk R, Hilf N, Schoor O, Fritsche J, Mahr A, Maurer D, Vass V, Trautwein C, Lewandrowski P, Flohr C, Pohla H, Stanczak JJ, Bronte V, Mandruzzato S, Biedermann T, Pawelec G, Derhovanessian E, Yamagishi H, Miki T, Hongo F, Takaha N, Hirakawa K, Tanaka H, Stevanovic S, Frisch J, Mayer-Mokler A, Kirner A, Rammensee HG, Reinhardt C, Singh-Jasuja H (2012) Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. Nat Med 18:1254–1261. doi:10.1038/nm.2883, nm.2883 [pii]

Wang J, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Toes RE (2007) Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. Eur J Immunol 37(1):129–138. doi:10.1002/eji.200636435

Warren RL, Freeman JD, Zeng T, Choe G, Munro S, Moore R, Webb JR, Holt RA (2011) Exhaustive T-cell

repertoire sequencing of human peripheral blood samples reveals signatures of antigen selection and a directly measured repertoire size of at least 1 million clonotypes. Genome Res 21(5):790–797. doi:10.1101/gr.115428.110

Weide B, Zelba H, Derhovanessian E, Pflugfelder A, Eigentler TK, Di Giacomo AM, Maio M, Aarntzen EH, de Vries IJ, Sucker A, Schadendorf D, Buttner P, Garbe C, Pawelec G (2012) Functional T cells targeting NY-ESO-1 or Melan-A are predictive for survival of patients with distant melanoma metastasis. J Clin Oncol 30(15):1835–1841. doi:10.1200/JCO.2011.40.2271

Weitz J, Koch M, Debus J, Hohler T, Galle PR, Buchler MW (2005) Colorectal cancer. Lancet 365(9454):153–165. doi:10.1016/S0140-6736(05)17706-X

Welters MJ, Kenter GG, de Vos van Steenwijk PJ, Lowik MJ, Berends-van der Meer DM, Essahsah F, Stynenbosch LF, Vloon AP, Ramwadhdoebe TH, Piersma SJ, van der Hulst JM, Valentijn AR, Fathers LM, Drijfhout JW, Franken KL, Oostendorp J, Fleuren GJ, Melief CJ, van der Burg SH (2010) Success or failure of vaccination for HPV16-positive vulvar lesions correlates with kinetics and phenotype of induced T-cell responses. Proc Natl Acad Sci U S A 107(26):11895–11899. doi:10.1073/pnas.1006500107, 1006500107 [pii]

Welters MJ, Gouttefangeas C, Ramwadhdoebe TH, Letsch A, Ottensmeier CH, Britten CM, van der Burg SH (2012) Harmonization of the intracellular cytokine staining assay. Cancer Immunol Immunother 61(7):967–978. doi:10.1007/s00262-012-1282-9

Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, Segal NH, Ariyan CE, Gordon RA, Reed K, Burke MM, Caldwell A, Kronenberg SA, Agunwamba BU, Zhang X, Lowy I, Inzunza HD, Feely W, Horak CE, Hong Q, Korman AJ, Wigginton JM, Gupta A, Sznol M (2013) Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med 369(2):122–133. doi:10.1056/NEJMoa1302369

Woo EY, Yeh H, Chu CS, Schlienger K, Carroll RG, Riley JL, Kaiser LR, June CH (2002) Cutting edge: regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. J Immunol 168(9):4272–4276

Yuan J, Adamow M, Ginsberg BA, Rasalan TS, Ritter E, Gallardo HF, Xu Y, Pogoriler E, Terzulli SL, Kuk D, Panageas KS, Ritter G, Sznol M, Halaban R, Jungbluth AA, Allison JP, Old LJ, Wolchok JD, Gnjatic S (2011) Integrated NY-ESO-1 antibody and CD8+ T-cell responses correlate with clinical benefit in advanced melanoma patients treated with ipilimumab. Proc Natl Acad Sci U S A 108(40):16723–16728. doi:10.1073/pnas.1110814108

Zhou X, Bailey-Bucktrout S, Jeker LT, Bluestone JA (2009) Plasticity of CD4(+) FoxP3(+) T cells. Curr Opin Immunol 21(3):281–285. doi:10.1016/j.coi.2009.05.007

Developing Cancer Immunotherapies as Drugs: Setting the Stage Through Methodological Progress

Axel Hoos

Background

Much progress has been made in basic oncologic science over the last decades, which has created high hopes to deliver more effective drugs and better success rates in clinical investigation. Nevertheless, clinical success rates did not keep up with reported scientific progress and thus did not meet expectations of academic or industry drug developers, patients, or society at large. A recent study by Begley and Ellis suggests the reproducibility of published landmark preclinical data from oncologic research to be as low as 11 % (Begley and Ellis 2012). The results were attributed to limited scrutiny regarding experimental controls and data interpretation as well as selection of nonrepresentative data for publication. While a similar analysis is not available for cancer immunotherapy, repeat observations such as limited reproducibility of data or limited correlation of findings between pharmacodynamic and clinical outcomes (e.g., immune monitoring) lead to the hypothesis that existing investigational methods used in cancer immunotherapy development would require adaptation, and the new methods would need to be added to the investigational toolbox to better reflect biology and achieve higher reproducibility (Hoos et al. 2007a).

A. Hoos, MD, PhD
Department of Oncology R&D,
Cancer Immunotherapy Consortium of the Cancer Research Institute, New York, NY, USA

GlaxoSmithKline, Collegeville, PA, USA
e-mail: axel.hoos@cri-cic.org

Indeed, looking back at the history of cancer immunotherapy, which arguably began in the late nineteenth century, reveals the following: The first regressions of cancerous tumors due to an immune intervention were observed by William B. Coley in 1890 after inducing inflammation in these tumors through local injection of a bacterial cocktail also known as Coley's toxins. In the subsequent 100 years, progress was limited to the scientific knowledge of its time, which was able to accelerate with the emergence of modern methods such as the process for making monoclonal antibodies. However, even in the modern era of controlled clinical trials between the 1970s and today, clinical progress in cancer immunotherapy trials remained rather disappointing, which has led to mostly negative assessments regarding the potential of this modality in the pharmaceutical, oncology, and investment communities (Lesterhuis et al. 2011; Parish 2003). It was only in the last decade that pivotal progress was made both on the basic science and methodological front, which culminated in 2010 and 2011 in the approval of two modern cancer immunotherapies, sipuleucel-T (Kantoff et al. 2010) and ipilimumab (Hodi et al. 2010), based on improved survival outcomes in randomized Phase 3 trials (Fig. 1).

Sipuleucel-T, a cell-based therapeutic cancer vaccine (Provenge®) approved for hormone-refractory prostate cancer (Kantoff et al. 2010), and ipilimumab, a T-cell potentiating monoclonal antibody blocking the cytotoxic T-cell antigen 4 (anti-CTLA-4; Yervoy) approved for unresectable

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_6, © Springer International Publishing Switzerland 2014

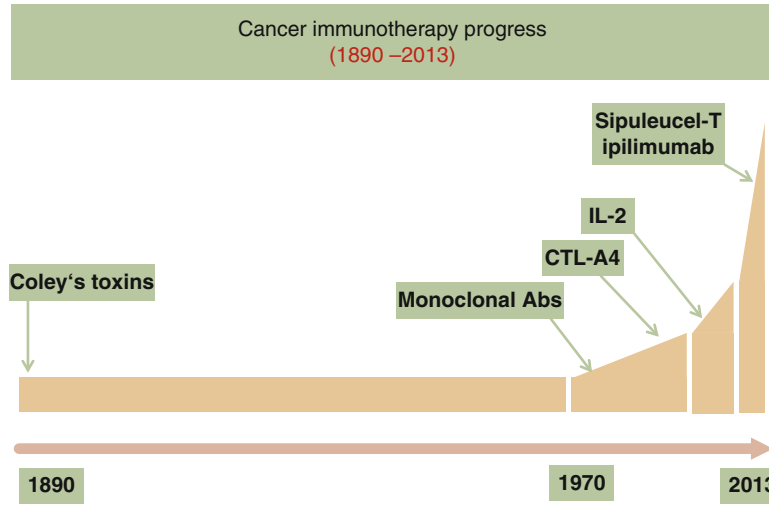

Fig. 1 History of progress in cancer immunotherapy

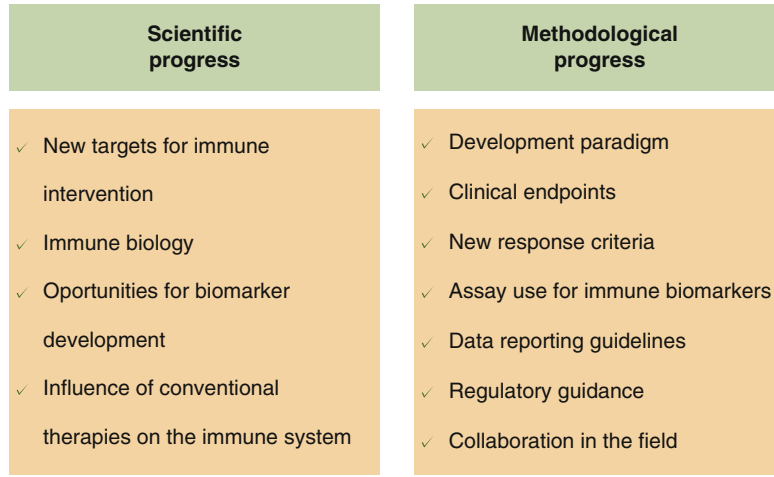

Fig. 2 Recent Scientific and methodological progress in cancer immunotherapy

or metastatic melanoma (Hodi et al. 2010; Hoos et al. 2010b), are two distinct types of immunotherapies, which achieved survival improvements for patients as monotherapies in two unrelated tumor entities. Clinical development of both agents were influenced by a new development paradigm (Hoos et al. 2011).

A key factor for this recent turn in the field is that – over the last decade – leading organizations in the cancer immunotherapy community began to systematically establish new methods for rational clinical investigation. These methods support increased data reproducibility and enabled clinical success (Goldman and DeFrancesco 2009; Finke et al. 2007) (Fig. 2).

This addresses the broader methodological concern for Oncology research raised by Begley and Ellis (2012). But it also addresses the more immunotherapy-specific concerns by Goldman and DeFrancesco that immunotherapy failures can be explained due to an inadequate approach to their development, suggesting "companies not doing their homework" and asking "what lessons from the list of failures will inform future practitioners in the field" (Goldman and DeFrancesco 2009) ? With the recent methodological advances, such lessons are now available (Finke et al. 2007; Hoos et al. 2007b, 2011) and are complementary with the basic scientific progress in the cancer immunotherapy field.

Methodological improvements were motivated by the struggle of the drug development industry and academic institutions devoted to inventing and developing cancer immunotherapies. Nonprofit groups such as the *Cancer Immunotherapy Consortium* (CIC; a program of the nonprofit Cancer Research Institute CRI) founded for the advancement of the cancer immunotherapy field systematically began to create a methodological framework that would provide the knowledge and tools needed for successful development programs. The US-based CIC created a partnership with the *Association for Cancer Immunotherapy* (CIMT) in Europe and, with broad contributions from the scientific and drug development communities, established this new framework encompassing the following: a biology-driven development paradigm for cancer immunotherapies (Hoos et al. 2007a), harmonized methods for detecting immune response to support immune biomarker development (Britten et al. 2007; van der Burg et al. 2011), improved clinical trial designs (Hoos et al. 2007b) and clinical endpoints (Hoos et al. 2010a; Wolchok et al. 2009), a publication framework for immune monitoring results from clinical trials (Janetzki et al. 2009; Britten et al. 2010), and scientific exchange and regulatory interactions to inform guidance document development by regulatory authorities (FDA 2009; EMA 2010).

This chapter provides a perspective on the recent methodological lessons in the immunotherapy space and summarizes the emerging framework that promises to enable greater and more reproducible success for future development programs (Fig. 3).

Immuno-oncology: An Evolving Area Within Oncology

Oncology, the clinical discipline of cancer therapy, has been an established medical specialty for several decades. Its hallmarks are the science of cancer biology as described by Hanahan and Weinberg (Hanahan and Weinberg 2011); a recognized clinical development paradigm (based on observations with chemotherapy) for investigation of new therapies in Phase 1, 2, and 3 clinical trials; defined criteria for measuring therapeutic effects such as RECIST (Response Evaluation Criteria in Solid Tumors) or WHO (World Health Organization) criteria for solid tumors; understood kinetics of therapeutic

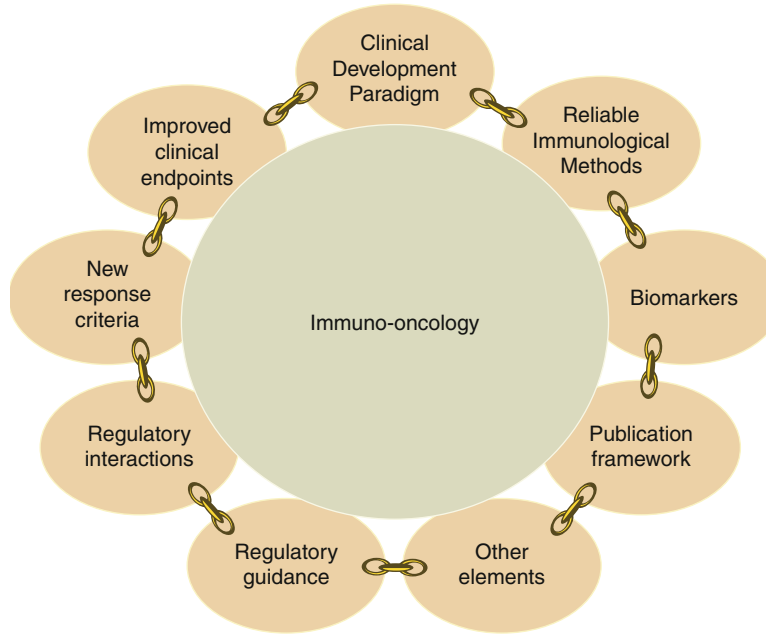

Fig. 3 The immuno-oncology framework (Adapted from [43] with permission)

effects; established standards for publication of new scientific data; and the availability of effective therapies paired with a clear understanding of their use. All this is anchored in a defined community represented by organizations such as the American Society of Clinical Oncology (ASCO) and the European Society of Medical Oncology (ESMO). Together, these hallmarks create a framework of credibility in which patient care, scientific discovery, publication, clinical development, and regulatory review can take place.

Despite clear evidence that the whole class of cancer immunotherapies has critical unique features that are different from those of the established classical therapeutic approaches in oncology, the field did not initially respond to the need of creating an appropriate alternative methodological framework accommodating these class-specific characteristics. Rather, to minimize controversy, keep shorter timelines, and build recognition in oncology, investigations of immunotherapies utilized the existing development paradigm based on cytotoxic drugs. This ultimately may have contributed to a high fraction of failures made in past developments (Goldman and DeFrancesco 2009).

Between 2004 and today, CIC and CIMT filled this void by creating a systematic framework using broad community knowledge and providing needed tools for successful development of immunotherapies (Table 1).

Table 1 Solutions for methodological challenges within the immuno-oncology framework

Challenge	Solution	Perspective	Refs.
Use of chemotherapy principles for clinical development of immunotherapy	New clinical development paradigm for immunotherapy with key components: (1) development phases for proof of principle and efficacy, (2) toxicity screening, (3) measurement of biologic activity, (4) immune response measurement in clinical trials, (5) dose and schedule, (6) developmental decision points, (7) trial design, (8) clinical endpoints, (9) combination therapy	A defined and reproducible path for adequate development of cancer immunotherapies	[2]
Clinical kinetics of immunotherapies not reflected by conventional endpoints	Adjustment of endpoints to immunotherapy biology	More complete detection of efficacy	[12]
No recognized system to measure all patterns of immunotherapy clinical activity	Immunotherapy response criteria derived from RECIST and WHO: Immune-related Response Criteria (irRC)	Capture all clinical activity patterns for a reliable assessment of activity signals in early trials	[13]
High data variability for immune monitoring in multicenter trials	Harmonization guidelines and quality assurance for immune monitoring assays	Enable reproducible investigation of immune response as biomarkers in clinical development. Subsequently, enable clinical qualification and investigate surrogacy	[11, 30–36]
Inconsistent reporting of immune monitoring results in scientific publications	Reporting framework for scientific publications: Minimal Information About T-Cell Assays (MIATA)	Transparency of results and comparability across centers and trials	[15, 17]
Absence of regulatory guidance for cancer immunotherapy development	Broad scientific exchange with participation of regulators to support guidance document development	Credible development criteria for prospective use	[18, 19]
Additional components	Based on community need	Continuous evolution of framework	

Adapted from [9], with permission

A Development Paradigm for Cancer Immunotherapies

The first step was the proposal of a clinical development paradigm in 2004. At the time, much knowledge around developmental problems and potential solutions existed in the field with little consensus on how to uniformly utilize and translate it into a comprehensive new paradigm. CIC and its partner organization the Society for Immunotherapy of Cancer (SITC) formed the Cancer Vaccine Clinical Trial Working Group (CVCTWG) with stakeholders from academia, the biotechnology and pharmaceutical industry and the US FDA. Together they built a paradigm for development of cancer vaccines and related immunotherapies (Hoos et al. 2007a). It recognizes differences between chemotherapy and immunotherapy such as (1) the optimal biologic dose is often not the maximum tolerated dose; (2) a treatment effect is not proportionally linked to toxicity; (3) conventional pharmacokinetics may not solely determine dose and schedule; (4) antitumor response may not be the only predictor of survival; and (5) clinical effects can be delayed in time and can occur after tumor volume increase (often categorized as progression). The new paradigm categorizes clinical development into proof-of-principle trials and efficacy trials, where efficacy trials are recommended to be randomized (Phases 2 and 3). It also provides considerations for toxicity screening in early trials, concepts for measurement of biologic activity, criteria for the use of immune monitoring assays, dose and schedule investigation, decision points in development, clinical study design, biology-based clinical endpoints, and combination therapy. The main value of this paradigm lies in the consensus between all main constituents involved with cancer immunotherapy development, namely, academicians, pharmaceutical/biotech industry, and the US FDA (Hoos et al. 2007b; FDA 2009).

Improved Clinical Endpoints

Chemotherapy and targeted therapy have direct effects on tumor cells and typically induce a measurable impact on tumor growth within a few weeks of administration or demonstrate not to be effective at all. In contrast, therapies utilizing the immune system induce indirect antitumor effects by initially stimulating the immune system followed by a broader spectrum of clinical responses including delayed effects. Delayed effects on tumors may include shrinkage after initial volume increase of existing lesions and appearance of new lesions, which may both be caused by immune infiltrates or prolonged stabilization of lesions without any shrinkage (Wolchok et al. 2009). With some immunotherapies, the latter patterns appear to be more common than the conventional response. They likely reflect the interplay between the immune system and the tumor described as immunoediting (Dunn et al. 2002).

Delayed effects and stabilization of tumor lesions influence the standard efficacy endpoints of antitumor response and overall survival (Finke et al. 2007; Hoos et al. 2010a). Both endpoints need adjustment to address this biology. For survival, Kaplan-Meier curves from randomized immunotherapy trials may show a delayed separation after months, which directly influences the statistical power to determine treatment effects observed over the entire length of a given curve (Fine 2007). Statistical models used in randomized oncology trials, where separation of Kaplan-Meier (KM) curves is expected early after treatment initiation, typically assume proportional hazards reflected in a constant hazard ratio over time. In order to address the delayed separation, alternative statistical models need to consider that all events prior to the separation do not contribute to the differentiation between study arms after the separation, thus causing reduced statistical power. Compensation for such power reduction can occur through a split of the hazard ratio into an early and a late component before and after the separation (Hoos et al. 2010a). When planning interim analyses in randomized studies, the absence of early effects would need to be accounted for to determine timing of the analysis and the value of testing for futility.

Immune-Related Response Criteria to Characterize Antitumor Effects

Standard response criteria based on WHO (1979) or RECIST (Eisenhauer et al. 2009) for assessing clinical effects of anticancer agents were created with the experience from cytotoxic drugs using tumor shrinkage as their measure of activity. With the altered biology of immunotherapies, their response patterns are broader than those of chemotherapy and may manifest after a period of stable disease or after initial tumor burden increase or appearance of new lesions. This may represent influx of lymphocytes into the tumor (Wolchok et al. 2009; Ribas et al. 2009). Such patterns were commonly noted in past trials but were never systematically described due to lack of suitable criteria (Kruit et al. 2005; van Baren et al. 2005). Principles for development of new response criteria were derived from the described development paradigm (Hoos et al. 2007b) and immune-related response criteria and were refined using large data sets from the ipilimumab (anti-CTLA-4) development program with 487 advanced melanoma patients from Phase 2 trials (Wolchok et al. 2009). Four patterns of response were described: *A*: immediate response, *B*: durable stable disease with possible slow decline in tumor burden, *C*: response after tumor burden increase (possible lymphocyte infiltration), and *D*: response in the presence of new lesions. Immune-related response criteria (irRC) are generally based on WHO and RECIST criteria, describe tumor burden as a continuous variable, account for new lesions in the overall tumor burden, and require confirmation of progression similar to the established confirmation of response at a subsequent time point after first detection. Ipilimumab data suggest that irRC identify patients with previously unrecognized benefit as indicated by favorable survival outcomes. Such patients displayed novel response patterns (Wolchok et al. 2009). Since their creation in 2009, irRC are undergoing prospective validation and are being tested in countless trials with a broad spectrum of cancer immunotherapies.

Managing Data Variability in Immune Biomarker Development

The monitoring of treatment-induced immune responses is important for understanding the mechanism of action and the description of early biologic effects prior to reaching clinical endpoints. Such immune biomarkers depend on reliable and reproducible assays and may provide data on (1) whether the biological target was hit, (2) how to dose the agent, (3) whether synergies exist for therapeutic combinations, (4) how patient populations may be defined, (5) how biologic activity can be characterized, and (6) whether they predict clinical outcomes as surrogates for patient benefit (Wagner 2002). Common immune response assays used to determine function, phenotype, and frequency of antigen-specific T cells such as ELISPOT, intracellular cytokine staining, and HLA-peptide multimer staining have inherently high data variability (Janetzki et al. 2009). This variability has contributed to the abundant challenge of developing biomarkers for the above applications. After extensive efforts across more than 120 academic, industry, and government laboratories over close to a decade, a solution for this data variability has emerged: immune assay harmonization. Harmonized use of immune assays across laboratories provides an external quality-control mechanism and guidance for assay conduct that – if followed – can substantially increase assay performance and decrease data variability.

Harmonization criteria were established through large international proficiency panel programs conducted by the CIC and CIMT (Janetzki et al. 2008; Britten et al. 2008, 2009; Attig et al. 2011; Mander et al. 2010; Moodie et al. 2010]. Harmonization is a tool to improve data reliability for immune monitoring and enhance clinical development of immune therapies at any stage of assay evolution (Janetzki and Britten 2012; van der Burg et al. 2011). It reminds of the successful initiatives of ICH-GCP for clinical protocols (ICH 1996) and has the potential to bring immune monitoring to the forefront of immune biomarker development and provide support in guiding decision making in clinical development (van der Burg 2008).

Consistent Reporting of Immune Monitoring Data

Variability is not limited to immune monitoring. It extends to the presentation of methods and results in scientific publications. To date, many publications of T-cell assay experiments lack information critical variables known to impact assay performance. In its absence scientists reading these publications are not enabled to fully understand the content or reproduce the experiment. The solution lies in creation of a publication framework that determines a minimum set of critical variables a publication must contain to transparently summarize what experiment was done under which conditions and with which results. The scientific community faced this challenge with a series of modern bioassays and responded with the creation of the Minimum Information About Biological and Biomedical Investigations (MIBBI) concept (Taylor et al. 2008). Over the last decade MIBBI created transparency measures for more than 30 biological assays such as DNA microarrays, RNAi experiments, or cellular assays. CIC and CIMT started the Minimal Information About T-Cell Assays (MIATA) project in 2009, which established a framework for publication of T-cell assay results from clinical trials (Janetzki et al. 2009). MIATA is based on an extensive community-wide vetting process over approximately 2 years incorporating the expertise and concerns of more than 120 experts from all areas of clinical immunology and achieved wide acceptance (Britten et al. 2010). The final version of MIATA became available in 2012 (Britten et al. 2012) and is being tested now as part of the *Materials and Methods* sections of several peer-reviewed journals. Its impact will depend on the breadth of use across the community.

Regulatory Guidance

The described methodological advances for the growing immune-oncology space evolved under the auspices of CIC and CIMT and with the participation of all major stakeholders from academia, biotechnology and pharmaceutical industries, and the US FDA. When a scientific area reaches the point of producing drug candidates ready for regulatory review and possible approval, there is an accompanying need for regulatory guidance documents clarifying a uniform view of regulatory authorities on the subject. In the case of cancer immunotherapy, the FDA utilized the scientific lessons from the community, hosted a workshop where these topics were reviewed, and published a draft guidance on "Clinical Considerations for Therapeutic Cancer Vaccines" (2009). The FDA draft document contained many of the topics summarized above, went through public consultation, and was finalized 2011. Similarly, the European Medicines Agency (EMA) issued a concept paper soliciting public feedback on a proposed revision of the guidance on "evaluation of anticancer medicinal products in man." EMA specifically requested community input regarding clinical endpoints for biologics and cancer vaccines (EMA 2010). CIMT and CIC jointly offered their integrated positions to EMA, which found inclusion in the updated guidance document. Overall, CIC and CIMT have created a process that addresses cutting-edge aspects of the field, create a uniform voice, and enable officials at FDA and EMA to more easily review and assess community positions.

Anti-CTLA-4 Antibody Development: Application of the Development Paradigm

The example of anti-CTLA-4 antibody development (Hoos et al. 2010b) illustrates the relevance of biology-based drug development as outlined in the new immunotherapy paradigm. Clinical trials with anti-CTLA-4 antibodies started at the biotechnology company Medarex in the year 2000 with Phase 1 and 2 trials suggesting an approximate 10 % response rate in patients with advanced melanoma. Interest from big pharmacy for developing anti-CTLA-4 blocking antibodies led to independent licensing deals with Pfizer and Bristol-Myers

Squibb (BMS) for different antibody isoforms and sparked two parallel development programs in advanced melanoma with tremelimumab (Pfizer) and ipilimumab (BMS), respectively. As was standard in the industry, both programs initially used chemotherapy criteria to guide development (Hoos et al. 2010a; Finke et al. 2007). By its design, the tremelimumab program conducted an early interim analysis using conventional futility criteria for survival in its Phase 3 study. A survival difference was not observed, and, consequently, the Phase 3 trial was terminated for futility as per Data Monitoring Committee recommendations (Ribas et al. 2008). Two years downstream extended follow-up on the study population revealed a separation of survival curves (Ascierto et al. 2011). To the contrary, interaction of the ipilimumab development program with CIC enabled the program to adapt to new scientific information. This resulted in the change of the primary endpoint for two pivotal Phase 3 trials in advanced melanoma from response or progression-free survival to overall survival with no early interim analyses that may mislead the assessment (Hodi et al. 2010; Hoos et al. 2010b). Both Phase 3 studies demonstrated improved survival (HR 0.66 and HR 0.72, respectively) in their final analyses, thus supporting the regulatory approval for patients with unresectable and metastatic melanoma. Based on the matured knowledge about immunotherapy development, BMS acquired Medarex in 2009 in a transaction valued $2.4 billion and is now developing a pipeline of immuno-oncology agents resulting from the acquisition (2011).

The development programs for ipilimumab and tremelimumab and their respective results illustrate the importance of science-driven clinical development for immunotherapies and of collaboration across various constituents to direct scientific progress. These observations also suggest that the prospective application of the new paradigm may help avoid critical pitfalls for future immunotherapy programs.

Summary

The last decade has brought many methodological improvements that accompany our growing scientific understanding of tumor immunology (Finn 2008). Their application has enabled success in the space of immuno-oncology and allowed it to emerge as a successful new subspecialty within oncology. By addressing the obvious weak spot in immunotherapy drug development, namely, the absence of a biology-based clinical development paradigm and other associated methodological advances, the foundation for future progress in immuno-oncology has been created. The resulting methodological framework will likely expand with the now rapidly growing space.

Acknowledgment CIC and CIMT thank all participants of its workshops and community-wide initiatives for their contribution of knowledge, which have enabled the creation of the immune-oncology framework.

References

Ascierto PA, Marincola FM, Ribas A (2011) Anti-CTLA4 monoclonal antibodies: the past and the future in clinical application. J Transl Med 9:196

Attig S, Price L, Janetzki S, Kalos M, Pride M, McNeil L, Clay T, Yuan J, Odunsi K, Hoos A, Romero P, Britten CM (2011) A critical assessment for the value of markers to gate-out undesired events in HLA-peptide multimer staining protocols. J Transl Med 9:108

Begley CG, Ellis LM (2012) Drug development: raise standards for preclinical cancer research. Nature 483: 531–533

BMS press release (2011) Bristol-Myers Squibb to acquire Medarex. July 22 2009. http://www.bms.com/news/press_releases/pages/default.aspx

Britten CM, Janetzki S, van der Burg SH, Gouttefangeas C, Hoos A (2007) Toward the harmonization of immune monitoring in clinical trials: Quo vadis? Cancer Immunol Immunother 57:285–288

Britten CM, Gouttefangeas C, Welters MJ, Pawelec G, Koch S, Ottensmeier C, Mander A, Walter S, Paschen A, Muller-Berghaus J, Haas I, Mackensen A, Kollgaard T, Thor SP, Schmitt M, Giannopoulos K, Maier R, Veelken H, Bertinetti C, Konur A, Huber C, Stevanovic S, Wolfel T, van der Burg SH (2008) The CIMT-monitoring panel: a two-step approach to harmonize the enumeration of antigen-specific CD8+ T lymphocytes by structural and functional assays. Cancer Immunol Immunother 57:289–302

Britten CM, Janetzki S, Ben Porat L, Clay TM, Kalos M, Maecker H, Odunsi K, Pride M, Old L, Hoos A, Romero P (2009) Harmonization guidelines for HLA-peptide multimer assays derived from results of a large scale international proficiency panel of the Cancer Vaccine Consortium. Cancer Immunol Immunother 58:1701–1713

Britten CM, Janetzki S, van der Burg SH, Huber C, Kalos M, Levitsky HI, Maecker HT, Melief CJ, O'Donnell-Tormey J, Odunsi K, Old LJ, Pawelec G, Roep BO, Romero P, Hoos A, Davis MM (2010) Minimal information about T cell assays: the process of reaching the community of T cell immunologists in cancer and beyond. Cancer Immunol Immunother 60:15–22

Britten CM, Janetzki S, Butterfield LH, Ferrari G, Gouttefangeas C, Huber C, Kalos M, Levitsky HI, Maecker HT, Melief CJ, O'Donnell-Tormey J, Odunsi K, Old LJ, Ottenhoff TH, Ottensmeier C, Pawelec G, Roederer M, Roep BO, Romero P, van der Burg SH, Walter S, Hoos A, Davis MM (2012) T cell assays and MIATA: the essential minimum for maximum impact. Immunity 37(1):1–2

Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD (2002) Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol 3:991–998

Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, Rubinstein L, Shankar L, Dodd L, Kaplan R, Lacombe D, Verweij J (2009) New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer 45:228–247

EMA (2010) Concept paper on the need to revise the guideline on the evaluation of anticancer medicinal products in man. European Medicines Agency. July 2010. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/09/WC500096730.pdf

FDA (2009) Guidance for industry: clinical considerations for therapeutic cancer vaccines. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research, September 2009. http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/UCM182826.pdf

Fine GD (2007) Consequences of delayed treatment effects on analysis of time-to-event endpoints. Drug Inf J 41:535–539

Finke LH, Wentworth K, Blumenstein B, Rudoplph NS, Hoos A (2007) Lessons from randomized phase III studies with active cancer immunotherapies-outcomes from the 2006 meeting of the Cancer Vaccine Consortium (CVC). Vaccine 25:B97–B109

Finn OJ (2008) Cancer immunology. N Engl J Med 358:2704–2715

Goldman B, DeFrancesco L (2009) The cancer vaccine roller coaster. Nat Biotechnol 27:129–139

Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144:646–674

Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbe C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ (2010) Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 363:711–723

Hoos A, Parmiani G, Hege K, Sznol M, Loibner H, Eggermont A, Urba W, Blumenstein B, Sacks N, Keilholz U, Nichol G (2007a) A clinical development paradigm for cancer vaccines and related biologics. J Immunother 30:1–15

Hoos A, Parmiani G, Hege K, Sznol M, Loibner H, Eggermont A, Urba W, Blumenstein B, Sacks N, Keilholz U, Nichol G (2007b) A clinical development paradigm for cancer vaccines and related biologics. J Immunother 30:1–15

Hoos A, Eggermont AM, Janetzki S, Hodi FS, Ibrahim R, Anderson A, Humphrey R, Blumenstein B, Old L, Wolchok J (2010a) Improved endpoints for cancer immunotherapy trials. J Natl Cancer Inst 102:1388–1397

Hoos A, Ibrahim R, Korman A, Abdallah K, Berman D, Shahabi V, Chin K, Canetta R, Humphrey R (2010b) Development of ipilimumab: contribution to a new paradigm for cancer immunotherapy. Semin Oncol 37:533–546

Hoos A, Britten CM, Huber C, O'Donnell-Tormey J (2011) A methodological framework to enhance the clinical success of cancer immunotherapy. Nat Biotechnol 29:867–870

ICH (1996) Harmonised tripartite guideline: guideline for good clinical practice E6(R1). 1996. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Efficacy/E6_R1/Step4/E6_R1__Guideline.pdf

Janetzki S, Britten CM (2012) The impact of harmonization on ELISPOT assay performance. Methods Mol Biol 792:25–36

Janetzki S, Panageas KS, Ben-Porat L, Boyer J, Britten CM, Clay TM, Kalos M, Maecker HT, Romero P, Yuan J, Kast WM, Hoos A (2008) Results and harmonization guidelines from two large-scale international Elispot proficiency panels conducted by the Cancer Vaccine Consortium (CVC/SVI). Cancer Immunol Immunother 57:303–315

Janetzki S, Britten CM, Kalos M, Levitsky HI, Maecker HT, Melief CJ, Old LJ, Romero P, Hoos A, Davis MM (2009) "MIATA"-minimal information about T cell assays. Immunity 31:527–528

Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, Redfern CH, Ferrari AC, Dreicer R, Sims RB, Xu Y, Frohlich MW, Schellhammer PF (2010) Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med 363:411–422

Kruit WH, van Ojik HH, Brichard VG, Escudier B, Dorval T, Dreno B, Patel P, van Baren N, Avril MF, Piperno S, Khammari A, Stas M, Ritter G, Lethe B, Godelaine D, Brasseur F, Zhang Y, van der Bruggen P, Boon T, Eggermont AM, March M (2005) Phase 1/2 study of subcutaneous and intradermal immunization with a recombinant MAGE-3 protein in patients with detectable metastatic melanoma. Int J Cancer 117:596–604

Lesterhuis WJ, Haanen JB, Punt CJ (2011) Cancer immunotherapy–revisited. Nat Rev Drug Discov 10:591–600

Mander A, Gouttefangeas C, Ottensmeier C, Welters MJ, Low L, van der Burg SH, Britten CM (2010) Serum is

not required for ex vivo IFN-gamma ELISPOT: a collaborative study of different protocols from the European CIMT Immunoguiding Program. Cancer Immunol Immunother 59:619–627

Moodie Z, Price L, Gouttefangeas C, Mander A, Janetzki S, Lower M, Welters MJ, Ottensmeier C, van der Burg SH, Britten CM (2010) Response definition criteria for ELISPOT assays revisited. Cancer Immunol Immunother 59:1489–1501

Parish CR (2003) Cancer immunotherapy: the past, the present and the future. Immunol Cell Biol 81:106–113

Ribas A, Hauschild A, Kefford R, Punt CJA, Haanen JB (2008) Phase III open-label, randomized, comparative study of tremelimumab (CP-675, 206) and chemotherapy (temozolomide [TMZ] or dacarbazine [DTIC]) in patients with advanced melanoma. American Society of Clinical Oncology, annual meeting 2008, abstract LBA 9011

Ribas A, Chmielowski B, Glaspy JA (2009) Do we need a different set of response assessment criteria for tumor immunotherapy? Clin Cancer Res 15:7116–7118

Taylor CF, Field D, Sansone SA, Aerts J, Apweiler R, Ashburner M, Ball CA, Binz PA, Bogue M, Booth T, Brazma A, Brinkman RR, Michael CA, Deutsch EW, Fiehn O, Fostel J, Ghazal P, Gibson F, Gray T, Grimes G, Hancock JM, Hardy NW, Hermjakob H, Julian RK Jr, Kane M, Kettner C, Kinsinger C, Kolker E, Kuiper M, Le Novere N, Leebens-Mack J, Lewis SE, Lord P, Mallon AM, Marthandan N, Masuya H, McNally R, Mehrle A, Morrison N, Orchard S, Quackenbush J, Reecy JM, Robertson DG, Rocca-Serra P, Rodriguez H, Rosenfelder H, Santoyo-Lopez J, Scheuermann RH, Schober D, Smith B, Snape J, Stoeckert CJ Jr, Tipton K, Sterk P, Untergasser A, Vandesompele J, Wiemann S (2008) Promoting coherent minimum reporting guidelines for biological and biomedical investigations: the MIBBI project. Nat Biotechnol 26:889–896

van Baren N, Bonnet MC, Dreno B, Khammari A, Dorval T, Piperno-Neumann S, Lienard D, Speiser D, Marchand M, Brichard VG, Escudier B, Negrier S, Dietrich PY, Maraninchi D, Osanto S, Meyer RG, Ritter G, Moingeon P, Tartaglia J, van der Bruggen P, Coulie PG, Boon T (2005) Tumoral and immunologic response after vaccination of melanoma patients with an ALVAC virus encoding MAGE antigens recognized by T cells. J Clin Oncol 23:9008–9021

van der Burg SH (2008) Therapeutic vaccines in cancer: moving from immunomonitoring to immunoguiding. Expert Rev Vaccines 7:1–5

van der Burg SH, Kalos M, Gouttefangeas C, Janetzki S, Ottensmeier C, Welters MJ, Romero P, Britten CM, Hoos A (2011) Harmonization of immune biomarker assays for clinical studies. Sci Transl Med 3:108ps44

Wagner JA (2002) Overview of biomarkers and surrogate endpoints in drug development. Dis Markers 18:41–46

WHO (1979) WHO handbook for reporting results of cancer treatment. World Health Organization Offset Publication No. 48, Geneva

Wolchok JD, Hoos A, O'Day S, Weber JS, Hamid O, Lebbe C, Maio M, Binder M, Bohnsack O, Nichol G, Humphrey R, Hodi FS (2009) Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. Clin Cancer Res 15:7412–7420

Part II

Immunotherapies

Reflections on Cancer Vaccines

Cornelis J.M. Melief

Introduction

Cancer vaccines have been vilified as futile attempts to impact on a grave disease that easily withstands the weak attempts by cancer vaccines to reduce, let alone to eradicate it. Fortunately, persistent investigators in the field of cancer immunology, among whom the Mainz group under the inspiring leadership of Cristoph Huber and the no less formidable group in Tübingen under the leadership of Hans-Georg Rammensee, the group of Jeff Schlom in Bethesda, and our own group in Leiden, have kept faith in cancer vaccines over the years. Indeed, the company Dendreon has succeeded in getting the first cancer vaccine approved by the FDA (Kantoff et al. 2010). The vaccine has been proven to prolong the life of patients with hormone-resistant prostate cancer and consists of dendritic cells loaded with prostate acid phosphatase as a tumor-associated antigen. Reportedly, the combination of gp100 peptide vaccination and interleukin-2 can prolong the life of patients with metastatic melanoma (Schwartzentruber et al. 2011). Indeed, specific T cell responses are needed to eradicate tumors. Despite the formidable success of checkpoint blockers such as monoclonal antibodies against CTLA-4 (Hodi et al. 2010), PD-1 (Topalian et al. 2012), or PDF-L1 (Brahmer et al. 2012), these antibodies rely on preexistent tumor-specific T cells. It seems possible to expand low numbers of T cells by combined treatment with CTLA-4 blocker and PD-1 blocker (Wolchok et al. 2013). Therefore, only those patients benefit most likely from these treatments, whose preexistent T cell immunity against their cancers has reached sufficient levels to benefit from checkpoint blocking. Specific vaccination can be used to greatly expand the proportion of patients benefiting from checkpoint blocking by inducing cancer-specific T cells in those patients who lack sufficient numbers of such cells. While cancer vaccines already perform much better in the presence of chemotherapeutics, additional combination with checkpoint blocking and with cytokines such as IL-7 and IL-15 is likely to lead to multimodality combination treatment of cancer with minimal toxicity and great specificity.

The Different Types of Cancer Vaccines

Cancer vaccines come in different varieties. The universal aim is to induce robust effector T cell responses. Historically many cancer vaccines consisted of exact HLA-binding peptides representing CTL epitopes (Feltkamp et al. 1993). We have demonstrated that these constitute suboptimal vaccines because such vaccines lack

C.J.M. Melief
ISA Pharmaceuticals,
J.H. Oortweg 19-21, Leiden 2333 CH,
The Netherlands
e-mail: melief@isa-pharma.com

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_7,

sufficient helper epitopes, precluding sufficient T cell memory formation. Also exact CTL epitope peptides can bind exogenously to all cells that express MHC class I, including nonprofessional antigen presenting cells without proper co-stimulatory molecule expression, therefore capable of causing tolerance (reviewed in Melief and van der Burg 2008; van der Burg and Melief 2011). This can be avoided by the use of longer peptides of 25–35 amino acids in length, called synthetic long peptides (SLP) (Zwaveling et al. 2002; Kenter et al. 2008, 2009; Welters et al. 2008, 2010, reviewed in Melief and van der Burg 2008; van der Burg and Melief 2011; Quakkelaar and Melief 2012, van der Burg et al. 2011). Such SLP need to go through an obligatory dendritic cell (DC) processing step, which is achieved much more efficiently by SLP than by proteins, achieving efficient MHC class I and II presentation. Addition of a potent adjuvant to the peptides is crucial. Clinically promising adjuvants are Montanide ISA-51 (Kenter et al. 2008, 2009), Poly I:C and CpG (Speiser et al. 2005; Sabbatini et al. 2012), which also perform well in preclinical mouse models (van Duikeren et al. 2012). Another very promising co-treatment that enhances Th1 T cell, CTL, and antibody responses to SLP p53 cancer vaccines is slow-acting pegylated interferon alpha-2b (Pegintron, Zeestraten et al. 2013). This cytokine may act equally well as the type I IFN inducer poly I:C. The effector T cell-enhancing effect of type I interferon has been amply documented. Mechanisms include promotion of cross-presentation of antigens by DC and activation of DC to express higher levels of co-stimulatory molecules (Belardelli et al. 2002; Hervas-Stubbs et al. 2011; Huber and Farrar 2011; Lattanzi et al. 2011; Spadaro et al. 2012; Sikora et al. 2009). The Schlom group at the National Cancer Institute of the USA in Bethesda, Md, has used recombinant pox virus vaccines with built-in co-stimulatory molecules. The problem, however, with recombinant virus vaccines is that most of the elicited T cell responses against such vaccines are directed against virus vector sequences and not against the inserted cancer antigens. This antigenic competition limits the potency of the cancer-specific immune response elicited by such vaccines. Despite these differences promising clinical responses have been elicited with all types of cancer vaccines just mentioned.

Comparison of the Clinical Effectiveness of Adoptive T Cell Transfer and Cancer Vaccines

Adoptive T cell transfer is supposed to be only effective if combined with lymphodepleting chemotherapy and in addition of total body irradiation, whereas cancer vaccines are asked to work without any additional combinatorial treatment. This is not fair! Lymphodepletion is obviously not a good idea with cancer vaccines because one then depletes the very cells that need to expand in response to the vaccine. Nevertheless, our recent preclinical and clinical experiments show that depletion of myeloid suppressor cells (Gabrilovich et al. 2012; Heusinkveld and van der Burg 2011) by certain chemotherapy combinations is highly capable of improving the expansion capacity of the T cells without affecting T cell numbers or T cell function in both mice and in patients with cancer (T. van der Sluis, M. Welters, S.H. van der Burg, H. van Meir, K. Burggraaf, C.J.M. Melief, unpublished observations). Cisplatin reportedly improves the result of DNA vaccination (Tseng et al. 2008). Additional improvements of the results of therapeutic vaccination are likely by combining vaccination with checkpoint blocking with monoclonal antibodies against PD-1 or PD-L1. The rationale for this comes from the observation that in cancer tissues PD-L1 is induced by interferon-γ produced by vaccine-induced tumor-infiltrating T cells (TILs). Such PD-L1 expression then interacts with PD-1 on the TILs to stop their proliferation. Thus, the attack by the TILs calls into action a self-tissue protective mechanism that relies on PD-L1/PD-1 interaction. Additional inhibition can be mediated by the inhibitory Lag-3 (Norde et al. 2012) and TIM3 (Ngiow et al. 2011) mechanisms. Thus, like in adoptive T cell transfer, vaccination as monotherapy is unlikely to achieve its goal. Future combination therapy needs to consist of

therapeutic vaccination, non-T-cell-suppressive chemotherapy, and checkpoint blocking. Further efficacy improvement is likely to be achieved by additional treatment with either of the cytokines IL-7 or IL-15 (Cheever et al. 2008), which can considerably enhance proliferation of tumor-specific T cells with much less toxicity as IL-2 treatment.

References

Belardelli F, Ferrantini M, Proietti E, Kirkwood JM (2002) Interferon-alpha in tumor immunity and immunotherapy. Cytokine Growth Factor Rev 13:119–134

Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsi K, Pitot HC, Hamid O, Bhatia S, Martins R, Eaton K, Chen S, Salay TM, Alaparthy S, Grosso JF, Korman AJ, Parker SM, Agrawal S, Goldberg SM, Pardoll DM, Gupta A, Wigginton JM (2012) Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med 366:2455–2465

Cheever MA, Schlom J, Weiner LM, Lyerly HK, Disis ML, Greenwood A, Grad O, Nelson WG (2008) Translational research working group developmental pathway for immune response modifiers. Clin Cancer Res 14:5692–5699

Feltkamp MC, Smits HL, Vierboom MP, Minnaar RP, de Jongh BM, Drijfhout JW, ter Schegget J, Melief CJ, Kast WM (1993) Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. Eur J Immunol 23:2242–2249

Gabrilovich DI, Ostrand-Rosenberg S, Bronte V (2012) Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol 12:253–268

Hervas-Stubbs S, Perez-Gracia JL, Rouzaut A, Sanmamed MF, Le Bon A, Melero I (2011) Direct effects of type I interferons on cells of the immune system. Clin Cancer Res 17:2619–2627

Heusinkveld M, van der Burg SH (2011) Identification and manipulation of tumor associated macrophages in human cancers. J Transl Med 9:216

Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbe C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ (2010) Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 363:711–723

Huber JP, Farrar JD (2011) Regulation of effector and memory T-cell functions by type I interferon. Immunology 132:466–474

Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, Redfern CH, Ferrari AC, Dreicer R, Sims RB, Xu Y, Frohlich MW, Schellhammer PF, Investigators IS (2010) Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med 363:411–422

Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP, Drijfhout JW, Wafelman AR, Oostendorp J, Fleuren GJ, Offringa R, van der Burg SH, Melief CJ (2008) Phase I immunotherapeutic trial with long peptides spanning the E6 and E7 sequences of high-risk human papillomavirus 16 in end-stage cervical cancer patients shows low toxicity and robust immunogenicity. Clin Cancer Res 14:169–177

Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP, Essahsah F, Fathers LM, Offringa R, Drijfhout JW, Wafelman AR, Oostendorp J, Fleuren GJ, van der Burg SH, Melief CJ (2009) Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. N Engl J Med 361:1838–1847

Lattanzi L, Rozera C, Marescotti D, D'Agostino G, Santodonato L, Cellini S, Belardelli F, Gavioli R, Ferrantini M (2011) IFN-alpha boosts epitope cross-presentation by dendritic cells via modulation of proteasome activity. Immunobiology 216:537–547

Melief CJM, van der Burg SH (2008) Immunotherapy of established (pre-)malignant disease by synthetic long peptide vaccines. Nat Rev Cancer 8:351–360

Ngiow SF, Teng MW, Smyth MJ (2011) Prospects for TIM3-targeted anti-tumor immunotherapy. Cancer Res 71:6567–6571

Norde WJ, Hobo W, van der Voort R, Dolstra H (2012) Coinhibitory molecules in hematological malignancies: targets for therapeutic intervention. Blood 120:728–736

Quakkelaar ED, Melief CJM (2012) Experience with synthetic vaccines for cancer and persistent virus infections in nonhuman primates and patients. Adv Immunol 114:77–106

Sabbatini P, Tsuji T, Ferran L, Ritter E, Sedrak C, Tuballes K, Jungbluth AA, Ritter G, Aghajanian C, Bell-McGuinn K, Hensley ML, Konner J, Tew W, Spriggs DR, Hoffman EW, Venhaus R, Pan L, Salazar AM, Diefenbach CM, Old LJ, Gnjatic S (2012) Phase I trial of overlapping long peptides from a tumor self-antigen and poly-ICLC shows rapid induction of integrated immune response in ovarian cancer patients. Clin Cancer Res 18:6497–6508

Schwartzentruber DJ, Lawson DH, Richards JM, Conry RM, Miller DM, Treisman J, Gailani F, Riley L, Conlon K, Pockaj B, Kendra KL, White RL, Gonzalez R, Kuzel TM, Curti B, Leming PD, Whitman ED, Balkissoon J, Reintgen DS, Kaufman H, Marincola FM, Merino MJ, Rosenberg SA, Choyke P, Vena D, Hwu P (2011) gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. N Engl J Med 364:2119–2127

Sikora AG, Jaffarzad N, Hailemichael Y, Gelbard A, Stonier SW, Schluns KS, Frasca L, Lou Y, Liu C,

Andersson HA, Hwu P, Overwijk WW (2009) IFN-alpha enhances peptide vaccine-induced CD8+ T cell numbers, effector function, and antitumor activity. J Immunol 182:7398–7407

Spadaro F, Lapenta C, Donati S, Abalsamo L, Barnaba V, Belardelli F, Santini SM, Ferrantini M (2012) IFN-alpha enhances cross-presentation in human dendritic cells by modulating antigen survival, endocytic routing, and processing. Blood 119:1407–1417

Speiser DE, Lienard D, Rufer N, Rubio-Godoy V, Rimoldi D, Lejeune F, Krieg AM, Cerottini JC, Romero P (2005) Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. J Clin Invest 115:739–746

Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Horn L, Drake CG, Pardoll DM, Chen L, Sharfman WH, Anders RA, Taube JM, McMiller TL, Xu H, Korman AJ, Jure-Kunkel M, Agrawal S, McDonald D, Kollia GD, Gupta A, Wigginton JM, Sznol M (2012) Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 366:2443–2454

Tseng CW, Hung CF, Alvarez RD, Trimble C, Huh WK, Kim D, Chuang CM, Lin CT, Tsai YC, He L, Monie A, Wu TC (2008) Pretreatment with cisplatin enhances E7-specific CD8+ T-Cell-mediated antitumor immunity induced by DNA vaccination. Clin Cancer Res 14:3185–3192

Van der Burg SH, Melief CJM (2011) Therapeutic vaccination against papilloma virus induced malignancies. Curr Opin Immunol 23:252–257

Van der Burg SH, Arens R, Melief CJM (2011) Immunotherapy for persistent viral infections and associated disease. Trends Immunol 32:97

van Duikeren S, Fransen MF, Redeker A, Wieles B, Platenburg G, Krebber WJ, Ossendorp F, Melief CJ, Arens R (2012) Vaccine-induced effector-memory CD8+ T cell responses predict therapeutic efficacy against tumors. J Immunol 189:3397–3403

Welters MJ, Kenter GG, Piersma SJ, Vloon AP, Lowik MJ, Berends-van der Meer DM, Drijfhout JW, Valentijn AR, Wafelman AR, Oostendorp J, Fleuren GJ, Offringa R, Melief CJ, van der Burg SH (2008) Induction of tumor-specific CD4+ and CD8+ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine. Clin Cancer Res 14:178–187

Welters MJ, Kenter GG, de Vos van Steenwijk PJ, Lowik MJ, Berends-van der Meer DM, Essahsah F, Stynenbosch LF, Vloon AP, Ramwadhdoebe TH, Piersma SJ, van der Hulst JM, Valentijn AR, Fathers LM, Drijfhout JW, Franken KL, Oostendorp J, Fleuren GJ, Melief CJ, van der Burg SH (2010) Success or failure of vaccination for HPV16-positive vulvar lesions correlates with kinetics and phenotype of induced T-cell responses. Proc Natl Acad Sci U S A 107:11895–11899

Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, Segal NH, Ariyan CE, Gordon RA, Reed K, Burke MM, Caldwell A, Kronenberg SA, Agunwamba BU, Zhang X, Lowy I, Inzunza HD, Feely W, Horak CE, Hong Q, Korman AJ, Wigginton JM, Gupta A, Sznol M (2013) Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med 369:122–133

Zeestraten EC, Speetjens FM, Welters MJ, Saadatmand S, Stynenbosch LF, Jongen R, Kapiteijn E, Gelderblom H, Nijman HW, Valentijn AR, Oostendorp J, Fathers LM, Drijfhout JW, van de Velde CJ, Kuppen PJ, van der Burg SH, Melief CJ (2013) Addition of interferon-alpha to the p53-SLP(R) vaccine results in increased production of interferon-gamma in vaccinated colorectal cancer patients: a phase I/II clinical trial. Int J Cancer 132:1581–1591

Zwaveling S, Ferreira Mota SC, Nouta J, Johnson M, Lipford GB, Offringa R, van der Burg SH, Melief CJ (2002) Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. J Immunol 169:350–358

Dendritic Cell-Based Cancer Vaccines

H. Westdorp, K.F. Bol, M. Coşkuntürk, G. Schreibelt, I.J.M. de Vries, and C.G. Figdor

Introduction on Dendritic Cells

Dendritic cells (DCs) are at the centre of the immune system. As professional antigen-presenting cells (APCs), they have the ability to control both tolerance and immune responses. DCs are capable of initiating primary T- and B-cell responses by presenting a captured and processed antigen to the specific arm of the immune system. Also they are well capable to stimulate naive resting T cells. These unique features make DCs an interesting drug candidate in cancer immunotherapy.

The immune system has the potential to eradicate cancer cells. It has been suggested that tumour growth and its tissue damage activate local DCs and subsequently the immune system (Finn 2003). In other words, the potency of the immune system to eliminate the cancer depends on the tumour size and its immunomodulatory characteristics. However, there is a weakness in this anticancer immunity. First, the expansion of the tumour is a slow and silent process that fails to provoke a 'danger signal' to activate the immune system. Second, neoplastic cells themselves are poor APCs, making the initiation of an immune response difficult. The aim of DC vaccination is restore this inattentiveness of the immune system by educating *ex vivo* DCs. These 'educated' cells are appropriately activated and loaded with tumour antigen.

The first clinical study of a dendritic cell vaccine was reported in Nature Medicine in 1996 (Hsu et al. 1996). Nowadays all over the world, DC-based immunotherapy is studied in clinical vaccination trials with cancer patients focused on inducing or augmenting an anti-tumour immune response.

Immunobiology of Dendritic Cells

Dendritic cells arise from haematopoietic bone marrow progenitor cells. These bone marrow-derived cells migrate into peripheral tissues, where they abide as resting immature DCs. They mainly reside in tissues such as the skin and mucosa, which are in close contact to the outside world. Tissue-resident DCs act as gatekeepers of the immune system that continuously sample the environment to capture antigen. Nonactivated DCs comprise a phenotype, which is mainly characterised by a low surface expression of major histocompatibility complex (MHC) class I and II molecules and co-stimulatory molecules

H. Westdorp • K.F. Bol • M. Coşkuntürk
Department of Tumor Immunology, Radboud Institute for Molecular Life Sciences, Geert Grooteplein 26, Nijmegen, GA 6525, The Netherlands

Department of Medical Oncology, Radboud University Medical Centre, Geert Grooteplein 8, Postbus 9101, Nijmegen, HB 6500, The Netherlands
e-mail: harm.westdorp@radboudumc.nl

G. Schreibelt • I.J.M. de Vries • C.G. Figdor (✉)
Department of Tumor Immunology, Radboud Institute for Molecular Life Sciences, Geert Grooteplein 26, Nijmegen, GA 6525, The Netherlands
e-mail: c.figdor@ncmls.ru.nl

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_8,

(Banchereau and Steinman 1998). These immature cells have an extreme efficient ability to capture and process antigens, a feature mediated by high endocytotic activity and expression of an array of cell surface receptors capable to uptake antigens that could harm the host (Sallusto and Lanzavecchia 1994; Jiang et al. 1995).

Antigens are divided into two main groups: exogenous antigens derived from extracellular pathogens such as bacteria and yeasts as well as endogenous antigens consisting of self-proteins or viral proteins. DCs capture these antigens through several complementary mechanisms (Trombetta and Mellman 2005). After internalisation they process antigenic proteins into peptides that bind to MHC class I and II molecules. Exogenous and endogenous antigens are then presented on the cell surface as MHC–peptide complexes of class II and I molecules, respectively. An interesting finding for DC-based immunotherapy is that exogenous antigens, like from apoptotic or necrotic neoplastic cells, may also be present in MHC class I molecules. This unique feature, a process called 'cross-presentation', allows DCs to present cancer antigens to $CD8^+$ T cells (Bevan 1976). The immune response of this entire process strongly depends on the maturation state of DCs. Immature DCs receiving inhibitory signals, such as interleukin-10 (IL-10) or corticosteroids, induce immune tolerance via T cell deletion or induction of regulatory T cells (T_{Regs}), whereas mature DCs induce immunity.

A strictly controlled series of events are important to convert an immature DC into a mature cell that is well antigen-loaded for peptide presentation and T cell activation. Preparatory to this process, a 'danger' signal, derived from tissue damage or microbial products, is required (Reis e Sousa 2004). Subsequently a process of downmodulation of endocytic and phagocytic receptors and upregulation of chemokine receptors CCR7 and CD62L takes place. Surface expression of MHC class I and II and co-stimulatory molecules CD40, CD58, CD80, CD83 and CD86 are upregulated, and morphology of the DC lysosomal compartment changes (Banchereau and Steinman 1998). Finally, the process of maturation goes in parallel with the migration of DCs into the draining lymph node.

In lymphoid tissues activated DCs present MHC–peptide complexes to naive T cells. The interaction between an MHC–peptide complex and T cell receptor (signal 1), stimulation of co-stimulatory molecules from DC to the T cell (signal 2) and cytokines in the microenvironment (signal 3) lead all together to the activation of T cells. Activated T cells proliferate, leave the lymph nodes and circulate through the body in search of cells that express antigen. In addition, DCs are also able to directly activate natural killer (NK) cells (Fernandez et al. 1999) and can produce large amounts of interferon in response to virus encounters (Kadowaki, Antonenko et al. 2000).

Dendritic Cell Subsets

DCs consist of a heterogeneous population of cells. In mice and human, two major subsets of DCs can be distinguished: myeloid DCs (mDCs; also known as conventional or classical DCs) and plasmacytoid DCs (pDCs). These subsets differ in function, localisation and phenotype. mDCs mainly migrate to or reside in the marginal zone of the lymph nodes (a primary entry point for blood-borne antigens), whereas pDCs mainly reside in the T cell areas (Banchereau and Steinman 1998; Liu 2001). Human subsets can be distinguished by the differential expression of three surface molecules: firstly, CD303, which is also known as BDCA2 or CLEC4C; secondly, CD1C, known as BDCA1; and finally, CD141, known as BDCA3 or thrombomodulin (Dzionek et al. 2000).

Natural DC subsets express various Toll-like receptors (TLRs) and therefore respond differently to pathogenic stimuli, i.e. each subset has a specialised function in directing immune responses (Schreibelt et al. 2010). A large quantity of data suggests that mDCs mainly recognise and respond to bacterial and fungal antigens, whereas pDCs represent a front line of immunity against viral infections (Siegal et al. 1999). More recent observations suggest that both pDCs and mDCs may be of importance for the induction of anti-tumour responses with and without DC-based immunotherapy.

The production of a DC vaccine requires a large amount of DCs. However, human peripheral blood leukocytes consist only about 0.2 % of natural DCs. The immunological community has developed several ways to generate DCs from precursors. In 1994 this resulted in the discovery that DCs can be generated from monocytes or $CD34^+$ progenitors by culture in the presence of IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF). This finding allowed the acquisition of these otherwise scarce cells in considerable numbers which were essential to conduct clinical trials (Romani et al. 1994).

Plasmacytoid Dendritic Cells

Human pDCs form a rare subpopulation of cells. They are a destitute of lineage markers and myeloid antigens and do not express CD11c. pDCs express CD303 and CD304 (also known as BDCA4 or neuropilin-1) (Dzionek et al. 2000). In the steady state they are round, non-dendritic and relatively long-lived cells. After receiving inflammatory stimuli, pDCs develop a dendritic cell morphology and function. Most notably, pDCs are the major producers of type I interferon (IFN), which is important in defence against viruses, and are therefore thought to be crucial to antiviral immunity (Colonna et al. 2004). Presynthesised stores of MHC class I molecules allow induction of a rapid $CD8^+$ T cell response to viral infections (Di Pucchio et al. 2008). pDCs can induce strong allogeneic T cell responses and prime $CD4^+$ and $CD8^+$ T cells against tumour and viral antigens (Cella et al. 2000; Fonteneau et al. 2003). Studies have shown that antigen-loaded pDCs induce inhibition of tumour growth and initiate T cell responses (Salio et al. 2004; Takagi et al. 2011). Furthermore, cytokines and surface signalling from activated pDCs can also induce the maturation of B cells into plasma cells (Jego et al. 2003; Shaw et al. 2010). pDCs reside in blood as well as in several lymphoid organs, and some studies suggest functional differentiation between different tissue-residing pDCs (Hochrein et al. 2004).

Myeloid Dendritic Cells

In peripheral blood mDCs are distinguished by the expression of myeloid markers, such as CD13 and CD33. They lack lineage-specific markers (CD3, CD14, CD19 and CD56) but express MHC and CD11c molecules. mDC can be further subdivided into three classes based on differential surface expression of CD1C, CD141 and CD16 (Schreibelt et al. 2010). All these subsets differ in their expression of cell surface markers and potency to stimulate T cells (MacDonald et al. 2002; Lindstedt et al. 2005; Piccioli et al. 2007). As an example, the C-type lectin receptor (CLR) CLEC9a is expressed only by $CD141^+$ mDCs (Huysamen et al. 2008). These $CD141^+$ mDCs have a high capacity to internalise exogenous antigens for presentation on MHC class I molecules (cross-presentation) and are essential for the generation of $CD8^+$ T cell-mediated immune responses (Bachem et al. 2010; Crozat et al. 2010). Further, mDCs express two extracellular TLRs on the cell surface that recognise exterior components of bacteria and fungi, e.g. cell wall components such as lipopolysaccharide (TLR4) and peptidoglycan (TLR2). TLR3 and TLR8 are expressed intracellularly to respond to viral RNA. Activated mDCs, especially $CD1C^+$ and $CD141^+$, are mainly producing IL-12, which enables the generation of INF-γ-secreting type 1 $CD4^+$ T cells and the priming of naive $CD8^+$ T cells, i.e. they regulate the differentiation of naive T cells into antigen-specific effector T cells to augment a cellular immune response (Meixlsperger et al. 2013; Schlitzer et al. 2013).

It has been suggested that mDCs and pDCs cooperate and act synergistically (Lou et al. 2007; Piccioli et al. 2009). This could open up the possibility to combine mDCs with pDCs in cancer immunotherapy. Future research will address whether mDC–pDC cross-talk could improve anti-tumour responses in cancer patients.

Ex Vivo-Generated Dendritic Cells

Up to now, most clinical studies have been conducted with *ex vivo*-generated 'monocyte-derived dendritic cells' (moDCs) (Sallusto and Lanzavecchia 1994). Monocytes are pre-DCs that originate from myeloid progenitor cells. They are easily obtained by leukapheresis. In vivo, monocytes are capable of transforming into DCs after sensing inflammatory signals and are important for the replenishment of dendritic cells in the

host. *Ex vivo*, stimulation with GM-CSF and IL-4 differentiates monocytes into immature moDCs over a period of 3–5 days (Romani et al. 1994; Sallusto and Lanzavecchia 1994). Subsequent maturation can be achieved by addition of cytokines such as tumour necrosis factor-α (TNF-α), prostaglandin E2 (PGE2), IL-1β, IL-6 or monocyte-conditioned medium (MCM), the supernatant of activated autologous monocytes (Jonuleit et al. 1997; de Vries et al. 2002). This allows the generation of large quantities ($>500 \times 10^6$) of clinical grade DCs from a single leukapheresis (Romani et al. 1994; Sallusto and Lanzavecchia 1994). Although *ex vivo*-generated moDCs share many phenotypic and functional characteristics with circulating mDCs, it remains unclear to what extent they resemble natural blood DCs.

In addition to monocytes, $CD34^+$ progenitors in blood are also used to generate DCs for vaccination of cancer patients. In the presence of GM-CSF, Flt3L and TNF-α, these progenitors are cultured in about a week (Banchereau et al. 2001). They consist of two populations: one with Langerhans cell-like properties and another with dermal DCs with properties resembling moDCs. Yields from leukapheresis after in vivo Flt3L expansion and negative selection *ex vivo* are much lower than for monocytes (Fong et al. 2001).

Most clinical DC vaccination studies use monocytes to generate DCs *ex vivo*. However, immunological and clinical responses have been observed in cancer patients vaccinated with monocyte-derived DCs as well as with $CD34^+$ progenitor-derived DCs (Banchereau et al. 2001; Figdor et al. 2004; Lesterhuis et al. 2008).

Dendritic Cell Maturation

Immature DCs are primarily involved in the recognition and internalisation of antigens. Certain changes take place when these immature cells receive maturation signals, e.g. they change their chemokine receptor repertoire, downmodulate endocytic and phagocytic receptors and upregulate their co-stimulatory molecules. During this maturation process immature DCs acquire the phenotype and functionality of mature DCs. Activated DCs are capable to migrate to the lymph nodes and activate T cells. In the absence of these signals, DCs would not upregulate their co-stimulatory molecules and thus remain anergy- or tolerance-inducing APCs.

DC maturation is a highly complex process. In order to elicit a productive immune response, DCs need a proper activation by adjuvants. Maturation should be regarded as a flexible process of which the outcome is depending on the type of signals a DC receives in the periphery. Different adjuvants activate DCs through different molecular pathways, resulting in various types of T cell responses (Maldonado-Lopez et al. 1999; Pulendran et al. 1999). Normally, in vivo, these signals primarily are derived from pathogens or tissue injury (Matzinger 2002; Skoberne et al. 2004). *Ex vivo* maturation can be achieved by coculturing the DCs with several stimuli such as cytokines (Thurner et al. 1999), pathogen-associated triggers (Reis e Sousa 2004) or endogenous ‘danger signals’, e.g. heat shock proteins (Singh-Jasuja et al. 2000).

In most clinical studies, immature or semi-mature monocyte-derived DCs have been used (Figdor et al. 2004). Worth mentioning is that studies show that when the immunogenicity of immature and mature DCs is compared, maturation is essential for the induction of immune responses in cancer patients (Jonuleit et al. 2001; de Vries et al. 2003b). Besides, the use of mature DCs appears to be associated with a better clinical outcome compared to patients receiving immature DCs (de Vries et al. 2003b; McIlroy and Gregoire 2003). This difference may partly be explained on the basis of some essential features of mature DCs. Firstly, after intradermal or subcutaneous injection, mature DCs have a better migratory capacity to the draining lymph nodes compared to their immature counterparts. Secondly, within the lymph node, mature DCs show a pronounced migration into the T cell areas where antigen presentation takes place, whereas immature DCs remain at the periphery (De Vries et al. 2003a). And lastly, mature DCs also have a higher expression of MHC and co-stimulatory molecules. Together this leads to superiority of mature DCs in antigen presentation and therefore in inducing T cell responses.

Cytokine Maturation Cocktails

Maturation of DCs can be induced by pro-inflammatory cytokines such as IL-1β or IL-6. Several maturation methods have been applied with maturation being defined by a high expression of mature DC-specific surface markers such as CD80, CD83, CD86 and MHC molecules. Since the maturation stage of DCs cannot be fully characterised by the expression of co-stimulatory molecules and surface MHC, it is therefore of crucial importance that the phenotypic and functional characteristics of the used DCs are carefully described when reporting clinical DC vaccination trials (Figdor et al. 2004). For example, functionality of DCs can be measured by the production of IL-12, a cytokine that plays an essential role in the differentiation of T cells into T helper 1 (T_H1) cells. The most widely used method to mature immature DCs is a cytokine cocktail that includes TNF-α, with any of the following cytokines in any combination: IL-1β, IL-6, PGE2 or MCM which was used in early clinical studies (Jonuleit et al. 1997; Thurner et al. 1999; Schuler-Thurner et al. 2000; de Vries et al. 2002; Schuler-Thurner et al. 2002). There is some evidence that culturing DC with IL-15 may lead to a type of mature DC that induces stronger T_H1 type of immune responses (Pulendran et al. 2004). However, no comparative studies have been reported yet. Lastly, also CD40 ligation has been used as a method of activation of DCs in a clinical setting (Davis et al. 2006; Palucka et al. 2006).

In addition, another level of complexity is added by the timing and duration of the maturation signal. Different cytokine cocktails require different lengths of maturation periods and can induce some dissimilarity in expression of co-stimulatory molecules and cytokine production of the DCs (Langenkamp et al. 2000; Camporeale et al. 2003). None of these different maturation methods has shown to be clearly superior, which is mainly due to the fact that there are no direct comparative studies, although the use of PGE2 for maturation may negatively affect DC function because of less IL-12 production (Kalinski et al. 2001; Kaka et al. 2008).

Maturation via Toll-Like Receptors

Besides cytokine maturation cocktails, another DC maturation method is triggering TLRs by using TLR ligands. TLRs are part of the pattern recognition receptors (PRRs). These receptors are sensors providing DCs to detect pathogens (Akira and Takeda 2004). Triggering of TLRs may be a more natural route to induce DC maturation. During evolution, the immune system has acquired various receptor families that recognise several crucial molecular components of pathogens. This set of pathogen-associated molecular patterns (PAMPs) recognised by the immune system is limited and constituted mostly of general molecular patterns that are absent on cells of the host and are essential for survival of the microbe. DCs sense their environment through both intracellular and surface receptors. These receptors comprise several families: CLRs, intracellular and surface TLRs and intracellular helicases (Zhang et al. 2011). The TLR family is best characterised as PRR and recognises the most diverse group of PAMPs. Nowadays 15 mammalian Toll-like receptors are found (TLR1–TLR15), of which 10 are expressed in humans (Iwasaki and Medzhitov 2004; Akira et al. 2006).

The Toll-like receptors TLR1–TLR9 can be divided into two main groups: extracellular TLRs that are found on the cell surface (TLR1, TLR2, TLR4, TLR5 and TLR6) and the intracellular TLRs that are located in endosomal compartments (TLR3, TLR7, TLR8 and TLR9). In general, intracellular TLRs recognise nucleotide-containing structures, for example, RNA molecules (TLR3, TLR7 and TLR8) and unmethylated CpG DNA (TLR9), originating from viruses and bacteria. Extracellular TLRs recognise exterior components of bacteria and fungi, e.g. cell wall components. Besides PAMPs derived from pathogens, TLRs have been proposed to recognise also endogenous ligands, such as heat shock proteins or necrotic cells (Asea et al. 2002; Vabulas et al. 2002). DC maturation varies according to different PAMPs that trigger different PPRs; thus the signalling pathways associated with ligation of the different TLRs are not identical and therefore distinct biological responses are initiated. Ligand binding of

TLRs recruits one or more adaptor molecules. The difference in signalling outcome (e.g. variation in cytokine production) can be explained in part by the use of different adaptor molecules by the TLRs. The binding partners of the recently discovered TLR10–TLR15 are less well known.

Both moDCs and mDCs express TLR1–TLR8 and not TLR9 (Krug et al. 2001; Ito et al. 2002) and only mDCs express TLR10. They respond to specific ligands of these TLRs, leading to a mature phenotype and production of pro-inflammatory cytokines (Krug et al. 2001; Ito et al. 2002; Matsumoto et al. 2003; Means et al. 2003; Renn et al. 2006). However, some significant differences between moDCs and mDCs in TLR expression and ligand reactivity were found. TLR1, TLR2 and TLR6 are expressed by both moDCs and mDCs (Kadowaki et al. 2001; Krug et al. 2001; Matsumoto et al. 2003; Renn et al. 2006). Through the formation of heterodimers with TLR1 or TLR6, TLR2 gains the capacity to bind a wide variety of bacterial and yeast-derived ligands. Consequently, this plays a central function in pathogen recognition by DCs. Activation of TLR1, TLR2 and TLR6 leads to DC maturation and secretion of several cytokines important in immune system activation, especially IL-6, IL-8, IL-10, IL-12 and TNF-α. TLR9 is only expressed by pDCs in the human setting and is responsible for a very high type I IFN response (Gilliet et al. 2008). Most notably, moDCs show negligible TLR10 expression, whereas blood mDCs do express TLR10. Unfortunately, the ligands and functionality of TLR10 are still unknown.

pDCs show a more distinct pattern of TLRs compared to moDCs and mDCs. They abundantly express TLR7 and TLR9 in their endosomal compartments. In addition, triggering of TLR7 and TLR9 on pDCs leads to high type I IFN secretion and a typical mature DC phenotype. Interestingly, it has been suggested that in human pDCs, TLR9 displays a unique feature that is not shared by the other described TLRs. Depending on the stimulus, activation of TLR9 on human pDCs can have different outcome. The dual function of TLR9 is attributed to the distinct intracellular locations where TLR9 can be triggered. It can either activate an innate immune response via IFN-α secretion after encountering nucleic acids via early endosomes or activate an adaptive immune response by IL-6 and TNF-α secretion in case of late endosomes (Guiducci et al. 2006).

With respect to the type of TLR ligands, it has been shown that combinations of different TLR ligands can have a synergistic effect on the immunogenic potential of DC *ex vivo* (Napolitani et al. 2005) and in vivo (Warger et al. 2006). It was identified that preventive vaccines against infectious diseases contain TLR ligands that are capable of inducing DC maturation. The combination of these clinical grade TLR ligands and PGE2 resulted in the generation of mature DCs that secrete high levels of IL-12, IFN-γ and TNF-α (Boullart et al. 2008). With the discovery of those promising *ex vivo* data, the potency of these TLR ligand-activated monocyte-derived and natural blood DCs is being explored in clinical trials. Despite their low number of natural blood DCs, the first results indicate that these cells are extremely potent in initiating immune responses in cancer patients (Schreibelt et al. 2010).

Dendritic Cell Antigen Loading

The MHC molecules of a mature DC must be loaded with relevant tumour antigens in order to initiate an anti-tumour immune response. Preferably, tumour antigens are presented by DCs to both $CD4^+$ T helper cells (in MHC class II) and $CD8^+$ cytotoxic T cells (in MHC class I). There is convincing preclinical evidence that targeting both T cells is important for the induction of a strong and sustained anti-tumour T cell response.

Peptide- or Protein-Pulsed Dendritic Cells

Several techniques have been developed to load human DCs with tumour-associated antigens (TAAs). The most widely used method is incubation of DCs with human leukocyte antigen (HLA)

class I-binding peptides, which can bind directly to MHC molecules on the cell surface (Nestle et al. 1998; de Vries et al. 2003b; Ribas et al. 2004; Banchereau et al. 2005). In some clinical vaccination studies, HLA class I-binding peptides are combined with class II-binding peptides to support the activation of T helper cells (Schuler-Thurner et al. 2002; Schadendorf et al. 2006).

The immune response, if it is induced, is restricted to the used epitope(s). On the opposite side, it has been described that death of tumour cells after vaccination against a single epitope results in a release of other tumour antigens, a phenomenon known as 'antigen-spreading'. These 'novel' antigens can subsequently be taken up by DCs and presented to T cells, resulting in T cell responses against antigens that were not included in the vaccine (Butterfield et al. 2003).

Alternatively to HLA-binding peptides, peptides can be endogenously loaded onto MHC molecules after proteolytic processing of recombinant protein or endocytosed tumour lysates. The DC processes the protein into peptides, which has the advantage that multiple epitopes are presented in both MHC class I and II and that it does not require upfront consideration of HLA restriction. Unfortunately, only few recombinant proteins are available in clinical grade (Gilboa 2007).

Autologous (Nestle et al. 1998; Griffioen et al. 2004; Hersey et al. 2004; Ridolfi et al. 2006) or allogeneic (Berard et al. 2000; Palucka et al. 2006; Salcedo et al. 2006) tumour cell lysates have also been applied as a source of antigens. This has several advantages: (1) the antigen expression by the tumour does not need to be defined and (2) a wide array of both MHC classes I and II epitopes are presented including tumour-unique antigens. Possible drawbacks of this method are the presentation of autoantigens, the requirement of a sufficient volume of tumour tissue for preparation of the lysate and difficulties in monitoring tumour-specific T cell responses since the antigens relevant to T cell responses are not known.

New prospects within this field include sequencing of tumours and targeting on mutated proteins that contain MHC-binding peptides, thus increasing the number of potentially immunogenic tumour-specific antigens (Dutoit et al. 2012).

mRNA Transfected Dendritic Cells

Transfection of DC with RNA is another antigen-loading technique (Sullenger and Gilboa 2002). This method uses tumour-derived RNA (Nair et al. 2002; Kyte et al. 2005) or synthetic RNA-encoding full-length tumour antigens (Schaft et al. 2005). A frequently applied technique of RNA transfection is RNA electroporation. Application of an electric field provides transient permeabilisation of the plasma membrane, thereby allowing entrance of RNA into the cell. A benefit of this technique lies in the presentation of several MHC class I and class II epitopes, depending on the presence of an endosomal targeting sequence (Bonehill et al. 2004). Also, it may lead to a more prolonged presentation of the antigen as compared to peptide loading which appears to be short-lived (Laverman et al. 2006). Disadvantages of RNA transfection include a variable expression and a low yield of viable cells after transfection. However, this goes without loss of phenotype and maturation potential of these viable cells. mRNA electroporation is more efficient compared to plasmid DNA electroporation, and since it is a nonviral method of transfection, the RNA lacks the potential to integrate in the host genome and thereby obviating safety concerns associated with clinical gene therapy trials.

Although tumour-derived RNA potentially harbours tumour-specific epitopes of mutated genes, it has the additional disadvantage that an unknown number of autoantigens are also presented. However, several studies have shown that this technique is feasible and results in highly efficient DC transfection (Ponsaerts et al. 2002; Ueno et al. 2004; Schaft et al. 2005). Furthermore, anti-tumour T cell responses and some evidence for clinical activity have been reported in patients vaccinated with DC electroporated with tumour-derived RNA (see Fig. 1) (Kyte et al. 2006; Kyte et al. 2007).

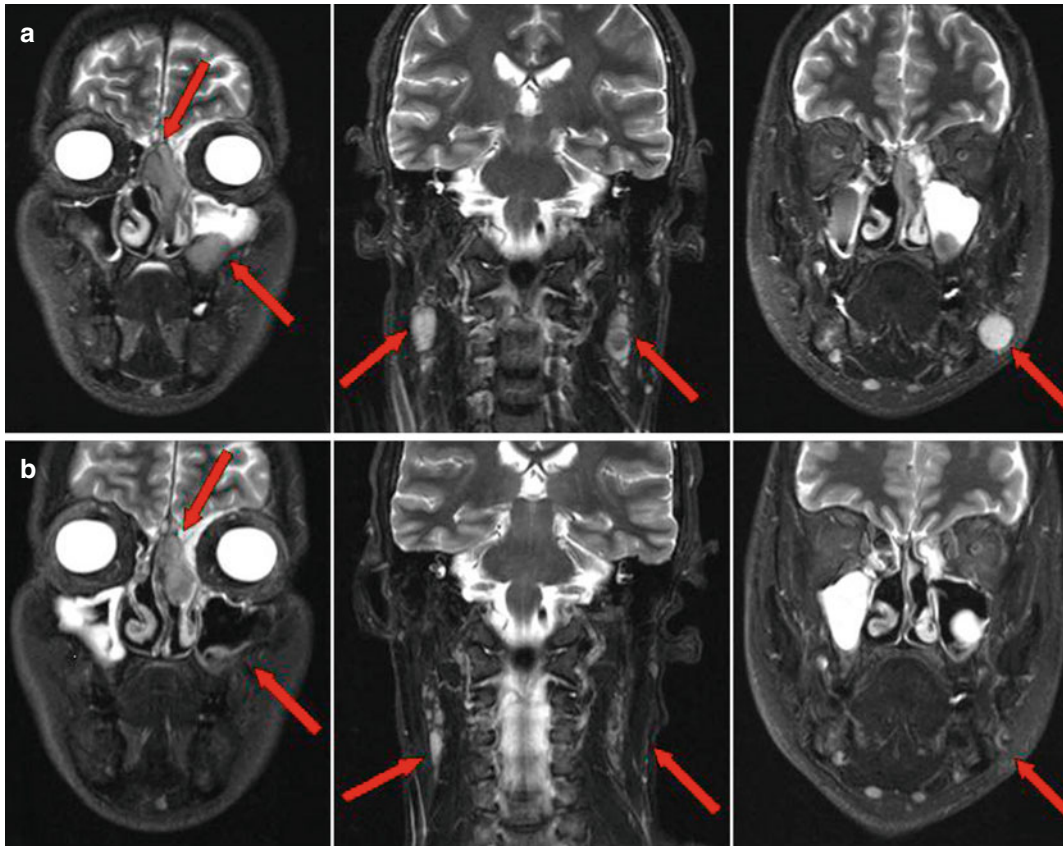

Fig. 1 Response after RNA-transfected moDC vaccinations: A patient with primary melanoma of the nasal mucosa (*left side*) with bilateral cervical lymph node metastases (*in the middle*) and a submandibular metastasis (*right side*). The localizations of the primary melanoma and the metastases are indicated with *red arrows*. (**a**) Shows the situation before moDC vaccinations. (**b**) After 1 cycle of moDC vaccinations, a significant reduction of the primary tumour and the metastases was seen. These radiological images are published with the permission of the patient

Furthermore, RNA technology can be exploited not only to improve antigen presentation but also to improve DC maturation and T cell stimulation. For example, it is shown that the T cell stimulatory capacity of peptide-pulsed DC can be greatly enhanced by providing them with three different molecular adjuvants. Through electroporation with mRNA encoding, the so-called TriMix of CD40 ligand (CD40L), CD70 and a constitutively active form of TLR4 (caTLR4) is generated, which induces T cell stimulation. The combination of CD40L and caTLR4 electroporation would mimic CD40 ligation and TLR4 signalling of the DC and generates phenotypically mature, cytokine-secreting DCs. Additionally, the introduction of CD70 into the DC would provide a co-stimulatory signal to $CD27^+$ naive T cells by inhibiting activated T cell apoptosis and by supporting T cell proliferation (Bonehill et al. 2008).

Applicability of Vaccines Against Cancer

Dendritic cell-based immunotherapy is explored worldwide in clinical vaccination trials (Lesterhuis et al. 2004). Most trials use autologous *ex vivo*-cultured, antigen-loaded, monocyte-derived or $CD34^+$ progenitor-derived DCs (see also Fig. 2). In recent years over 100 clinical studies have been carried out in cancer patients. The most studied cancer type in DC immunotherapy is melanoma, consisting of tumour differentiation antigens such

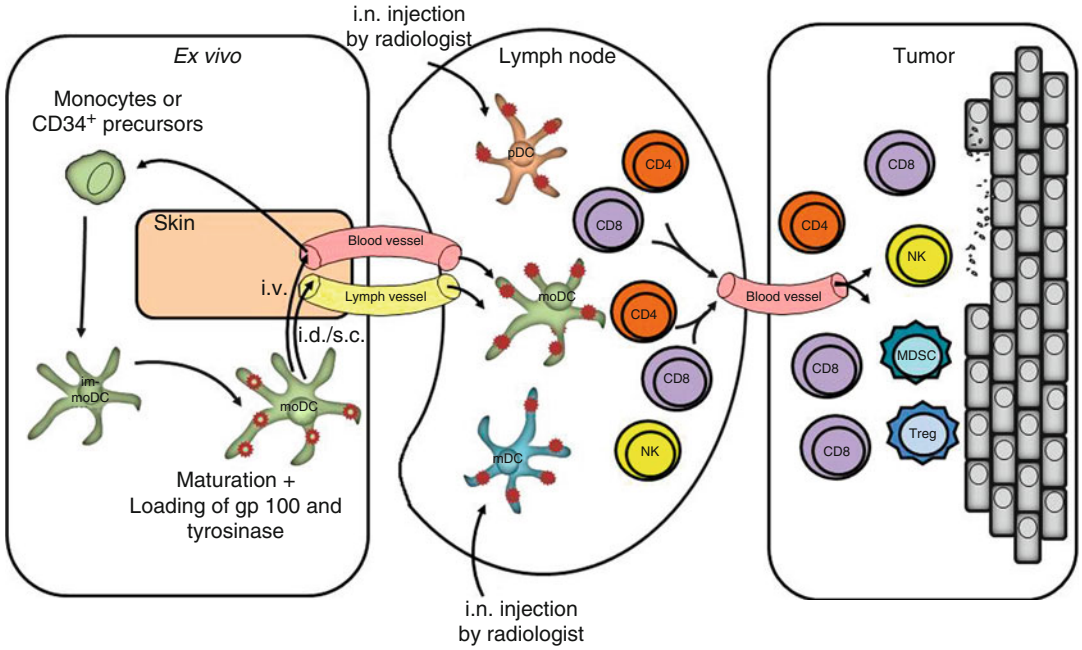

Fig. 2 The induction of tumour antigen-specific T cells via dendritic cell vaccination: DCs cultured from monocytes or CD34+ progenitor cells can be loaded with tumour antigen *ex vivo* and administered to cancer patients via different routes, after culture in the presence of pro-inflammatory cytokines for maturation. Within the lymph node DCs present antigens to T cells, in combination with a co-stimulatory signal, to initiate an immune response. The activated tumour antigen-specific T cells proliferate and migrate out of the lymph node towards the site of the antigen, the tumour site. At the tumour site MDSCs and T_{Regs} are able to create an immunosuppressive microenvironment, inducing peripheral tolerance and complicating tumour clearance by T cells. Exploiting natural DC subsets can be performed by isolating either pDCs or mDCs and stimulating them *ex vivo* with adjuvant and antigen (not shown). Both pDCs and mDCs are administered intranodally in a benign lymph node by the radiologist in order to stimulate T cells. Cross-talk between both DC subsets may also stimulate other immune cells such as NK cells. *Im-moDC* immature monocyte-derived dendritic cell, *moDC* mature monocyte-derived dendritic cell, *i.d.* intradermal, *s.c.* subcutaneous, *i.v.* intravenous, *i.n.* intranodal, *mDC* myeloid dendritic cell, *pDC* plasmacytoid dendritic cell, *CD4, CD4+* T helper cell, *CD8, CD8+* cytotoxic T cell, *NK* natural killer cell, *MDSC* myeloid-derived suppressor cell, T_{Reg} regulatory T cell

as gp100 and tyrosinase and tumour-specific antigens such as melanoma-associated antigen 3 (MAGE-3) (Rosenberg 1999). Other reasons are that melanoma is considered as one of the most immunogenic tumours and that no first-line treatment is available which improves overall survival in case of metastatic disease (Balch al. 2001; Eggermont and Kirkwood 2004; Ives et al. 2007).

Tumour types that also have been investigated using DC vaccines include colon cancer, renal cell carcinoma, breast cancer, ovarian cancer, nasopharyngeal carcinoma, hepatocellular carcinoma, pancreatic tumours, adrenal carcinoma, cholangiocarcinoma, parathyroid carcinoma, non-small cell lung cancer, head and neck cancer, sarcoma, bladder cancer, glioma and paediatric malignancies (Schott et al. 1999; Brossart et al. 2000; Geiger et al. 2001; Toungouz et al. 2001; Holtl et al. 2002; Lin et al. 2002; Stift et al. 2003; Yamanakaet al. 2003). In most of these studies, DCs were cultured without maturation stimuli. Because of the highly individual DC vaccines in these cancer types, there is a large interest in developing strategies to target DCs in vivo.

Clinical Results

The first proof of principle studies exploring DC vaccination was performed in the late nineties of the last centuries, showing the feasibility and the potential efficacy of DC vaccination in cancer

patients (Hsu et al. 1996; Nestle et al. 1998; Thurner et al. 1999). DC vaccines have proven to be safe with only minimal side effects in multiple phase I and/or II trials in adults (Banchereau et al. 2001; Schuler-Thurner et al. 2002; de Vries et al. 2003b) as well as in children (Carusoet al. 2005). Side effects were mostly limited to transient chills, fever, fatigue, nausea and headache. Although immunological responses are often reported using several immune monitoring methods and different culture protocols, objective clinical responses remain anecdotal with objective response rates not exceeding 5–15 %, with disease stabilisation and mixed responses being observed more often (Lesterhuis et al. 2008). Interestingly however, in cases where clinical responses were induced, these were often long-lasting (Rosenberg et al. 2004).

Previously very few phase III DC-based studies were performed, mainly because it was thought that the vaccines had not yet reached their full potential, but also because financial support was hard to obtain since most companies were not interested in producing laborious patient-specific vaccines. Less than 2 years after one of the first publications on dendritic cell therapy was published (Nestle et al. 1998), a prospective phase III trial was initiated in 2000 that compared standard dacarbazine chemotherapy with a dendritic cell vaccine as first-line treatment of patients with metastatic melanoma (Schadendorf et al. 2006). The trial was prematurely discontinued at the first interim analysis after the inclusion of 103 patients owing to lack of efficacy. The authors identified several possible negative contributing factors, including a variable quality of the dendritic cell vaccine among participating centres and a suboptimal maturation state, dose and route of administration of the DCs. In retrospect, this trial was carried out too soon and was performed at a time when DC vaccination was too early in its development. Although this trial could be interpreted as a negative trial for DC vaccination in melanoma patients, it was perhaps not a bad starting point.

Few years back, sipuleucel-T (Provenge, Dendreon) was introduced, a cell-based vaccine composed of enriched blood APCs that are cultured with fusion protein of prostatic acid phosphatase (PAP) and GM-CSF. The United States Food and Drug Administration (FDA) has approved sipuleucel-T for the treatment of metastatic prostate cancer. Sipuleucel-T is the first cellular immunotherapy ever to be approved for any malignancy. This decision of the FDA was made after three placebo-controlled phase III randomised trials (Small et al. 2006; Higano et al. 2009; Kantoff et al. 2010). Sipuleucel-T *ex vivo* activation of APCs generates long-lived immune responses in vivo, and antigen-specific memory T cell responses have been assessed by IFN gamma ELISPOT. The proposed mechanism of sipuleucel-T is inducing these antigen-specific immune responses and thereby targeting prostate cancer (Sheikh et al. 2013). Treatment with this vaccine prolonged median overall survival (OS) with approximately 4 months (Small et al. 2006). However, no difference could be shown in time to prostate-specific antigen increase (Beer et al. 2011) or in terms of improvement in progression-free survival (PFS) (Small et al. 2006; Kantoff et al. 2010). The OS benefit of sipuleucel-T cannot be fully explained by the recorded immune responses, and further research is currently ongoing to define additional biomarkers that could be related with increased survival.

Within the next decade the field will have to demonstrate maturity and not only yield a higher percentage of clinically responding patients but preferentially also develop means to predict in an early stage which patients will likely benefit from DC-based vaccines and which will not. Only this will warrant the implementation of DC vaccination in the long run as the preferred form of standard therapy. So, we will not only have to improve our vaccines but also our monitoring tools. The latter will not only be beneficial for DC vaccination but also for other forms of immunotherapy.

Novel Concepts and Future Perspectives

The immunological and clinical responses in clinical trials thus far support the concept of DC-based immunotherapy. Nevertheless, a number of

variables need to be evaluated and controlled to further improve clinical outcome in DC vaccination in more patients. Among these are optimisation of the *ex vivo* generation of DCs, the use of different natural DC subsets, route of administration, maturation stimuli for DCs (Boullart et al. 2008) and antigen loading of DCs (Figdor et al. 2004). These variables are still subject of an ongoing debate, but one can clearly conclude that the potential of DC-based immunotherapy has not yet been fully exploited. The next hurdle to take is the local immune suppressive environment created by the tumour. No matter how effective a DC vaccine is developed, an immunosuppressive tumour microenvironment is considered catastrophic. To circumvent or tackle these hurdles, novel concepts are under development to further improve DC-based immunotherapy.

Natural Dendritic Cell Subsets

It remains unclear whether DCs, differentiated *ex vivo* from precursor cells, are the optimal source of DCs for the induction of potent immune responses. It may be possible that the extensive culture period (7–9 days) and compounds required to differentiate DCs negatively affect their functionality. For example, exhaustion of cells may affect the migration capacity towards the site of T cell interaction (Soruri et al. 2003; Breckpot et al. 2005). Therefore, it is attractive to consider alternative DC sources, such as mDCs and pDCs. These natural DCs may not require extensive culture. However, in order to be effective, they must be activated through molecularly defined triggers, such as TLRs or CD40 ligand, prior to reinfusion (Krug et al. 2001). This is important, because nonactivated or improperly activated DCs might result in T cell tolerance (Steinman and Nussenzweig 2002).

A recent study used natural pDCs as a therapeutic vaccine against cancer. Fifteen patients with stage IV metastatic melanoma received intranodal injections of *ex vivo* tumour antigen-loaded pDCs (see also Fig. 2). Scintigraphic imaging showed that administered pDCs were capable to distribute over multiple lymph nodes. The pDCs were also capable to induce tumour antigen-specific $CD4^+$ and $CD8^+$ T cell responses. Although the number of patients did not allow drawing significant conclusions, the observed clinical outcomes were still interesting. Two subjects showed stable disease and were eligible for two additional vaccination cycles. One patient showed regression of lung metastasis, but with progression of a nodal metastasis. All subjects were compared with matched control patients who received standard dacarbazine. The median PFS in the vaccine group was 4.0 versus 2.1 months in the control group. The median OS was 22.0 versus 7.6 months for the pDC treatment group and the control group, respectively (see Fig. 3). In summary, this study showed that vaccination with natural pDCs is feasible with minimal toxicity and capable to induce both immune responses and preliminary signs of clinical efficacy in patients with metastatic melanoma (Tel et al. 2013).

Natural DCs might represent the next generation of anticancer immunotherapy to induce antitumour responses and improve clinical efficacy. Several studies even suggest that pDCs and mDCs may cooperate and act synergistically, generating stronger cancer-specific responses than moDC-based vaccinations.

Combating Tumour Escape Mechanisms

The accrual of immunosuppressive cells at the tumour site, and creating an immunosuppressive niche, is another mechanism contributing to tumour escape (see Fig. 2). This well-known type of cell that can suppress the immune system and plays a key factor in peripheral tolerance is the regulatory T cell. T_{Regs} are specific T cells that control immune responses and maintain immunological self-tolerance by downregulating the activation and expansion of self-reactive T cells without killing them. By this, autoreactivity and immune-mediated responses by effector T cells are in balance. A deficiency of effector T cells may lead to serious infections, while a deficiency of T_{Regs} can cause autoimmunity. T_{Regs} are not only capable of infiltrating a site of infection,

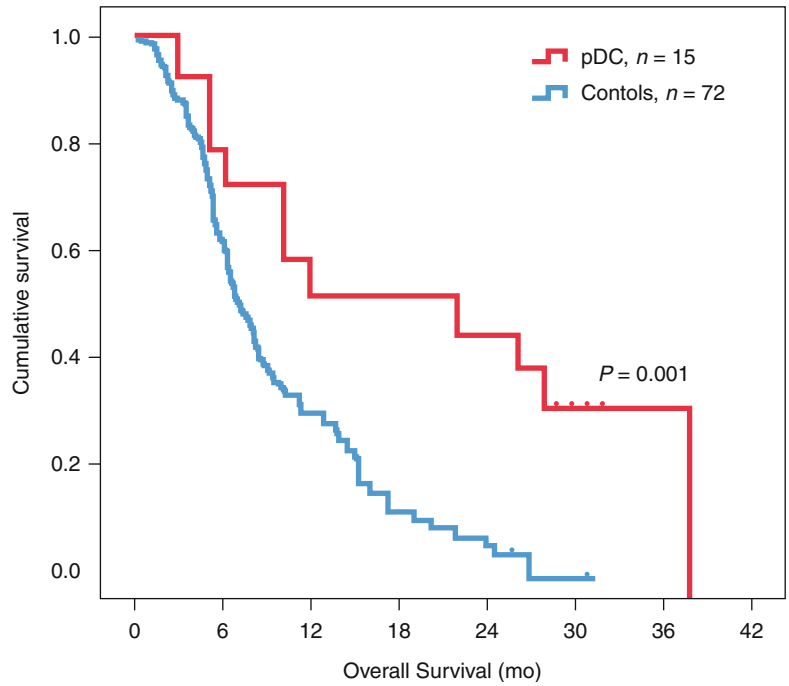

Fig. 3 Overall survival benefit after pDC vaccination: pDC vaccination improves OS. Clinical outcome to pDC vaccination was compared with a group of carefully matched historical control patients who received dacarbazine as first-line treatment. Median OS data showed a significant improvement compared with matched control patients: 22.0 versus 7.6 months. Statistical significance between the survival of the groups was determined by a log-rank test, $P = 0.001$ (Reproduced from Tel et al. (2013))

it is also a well-known phenomenon that they can infiltrate tumours (Miller et al. 2006; Ling et al. 2007). T_{Regs} can affect immune responses at the level of antigen presentation and during the effector phase of T cells at the site of inflammation or tumour growth (Fehervari and Sakaguchi 2004). The suppression T_{Regs} exert is antigen nonspecific, and these cells could therefore inhibit immune responses to all tumour antigens regardless of whether these antigens are self-antigens or not (Takahashi et al. 1998). In most cancer types their presence correlates negatively with survival (Curiel et al. 2004; Petersen et al. 2006; Wang 2006; El Andaloussi and Lesniak 2007). T_{Reg} frequencies are elevated in the blood of cancer patients but are also significantly enriched within the tumour microenvironment. This generates new therapeutic possibilities in order to manipulate the balance of anti-tumour infiltrating lymphocytes and immunosuppressive cells (Ondondo et al. 2013). Besides T_{Regs}, myeloid-derived suppressor cells (MDSCs) expand in cancer-bearing hosts and also have immunosuppressive properties (Kusmartsev and Gabrilovich 2006; Serafini et al. 2006). MDSCs are immature myeloid progenitor cells that suppress T cell effector functions and promote angiogenesis (Olson and McNeel 2013). Studies in tumour-bearing mice and cancer patients showed the critical role of MDSCs in the regulation of immune responses in cancer (Youn et al. 2008, 2012, 2013; Gabrilovich et al. 2012). Fibrocytes, haematopoietic stem cell-derived fibroblast precursors, represent a novel cancer-induced MDSC subset circulating in patients with metastatic cancer. They mediate immune suppression by prevention of $CD3^+$ T cell proliferation (Zhang et al. 2013). Therefore suppression of MDSCs appears like a promising measure in cancer immunotherapy.

Other Negative Regulatory Pathways

Cytotoxic T lymphocyte antigen 4 (CTLA-4), also known as CD152, is a protein receptor that also downregulates the immune system. CTLA-4 is found on the surface of T cells, which lead the cellular immune attack on antigens. Blockade of CTLA-4 was thought to deplete T_{Regs}. However, data indicate that CTLA-4-blocking antibodies did not result in depletion or decreased suppressive activity of T_{Regs}, but executes its immunostimulatory effect by preventing normal downregulation of activated T cells by blocking CTLA-4/B7 interaction (Chambers et al. 2001; Maker al. 2005; Khan et al. 2011).

Another important pathway through which tolerance or anergy might be induced involves modulation of pathways regulating cell death, such as the Fas/Fas ligand (FasL) pathway and the inhibitory co-stimulatory molecule programmed death 1 (PD-1) and its ligands, PD-L1 and PD-L2 (Khoury and Sayegh 2004; Lang et al. 2006). The membrane receptor PD-1 is, as CD28 and CTLA-4, a member of the B7 family of co-stimulatory/inhibitory molecules. Engagement of this receptor by a B7 family molecule leads to a negative regulation of T cell activation (Ishida et al. 1992; Blank and Mackensen 2007; Fife and Pauken 2011). PD-1 is expressed on activated T cells, B cells and macrophages, suggesting that compared to CTLA-4, PD-1 has a more extended negatively regulation on different immune responses. Two ligands have been described for PD-1. PD-L1 is upregulated on APCs, macrophages and T cells upon activation (Freeman et al. 2000). PD-L2 is not that widely expressed and is restricted to DCs and a few tumour cell lines (Latchmanet al. 2001). For several cancers, it was shown that the expression of PD-L1 is correlated with poor clinical outcome (Thompson et al. 2006).

Immune Checkpoint Antibodies

Promising results have already been generated in immunotherapy with human antibodies directed against the immune checkpoint proteins CTLA-4 (Hodi et al. 2010; Wolchok et al. 2010; Robert et al. 2011), PD-1 (Topalian et al. 2012) and PD-L1 (Brahmer et al. 2012). Since treatment with anti-CTLA-4 is antigen nonspecific, the combination with a vaccine could potentially direct the T cell response in a more specific manner, thereby probably diminishing autoimmune side effects. There is anecdotal information that anti-CTLA-4 treatment after DC vaccination may indeed enhance DC vaccine-induced T cell responses (Ribaset al. 2005); however, clinical trials that are specifically designed to answer this question have not yet been published. Also combination therapy with anti-CTLA-4 and DC vaccination, instead of consecutive use, may have a synergistic effect. In advanced melanoma anti-CTLA-4 antibodies showed pivotal data (Wolchok et al. 2010; Robert et al. 2011), and this led to the registration of ipilimumab as an immunotherapeutic agent. However, in other solid tumour types, the place of anti-CTLA-4 antibody therapy is less determined, and more studies are needed to determine its position.

Further research is needed to combine these approaches with DC vaccination. Trials will have to answer the question whether DC vaccination can elicit sustainable clinical responses in a substantial percentage of treated patients or can add to the clinical efficacy of other anticancer treatment modalities. With the wealth of information currently available on the molecular mechanisms that control the immune system, there is no doubt that these are thrilling times for immunotherapy.

Summary and Conclusions

During the past decade considerable progress was made in understanding the biology of DCs, and the quality of DC-derived vaccines has been improved significantly since these first clinical trials. We have by now proven that DC vaccination is amongst the safest types of immunotherapy, and with the current development towards natural DCs, which require only minimal in vitro culturing, also the reproducibility and quality of the vaccines will further improve. Next-generation DC vaccines need to be based on those DC subsets that are best equipped to elicit

$CD8^+$ T cells, but also $CD4^+$ T cells regulating $CD8^+$ T cell immunity in both the priming and the effector phases are equally important. Ahead of us lies the introduction of strategies aiming at in vivo targeting of DC subsets, which would further widen their applicability in the clinic.

It remains of utmost importance to resolve the discrepancy between the immune and clinical efficacy as measured by overall survival and the rate of cancer rejection. Therefore, it would be a tremendous step forwards if we could develop biomarkers that could predict which patients benefit most of DC vaccination. It is clear that we have to remove the barriers for cytotoxic T cells induced by DC vaccines, to reach the tumour and properly exert their effector functions. For that, immunosuppressive networks as discussed above must be eradicated. A logical approach to address these issues is the combination of DC vaccine candidates and immune checkpoint inhibitors.

We strongly believe that ultimately antigen-specific vaccination strategies will remain important next to less specific checkpoint inhibitors, to obtain curative immunotherapies. We believe that DC vaccines will find their way in the standard repertoire of therapeutic options of the oncologist.

References

Akira S, Takeda K (2004) Toll-like receptor signalling. Nat Rev Immunol 4(7):499–511

Akira S, Uematsu S et al (2006) Pathogen recognition and innate immunity. Cell 124(4):783–801

Asea A, Rehli M et al (2002) Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. J Biol Chem 277(17):15028–15034

Bachem A, Guttler S et al (2010) Superior antigen cross-presentation and XCR1 expression define human CD11c + CD141+ cells as homologues of mouse CD8+ dendritic cells. J Exp Med 207(6):1273–1281

Balch CM, Soong SJ et al (2001) Prognostic factors analysis of 17,600 melanoma patients: validation of the American Joint Committee on Cancer melanoma staging system. J Clin Oncol 19(16):3622–3634

Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. Nature 392(6673):245–252

Banchereau J, Palucka AK et al (2001) Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. Cancer Res 61(17):6451–6458

Banchereau J, Ueno H et al (2005) Immune and clinical outcomes in patients with stage IV melanoma vaccinated with peptide-pulsed dendritic cells derived from CD34+ progenitors and activated with type I interferon. J Immunother 28(5):505–516

Beer TM, Bernstein GT et al (2011) Randomized trial of autologous cellular immunotherapy with sipuleucel-T in androgen-dependent prostate cancer. Clin Cancer Res 17(13):4558–4567

Berard F, Blanco P et al (2000) Cross-priming of naive CD8 T cells against melanoma antigens using dendritic cells loaded with killed allogeneic melanoma cells. J Exp Med 192(11):1535–1544

Bevan MJ (1976) Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. J Exp Med 143(5):1283–1288

Blank C, Mackensen A (2007) Contribution of the PD-L1/PD-1 pathway to T-cell exhaustion: an update on implications for chronic infections and tumor evasion. Cancer Immunol Immunother 56(5):739–745

Bonehill A, Heirman C et al (2004) Messenger RNA-electroporated dendritic cells presenting MAGE-A3 simultaneously in HLA class I and class II molecules. J Immunol 172(11):6649–6657

Bonehill A, Tuyaerts S et al (2008) Enhancing the T-cell stimulatory capacity of human dendritic cells by co-electroporation with CD40L, CD70 and constitutively active TLR4 encoding mRNA. Mol Ther 16(6):1170–1180

Boullart AC, Aarntzen EH et al (2008) Maturation of monocyte-derived dendritic cells with Toll-like receptor 3 and 7/8 ligands combined with prostaglandin E2 results in high interleukin-12 production and cell migration. Cancer Immunol Immunother 57(11):1589–1597

Brahmer JR, Tykodi SS et al (2012) Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med 366(26):2455–2465

Breckpot K, Corthals J et al (2005) Dendritic cells differentiated in the presence of IFN-{beta} and IL-3 are potent inducers of an antigen-specific CD8+ T cell response. J Leukoc Biol 78(4):898–908

Brossart P, Wirths S et al (2000) Induction of cytotoxic T-lymphocyte responses in vivo after vaccinations with peptide-pulsed dendritic cells. Blood 96(9):3102–3108

Butterfield LH, Ribas A et al (2003) Determinant spreading associated with clinical response in dendritic cell-based immunotherapy for malignant melanoma. Clin Cancer Res 9(3):998–1008

Camporeale A, Boni A et al (2003) Critical impact of the kinetics of dendritic cells activation on the in vivo induction of tumor-specific T lymphocytes. Cancer Res 63(13):3688–3694

Caruso DA, Orme LM et al (2005) Results of a Phase I study utilizing monocyte-derived dendritic cells pulsed with tumor RNA in children with Stage 4 neuroblastoma. Cancer 103(6):1280–1291

Cella M, Facchetti F et al (2000) Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent TH1 polarization. Nat Immunol 1(4):305–310

Chambers CA, Kuhns MS et al (2001) CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. Annu Rev Immunol 19:565–594

Colonna M, Trinchieri G et al (2004) Plasmacytoid dendritic cells in immunity. Nat Immunol 5(12):1219–1226

Crozat K, Guiton R et al (2010) The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8alpha+ dendritic cells. J Exp Med 207(6):1283–1292

Curiel TJ, Coukos G et al (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 10(9):942–949

Davis ID, Chen Q et al (2006) Blood dendritic cells generated with Flt3 ligand and CD40 ligand prime CD8+ T cells efficiently in cancer patients. J Immunother 29(5): 499–511

de Vries IJ, Eggert AA et al (2002) Phenotypical and functional characterization of clinical grade dendritic cells. J Immunother 25(5):429–438

De Vries IJ, Krooshoop DJ et al (2003a) Effective migration of antigen-pulsed dendritic cells to lymph nodes in melanoma patients is determined by their maturation state. Cancer Res 63(1):12–17

de Vries IJ, Lesterhuis WJ et al (2003b) Maturation of dendritic cells is a prerequisite for inducing immune responses in advanced melanoma patients. Clin Cancer Res 9(14):5091–5100

Di Pucchio T, Chatterjee B et al (2008) Direct proteasome-independent cross-presentation of viral antigen by plasmacytoid dendritic cells on major histocompatibility complex class I. Nat Immunol 9(5):551–557

Dutoit V, Herold-Mende C et al (2012) Exploiting the glioblastoma peptidome to discover novel tumour-associated antigens for immunotherapy. Brain 135(Pt 4):1042–1054

Dzionek A, Fuchs A et al (2000) BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. J Immunol 165(11):6037–6046

Eggermont AM, Kirkwood JM (2004) Re-evaluating the role of dacarbazine in metastatic melanoma: what have we learned in 30 years? Eur J Cancer 40(12): 1825–1836

El Andaloussi A, Lesniak MS (2007) CD4(+) CD25(+) FoxP3(+) T-cell infiltration and heme oxygenase-1 expression correlate with tumor grade in human gliomas. J Neurooncol 83(2):145–152

Fehervari Z, Sakaguchi S (2004) CD4+ Tregs and immune control. J Clin Invest 114(9):1209–1217

Fernandez NC, Lozier A et al (1999) Dendritic Cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. Nat Med 5(4):405–411

Fife BT, Pauken KE (2011) The role of the PD-1 pathway in autoimmunity and peripheral tolerance. Ann N Y Acad Sci 1217:45–59

Figdor CG, de Vries IJ et al (2004) Dendritic cell immunotherapy: mapping the way. Nat Med 10(5):475–480

Finn OJ (2003) Cancer vaccines: between the idea and the reality. Nat Rev Immunol 3(8):630–641

Fong L, Hou Y et al (2001) Altered peptide ligand vaccination with Flt3 ligand expanded dendritic cells for tumor immunotherapy. Proc Natl Acad Sci U S A 98(15):8809–8814

Fonteneau JF, Gilliet M et al (2003) Activation of influenza virus-specific CD4+ and CD8+ T cells: a new role for plasmacytoid dendritic cells in adaptive immunity. Blood 101(9):3520–3526

Freeman GJ, Long AJ et al (2000) Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 192(7):1027–1034

Gabrilovich DI, Ostrand-Rosenberg S et al (2012) Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol 12(4):253–268

Geiger JD, Hutchinson RJ et al (2001) Vaccination of pediatric solid tumor patients with tumor lysate-pulsed dendritic cells can expand specific T cells and mediate tumor regression. Cancer Res 61(23): 8513–8519

Gilboa E (2007) DC-based cancer vaccines. J Clin Invest 117(5):1195–1203

Gilliet M, Cao W et al (2008) Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. Nat Rev Immunol 8(8):594–606

Griffioen M, Borghi M et al (2004) Analysis of T-cell responses in metastatic melanoma patients vaccinated with dendritic cells pulsed with tumor lysates. Cancer Immunol Immunother 53(8):715–722

Guiducci C, Ott G et al (2006) Properties regulating the nature of the plasmacytoid dendritic cell response to Toll-like receptor 9 activation. J Exp Med 203(8): 1999–2008

Hersey P, Menzies SW et al (2004) Phase I/II study of treatment with dendritic cell vaccines in patients with disseminated melanoma. Cancer Immunol Immunother 53(2):125–134

Higano CS, Schellhammer PF et al (2009) Integrated data from 2 randomized, double-blind, placebo-controlled, phase 3 trials of active cellular immunotherapy with sipuleucel-T in advanced prostate cancer. Cancer 115(16):3670–3679

Hochrein H, Schlatter B et al (2004) Herpes simplex virus type-1 induces IFN-alpha production via Toll-like receptor 9-dependent and -independent pathways. Proc Natl Acad Sci U S A 101(31):11416–11421

Hodi FS, O'Day SJ et al (2010) Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 363(8):711–723

Holtl L, Zelle-Rieser C et al (2002) Immunotherapy of metastatic renal cell carcinoma with tumor lysate-pulsed autologous dendritic cells. Clin Cancer Res 8(11):3369–3376

Hsu FJ, Benike C et al (1996) Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. Nat Med 2(1):52–58

Huysamen C, Willment JA et al (2008) CLEC9A is a novel activation C-type lectin-like receptor expressed

on BDCA3+ dendritic cells and a subset of monocytes. J Biol Chem 283(24):16693–16701
Ishida Y, Agata Y et al (1992) Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. EMBO J 11(11):3887–3895
Ito T, Amakawa R et al (2002) Interferon-alpha and interleukin-12 are induced differentially by Toll-like receptor 7 ligands in human blood dendritic cell subsets. J Exp Med 195(11):1507–1512
Ives NJ, Stowe RL et al (2007) Chemotherapy compared with biochemotherapy for the treatment of metastatic melanoma: a meta-analysis of 18 trials involving 2,621 patients. J Clin Oncol 25(34):5426–5434
Iwasaki A, Medzhitov R (2004) Toll-like receptor control of the adaptive immune responses. Nat Immunol 5(10):987–995
Jego G, Palucka AK et al (2003) Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6. Immunity 19(2):225–234
Jiang W, Swiggard WJ et al (1995) The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. Nature 375(6527):151–155
Jonuleit H, Kuhn U et al (1997) Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. Eur J Immunol 27(12):3135–3142
Jonuleit H, Giesecke-Tuettenberg A et al (2001) A comparison of two types of dendritic cell as adjuvants for the induction of melanoma-specific T-cell responses in humans following intranodal injection. Int J Cancer 93(2):243–251
Kadowaki N, Antonenko S et al (2000) Natural interferon alpha/beta-producing cells link innate and adaptive immunity. J Exp Med 192(2):219–226
Kadowaki N, Ho S et al (2001) Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J Exp Med 194(6):863–869
Kaka AS, Foster AE et al (2008) Using dendritic cell maturation and IL-12 producing capacity as markers of function: a cautionary tale. J Immunother 31(4):359–369
Kalinski P, Vieira PL et al (2001) Prostaglandin E(2) is a selective inducer of interleukin-12 p40 (IL-12p40) production and an inhibitor of bioactive IL-12p70 heterodimer. Blood 97(11):3466–3469
Kantoff PW, Higano CS et al (2010) Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med 363(5):411–422
Khan S, Burt DJ et al (2011) Tremelimumab (anti-CTLA4) mediates immune responses mainly by direct activation of T effector cells rather than by affecting T regulatory cells. Clin Immunol 138(1):85–96
Khoury SJ, Sayegh MH (2004) The roles of the new negative T cell costimulatory pathways in regulating autoimmunity. Immunity 20(5):529–538
Krug A, Towarowski A et al (2001) Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. Eur J Immunol 31(10):3026–3037
Kusmartsev S, Gabrilovich DI (2006) Role of immature myeloid cells in mechanisms of immune evasion in cancer. Cancer Immunol Immunother 55(3):237–245
Kyte JA, Kvalheim G et al (2005) Preclinical full-scale evaluation of dendritic cells transfected with autologous tumor-mRNA for melanoma vaccination. Cancer Gene Ther 12(6):579–591
Kyte JA, Mu L et al (2006) Phase I/II trial of melanoma therapy with dendritic cells transfected with autologous tumor-mRNA. Cancer Gene Ther 13(10):905–918
Kyte JA, Kvalheim G et al (2007) T cell responses in melanoma patients after vaccination with tumor-mRNA transfected dendritic cells. Cancer Immunol Immunother 56(5):659–675
Lang K, Entschladen F et al (2006) Tumor immune escape mechanisms: impact of the neuroendocrine system. Cancer Immunol Immunother 55(7):749–760
Langenkamp A, Messi M et al (2000) Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. Nat Immunol 1(4):311–316
Latchman Y, Wood CR et al (2001) PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat Immunol 2(3):261–268
Laverman P, de Vries IJ et al (2006) Development of 111In-labeled tumor-associated antigen peptides for monitoring dendritic-cell-based vaccination. Nucl Med Biol 33(4):453–458
Lesterhuis WJ, de Vries IJ et al (2004) Dendritic cell-based vaccines in cancer immunotherapy: an update on clinical and immunological results. Ann Oncol 15(Suppl 4):iv145–iv151
Lesterhuis WJ, Aarntzen EH et al (2008) Dendritic cell vaccines in melanoma: from promise to proof? Crit Rev Oncol Hematol 66(2):118–134
Lin CL, Lo WF et al (2002) Immunization with Epstein-Barr Virus (EBV) peptide-pulsed dendritic cells induces functional CD8+ T-cell immunity and may lead to tumor regression in patients with EBV-positive nasopharyngeal carcinoma. Cancer Res 62(23): 6952–6958
Lindstedt M, Lundberg K et al (2005) Gene family clustering identifies functionally associated subsets of human in vivo blood and tonsillar dendritic cells. J Immunol 175(8):4839–4846
Ling KL, Pratap SE et al (2007) Increased frequency of regulatory T cells in peripheral blood and tumour infiltrating lymphocytes in colorectal cancer patients. Cancer Immun 7:7
Liu YJ (2001) Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. Cell 106(3):259–262
Lou Y, Liu C et al (2007) Plasmacytoid dendritic cells synergize with myeloid dendritic cells in the induction of antigen-specific antitumor immune responses. J Immunol 178(3):1534–1541
MacDonald KP, Munster DJ et al (2002) Characterization of human blood dendritic cell subsets. Blood 100(13): 4512–4520

Maker AV, Attia P et al (2005) Analysis of the cellular mechanism of antitumor responses and autoimmunity in patients treated with CTLA-4 blockade. J Immunol 175(11):7746–7754

Maldonado-Lopez R, De Smedt T et al (1999) CD8alpha+ and CD8alpha- subclasses of dendritic cells direct the development of distinct T helper cells in vivo. J Exp Med 189(3):587–592

Matsumoto M, Funami K et al (2003) Subcellular localization of Toll-like receptor 3 in human dendritic cells. J Immunol 171(6):3154–3162

Matzinger P (2002) The danger model: a renewed sense of self. Science 296(5566):301–305

McIlroy D, Gregoire M (2003) Optimizing dendritic cell-based anticancer immunotherapy: maturation state does have clinical impact. Cancer Immunol Immunother 52(10):583–591

Means TK, Hayashi F et al (2003) The Toll-like receptor 5 stimulus bacterial flagellin induces maturation and chemokine production in human dendritic cells. J Immunol 170(10):5165–5175

Meixlsperger S, Leung CS et al (2013) CD141+ dendritic cells produce prominent amounts of IFN-alpha after dsRNA recognition and can be targeted via DEC-205 in humanized mice. Blood 121(25):5034–5044

Miller AM, Lundberg K et al (2006) CD4+ CD25high T cells are enriched in the tumor and peripheral blood of prostate cancer patients. J Immunol 177(10): 7398–7405

Nair SK, Morse M et al (2002) Induction of tumor-specific cytotoxic T lymphocytes in cancer patients by autologous tumor RNA-transfected dendritic cells. Ann Surg 235(4):540–549

Napolitani G, Rinaldi A et al (2005) Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. Nat Immunol 6(8):769–776

Nestle FO, Alijagic S et al (1998) Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med 4(3):328–332

Olson BM, McNeel DG (2013) Monitoring regulatory immune responses in tumor immunotherapy clinical trials. Front Oncol 3:109

Ondondo B, Jones E et al (2013) Home sweet home: the tumor microenvironment as a haven for regulatory T cells. Front Immunol 4:197

Palucka AK, Ueno H et al (2006) Dendritic cells loaded with killed allogeneic melanoma cells can induce objective clinical responses and MART-1 specific CD8+ T-cell immunity. J Immunother 29(5):545–557

Petersen RP, Campa MJ et al (2006) Tumor infiltrating Foxp3+ regulatory T-cells are associated with recurrence in pathologic stage I NSCLC patients. Cancer 107(12):2866–2872

Piccioli D, Tavarini S et al (2007) Functional specialization of human circulating CD16 and CD1c myeloid dendritic-cell subsets. Blood 109(12):5371–5379

Piccioli D, Sammicheli C et al (2009) Human plasmacytoid dendritic cells are unresponsive to bacterial stimulation and require a novel type of cooperation with myeloid dendritic cells for maturation. Blood 113(18):4232–4239

Ponsaerts P, Van Tendeloo VF et al (2002) MRNA-electroporated mature dendritic cells retain transgene expression, phenotypical properties and stimulatory capacity after cryopreservation. Leukemia 16(7): 1324–1330

Pulendran B, Smith JL et al (1999) Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. Proc Natl Acad Sci U S A 96(3):1036–1041

Pulendran B, Dillon S et al (2004) Dendritic cells generated in the presence of GM-CSF plus IL-15 prime potent CD8+ Tc1 responses in vivo. Eur J Immunol 34(1):66–73

Reis e Sousa C (2004) Activation of dendritic cells: translating innate into adaptive immunity. Curr Opin Immunol 16(1):21–25

Renn CN, Sanchez DJ et al (2006) TLR activation of Langerhans cell-like dendritic cells triggers an antiviral immune response. J Immunol 177(1):298–305

Ribas A, Glaspy JA et al (2004) Role of dendritic cell phenotype, determinant spreading, and negative costimulatory blockade in dendritic cell-based melanoma immunotherapy. J Immunother 27(5): 354–367

Ribas A, Camacho LH et al (2005) Antitumor activity in melanoma and anti-self responses in a phase I trial with the anti-cytotoxic T lymphocyte-associated antigen 4 monoclonal antibody CP-675,206. J Clin Oncol 23(35):8968–8977

Ridolfi R, Petrini M et al (2006) Improved overall survival in dendritic cell vaccination-induced immunoreactive subgroup of advanced melanoma patients. J Transl Med 4:36

Robert C, Thomas L et al (2011) Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. N Engl J Med 364(26):2517–2526

Romani N, Gruner S et al (1994) Proliferating dendritic cell progenitors in human blood. J Exp Med 180(1):83–93

Rosenberg SA (1999) A new era for cancer immunotherapy based on the genes that encode cancer antigens. Immunity 10(3):281–287

Rosenberg SA, Yang JC et al (2004) Cancer immunotherapy: moving beyond current vaccines. Nat Med 10(9):909–915

Salcedo M, Bercovici N et al (2006) Vaccination of melanoma patients using dendritic cells loaded with an allogeneic tumor cell lysate. Cancer Immunol Immunother 55(7):819–829

Salio M, Palmowski MJ et al (2004) CpG-matured murine plasmacytoid dendritic cells are capable of in vivo priming of functional CD8 T cell responses to endogenous but not exogenous antigens. J Exp Med 199(4):567–579

Sallusto F, Lanzavecchia A (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated

by tumor necrosis factor alpha. J Exp Med 179(4):1109–1118
Schadendorf D, Ugurel S et al (2006) Dacarbazine (DTIC) versus vaccination with autologous peptide-pulsed dendritic cells (DC) in first-line treatment of patients with metastatic melanoma: a randomized phase III trial of the DC study group of the DeCOG. Ann Oncol 17(4):563–570
Schaft N, Dorrie J et al (2005) Generation of an optimized polyvalent monocyte-derived dendritic cell vaccine by transfecting defined RNAs after rather than before maturation. J Immunol 174(5):3087–3097
Schlitzer A, McGovern N et al (2013) IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. Immunity 38(5):970–983
Schott M, Feldkamp J et al (1999) Dendritic cell immunotherapy in disseminated parathyroid carcinoma. Lancet 353(9159):1188–1189
Schreibelt G, Tel J et al (2010) Toll-like receptor expression and function in human dendritic cell subsets: implications for dendritic cell-based anti-cancer immunotherapy. Cancer Immunol Immunother 59(10):1573–1582
Schuler-Thurner B, Dieckmann D et al (2000) Mage-3 and influenza-matrix peptide-specific cytotoxic T cells are inducible in terminal stage HLA-A2.1+ melanoma patients by mature monocyte-derived dendritic cells. J Immunol 165(6):3492–3496
Schuler-Thurner B, Schultz ES et al (2002) Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells. J Exp Med 195(10):1279–1288
Serafini P, Borrello I et al (2006) Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. Semin Cancer Biol 16(1):53–65
Shaw J, Wang YH et al (2010) Plasmacytoid dendritic cells regulate B-cell growth and differentiation via CD70. Blood 115(15):3051–3057
Sheikh NA, Petrylak D et al (2013) Sipuleucel-T immune parameters correlate with survival: an analysis of the randomized phase 3 clinical trials in men with castration-resistant prostate cancer. Cancer Immunol Immunother 62(1):137–147
Siegal FP, Kadowaki N et al (1999) The nature of the principal type 1 interferon-producing cells in human blood. Science 284(5421):1835–1837
Singh-Jasuja H, Scherer HU et al (2000) The heat shock protein gp96 induces maturation of dendritic cells and down-regulation of its receptor. Eur J Immunol 30(8):2211–2215
Skoberne M, Beignon AS et al (2004) Danger signals: a time and space continuum. Trends Mol Med 10(6): 251–257
Small EJ, Schellhammer PF et al (2006) Placebo-controlled phase III trial of immunologic therapy with sipuleucel-T (APC8015) in patients with metastatic, asymptomatic hormone refractory prostate cancer. J Clin Oncol 24(19):3089–3094
Soruri A, Kiafard Z et al (2003) IL-4 down-regulates anaphylatoxin receptors in monocytes and dendritic cells and impairs anaphylatoxin-induced migration in vivo. J Immunol 170(6):3306–3314
Steinman RM, Nussenzweig MC (2002) Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. Proc Natl Acad Sci U S A 99(1):351–358
Stift A, Friedl J et al (2003) Dendritic cell-based vaccination in solid cancer. J Clin Oncol 21(1):135–142
Sullenger BA, Gilboa E (2002) Emerging clinical applications of RNA. Nature 418(6894):252–258
Takagi H, Fukaya T et al (2011) Plasmacytoid dendritic cells are crucial for the initiation of inflammation and T cell immunity in vivo. Immunity 35(6): 958–971
Takahashi T, Kuniyasu Y et al (1998) Immunologic self-tolerance maintained by CD25+ CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. Int Immunol 10(12):1969–1980
Tel J, Aarntzen EH et al (2013) Natural human plasmacytoid dendritic cells induce antigen-specific T-cell responses in melanoma patients. Cancer Res 73(3):1063–1075
Thompson RH, Kuntz SM et al (2006) Tumor B7-H1 is associated with poor prognosis in renal cell carcinoma patients with long-term follow-up. Cancer Res 66(7):3381–3385
Thurner B, Haendle I et al (1999) Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. J Exp Med 190(11):1669–1678
Topalian SL, Hodi FS et al (2012) Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 366(26):2443–2454
Toungouz M, Libin M et al (2001) Transient expansion of peptide-specific lymphocytes producing IFN-gamma after vaccination with dendritic cells pulsed with MAGE peptides in patients with mage-A1/A3-positive tumors. J Leukoc Biol 69(6):937–943
Trombetta ES, Mellman I (2005) Cell biology of antigen processing in vitro and in vivo. Annu Rev Immunol 23:975–1028
Ueno H, Tcherepanova I et al (2004) Dendritic cell subsets generated from CD34+ hematopoietic progenitors can be transfected with mRNA and induce antigen-specific cytotoxic T cell responses. J Immunol Methods 285(2):171–180
Vabulas RM, Braedel S et al (2002) The endoplasmic reticulum-resident heat shock protein Gp96 activates dendritic cells via the Toll-like receptor 2/4 pathway. J Biol Chem 277(23):20847–20853
Wang RF (2006) Immune suppression by tumor-specific CD4+ regulatory T-cells in cancer. Semin Cancer Biol 16(1):73–79
Warger T, Osterloh P et al (2006) Synergistic activation of dendritic cells by combined Toll-like receptor ligation induces superior CTL responses in vivo. Blood 108(2):544–550

Wolchok JD, Neyns B et al (2010) Ipilimumab monotherapy in patients with pretreated advanced melanoma: a randomised, double-blind, multicentre, phase 2, dose-ranging study. Lancet Oncol 11(2):155–164

Yamanaka R, Abe T et al (2003) Vaccination of recurrent glioma patients with tumour lysate-pulsed dendritic cells elicits immune responses: results of a clinical phase I/II trial. Br J Cancer 89(7):1172–1179

Youn JI, Nagaraj S et al (2008) Subsets of myeloid-derived suppressor cells in tumor-bearing mice. J Immunol 181(8):5791–5802

Youn JI, Collazo M et al (2012) Characterization of the nature of granulocytic myeloid-derived suppressor cells in tumor-bearing mice. J Leukoc Biol 91(1): 167–181

Youn JI, Kumar V et al (2013) Epigenetic silencing of retinoblastoma gene regulates pathologic differentiation of myeloid cells in cancer. Nat Immunol 14(3): 211–220

Zhang Z, Yuan B et al (2011) The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells. Nat Immunol 12(10):959–965

Zhang H, Maric I et al (2013) Fibrocytes represent a novel MDSC subset circulating in patients with metastatic cancer. Blood 122(7):1105–1113

mRNA Vaccination and Personalized Cancer Therapy

S. Kreiter, M. Diken, and U. Sahin

A Short History of mRNA-Based Vaccines

Being discovered in 1961, mRNA is a subject of consistent basic as well as application-oriented research for a diversity of diseases. The use of in vitro-transcribed mRNA for vaccination was first suggested in 1986 when infectious poliovirus mRNA was produced by in vitro transcription with T7 polymerase (van der Werf et al. 1986). The concept of in vivo application of nucleic acid-encoded drugs was launched when Wolff et al. demonstrated the expression of the encoded protein in mice after direct injection of messenger RNA (mRNA) or plasmid DNA (pDNA) to the skeletal muscle (Wolff et al. 1990). The first reported T-cell response upon mRNA vaccination is documented by induction of influenza virus-specific immune responses in mice upon in vivo delivery of liposome-entrapped influenza nucleoprotein-coding mRNA (Martinon et al. 1993).

The development of anticancer mRNA vaccines was initiated in 1995 when Conry and coworkers demonstrated tumor antigen-specific antibody responses upon intramuscular injection of carcinoembryonic antigen (CEA) mRNA (Conry et al. 1995). A year later, Gilboa and colleagues demonstrated T-cell responses and antitumoral immunity by immunization of tumor-bearing mice with murine dendritic cells (DC) transfected either with antigen-specific mRNA or total mRNA extracted from tumor cells. At that time dendritic cells emerged as the key antigen presenters for priming of T cells and crucial cell population for the induction of potent antigen-specific immune response (Inaba et al. 1990; Rock et al. 1990). This together with the discovery of cancer vaccine targets by novel tumor antigen-cloning approaches (van der Bruggen et al. 1991; Sahin et al. 1995) stimulated the rapid development and clinical translation of the mRNA-transfected dendritic cell approach with entry into clinical testing more than one decade ago (Morse et al. 1998; Rains et al. 2001). Since then, many clinical trials using mRNA-transfected dendritic cell vaccines were performed in cancer patients and firmly established the feasibility, safety, and antitumoral activity of this approach (Van Lint et al. 2013; Kreiter et al. 2011b).

The clinical translation of cell therapies is expensive and cumbersome. Constraining the use of mRNA to cell therapy approaches limits the major advantages mRNA as a drug may have (e.g., simple platform-type production process, low cost of goods). Therefore, direct in vivo administration of tumor antigen mRNA was revisited by various groups. Between 1990

S. Kreiter • U. Sahin (✉)
TRON-Translational Oncology University Medical Center, Johannes Gutenberg University, Mainz 55131, Germany

BioNTech AG, Mainz 55131, Germany
e-mail: sahin@uni-mainz.de

M. Diken
Department of Immunotherapy Development Center, TRON-Translational Oncology University Medical Center, Johannes Gutenberg University, Mainz 55131, Germany

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_9,

and 2000, scientists already set out to utilize mRNA in different formulations and also tested self-amplifying mRNA vectors obtained from the genome of positive-strand RNA viruses. To improve delivery gene gun approaches, complexing of mRNA with positively charged lipids or liposomal encapsulation were investigated (Qiu et al. 1996; Martinon et al. 1993; Zhou et al. 1994, 1999; Ying et al. 1999). Whereas most of these approaches remained preclinical, two concepts employing either the intradermal delivery of naked mRNA (Hoerr et al. 2000) or the in vivo mRNA delivery into lymphatic dendritic cells in situ (Kreiter et al. 2010) advanced to clinical testing in cancer patients. Currently, clinical trials are running employing the intradermal as well as the intranodal application route for mRNA in different cancer entities, and a rising number of studies are expected to be launched in the coming years.

Concept and Molecular Design of mRNA Vaccines

Synthetic mRNA is produced by in vitro transcription from plasmid-based vectors. The resulting transcript structurally resembles mature and processed mRNA molecules as they occur naturally in the cytoplasm of eukaryotic cells. The concept behind using mRNA vaccines is to introduce the mRNA into cells of interest for translation of a corresponding protein antigen that acts as an immunogen for the induction of antigen-specific immune responses. Upon cellular mRNA uptake the most critical factors determining the antigen yield are the translational efficacy and the cytoplasmic half-life of the mRNA. The stability and translation of the mRNA are controlled by precisely regulated and complex mechanisms involving various pathways and molecules (Garneau et al. 2007; Jacobson and Peltz 1996; Meyer et al. 2004). Intracellular mRNA is metabolized to a great extent by exonucleases in 5′–3′or reverse direction (Balagopal et al. 2012; Houseley and Tollervey 2009). The initial decay step is mostly removal of the poly(A) sequence at the 3′ end (deadenylation) followed by degradation through the cytoplasmic exosome and the scavenger enzyme DcpS (Balagopal et al. 2012; Lykke-Andersen et al. 2011). Alternatively, the deadenylation is followed by decapping via the enzymes Dcp1 and Dcp2 (Song et al. 2010). This is followed by 5′–3′ exonucleolytic degradation by Xrn1 (Jinek et al. 2011; Chang et al. 2011). The main features of an mRNA that determine its susceptibility to mRNA decay mechanisms are therefore the 5′-cap, the 5′-, and 3′-untranslated regions (UTR) and the poly(A)-tail. Modification of vector design or components used for in vitro synthesis of mRNA allows modulation of mRNA pharmacology. In order to achieve a synergy and maximize antigen yield, several beneficial modifications can be combined (Fig. 1). The main determinants of mRNA-mediated protein biosynthesis are described in the following paragraph.

The natural 5′ cap is an m^7GpppN structure that is recognized by the eukaryotic translation initiation factor eIF4E and is mandatory for efficient translation. Synthetic mRNA is mostly produced by adding a dinucleotide of the general form m^7GpppG as structural homolog of the endogenous cap directly to the reaction mixture (Pascolo 2008). Unfortunately this cap analogue can be incorporated in both directions into the mRNA with the one incorporated in the reverse orientation not being recognizable by the cellular translational machinery (Pasquinelli et al. 1995). Therefore, the development of anti-reverse caps (ARCA, $m_2^{7,2'\text{-O}}$GpppG, $m_2^{7,3'\text{-O}}$GpppG among other modifications) that can only be incorporated in the correct orientation was a major improvement albeit no clinical data are yet reported (Jemielity et al. 2003; Peng et al. 2002; Stepinski et al. 2001; Kuhn et al. 2010). Resistance against cleavage by decapping enzyme Dcp2 was striven for by imido-phosphates, where the bridging oxygen is replaced by NH and S-ARCA in which a non-bridging oxygen is changed to a sulfur (Rydzik et al. 2012; Kowalska et al. 2008; Grudzien-Nogalska et al. 2007; Warminski et al. 2013). Administering antigen-encoding mRNA containing phosphorothioate-modified cap into mice has been shown to greatly enhance antigen production in immature dendritic cells and to induce potent antigen-specific immune responses superior to a control cap (Kuhn et al. 2010).

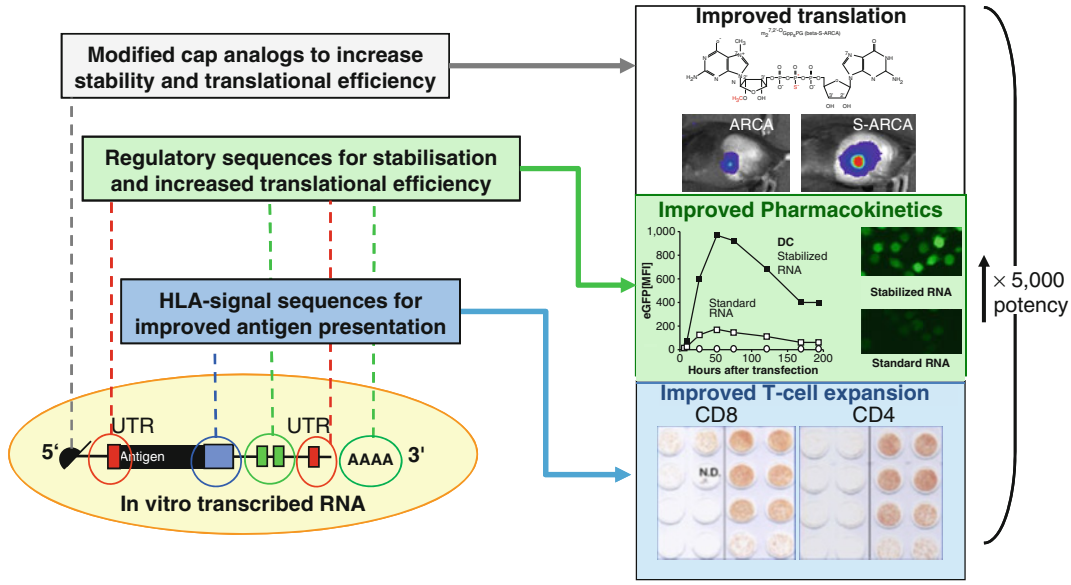

Fig. 1 Improving the RNA: Different strategies to develop an RNA molecule with higher potency. *UTR* untranslated region, *ND* not detected, *DC* dendritic cell, *HLA* human leukocyte antigen

3′-UTRs can have a deep impact on mRNA stability. Many mRNA vectors incorporate the 3′ UTRs of alpha- and beta-globin mRNAs that harbor sequence elements increasing stability and translation of mRNA (Yu and Russell 2001). This effect is mainly dependent on a phylogenetically conserved pyrimidine-rich element (Waggoner and Liebhaber 2003; Yu and Russell 2001). Interestingly Holtkamp et al. showed in a comparative study that two copies of beta-globin 3′-UTRs compared to one copy resulted synergistically in increased mRNA stability and functional half-life (Holtkamp et al. 2006). Another option to enhance mRNA stability and translational efficacy is to utilize structural elements found in viral mRNAs (Bergman et al. 2007; Sjoberg et al. 1994; Pogue et al. 1993; Garneau et al. 2008; Chiu et al. 2005). Moreover, mRNA translation can be improved by species-specific tailoring of the codon usage that differs from organism to organism (Grantham et al. 1980) as well as by considering that defined codon pairs as well as other sequence elements (e.g., UA dinucleotides, AU-rich elements) may have an impact on mRNA stability and translational efficacy (Moura et al. 2011; Duan and Antezana 2003; Khabar 2005).

Finally the poly(A) tail can be designed to maximize translational output. Its mechanistic impact is explained by the association of multiple copies of poly(A)-binding protein (PABP) which in complex interaction network enables mRNA circularization (Bernstein et al. 1989; Sachs et al. 1987; Baer and Kornberg 1980). This process has been proposed to stabilize mRNA by minimizing access of decapping and deadenylating enzymes, as well as promoting translation. A poly(A)-tail can be added either directly in the in vitro transcription if it is encoded in the template vector or by a two-step reaction extending the in vitro-transcribed RNA enzymatically using recombinant poly(A)-polymerase. For clinical translation the latter approach seems not appropriate as for enzymatic polyadenylation reproducibility from one reaction to another is poor and each mRNA preparation consists of a mixture of RNA species differing in length of the poly(A) (Holtkamp et al. 2006). For human DCs, it was shown that for template-encoded poly(A)-tails the protein expression increases with increasing length of the poly(A)-tail until around 120 adenosines (Holtkamp et al. 2006). Importantly, only an unmasked 3′ ending guarantees maximum translational efficacy (Holtkamp et al. 2006).

Production of mRNA Drugs

Messenger RNA can be generated from a plasmid-based template by in vitro transcription. The plasmid vector is equipped with a bacteriophage RNA polymerase promoter (e.g., T3, T7, or SP6) located upstream of the transcription start side. Usually, the protein-coding open-reading frame is flanked by regulatory sequences that ensure efficient mRNA translation and stability (e.g., poly(A) tail, 5′-, 3′-UTRs) (Kuhn et al. 2011; Grudzien-Nogalska et al. 2013; Kuhn et al. 2012). Prior to in vitro transcription, the plasmid is linearized by cutting at a restriction site downstream of the mRNA transcription cassette thereby ensuring that the resulting mRNA has a defined length. Alternatively, instead of plasmid vectors, a polymerase chain reaction-amplified cDNA template can be used for in vitro transcription. Capping of the mRNA can be achieved co-transcriptionally by adding a cap analogue or post-transcriptionally by usage of a recombinant capping enzyme. In vitro transcription is followed by purification of the mRNA. To this end residual plasmid DNA is degraded by treatment with RNAse-free DNAse. Afterwards, bead-based methods, precipitation, or chromatographic methods are used to purify the mRNA. The purified mRNA can then be subject of formulation after sterile filtration.

The mRNA production and purification process is robust and largely template independent. When a work flow is established, the same process can be used with relatively minor adaptations for production of virtually any individual mRNA sequence of similar size. The mRNA synthesis and purification take only a few days and can be scaled from a few milligrams and large gram quantities of drug substance. This opens up the opportunity to produce mRNA quickly in an "on-demand" fashion for personalized use. On top of various in-process analytics, GMP release of the mRNA drug substance and of the formulated drug products requires extensive testing and characterization, such as tests confirming identity, appearance, content, integrity, residual DNA, endotoxin contamination, and sterility.

The rising interest in mRNA as drug format will probably drive further process improvements enabling to decrease the production costs, the production time, and thereby attract more groups to the field of mRNA-based cancer vaccination.

Delivery of mRNA and Induction of Antigen-Specific Immune Responses in the Preclinical and Clinical Setting

Different approaches are utilized for the delivery of mRNA vaccines. The seminal article of Boczkowski et al. showed for the first time that transfecting DCs via incubating them with mRNA is feasible, and these mRNA-transfected cells can be used for presentation of the antigen in vitro and in vivo (Boczkowski et al. 1996). Subsequent studies proved electroporation as an efficient method to transfect DCs (Van Tendeloo et al. 2001; Ponsaerts et al. 2002). Motivated by promising findings in animal models, mRNA-transfected DCs were tested in several clinical trials, the results of which are reviewed elsewhere (Kreiter et al. 2011b; Van Lint et al. 2013) (see also Chap. 8).

Cationic liposomes are also employed for mRNA delivery to trap the negatively charged mRNA. Liposomal formulations of mRNA were used for induction of T-cell responses by subcutaneous or intravenous delivery of liposome containing influenza protein-coding mRNA (Martinon et al. 1993). Further support for this approach came from another study in which the injection of liposome encapsulated melanoma antigen gp100 coding mRNA by direct injection into spleens of mice resulting in antitumor responses (Zhou et al. 1999). Moreover, systemic injection of MART1-mRNA containing histidylated and mannosylated lipopolyplexes were shown to increase the efficiency of internalization by antigen-presenting cells leading to a better inhibition of tumor growth in a mouse melanoma mode I (Perche et al. 2011; Mockey et al. 2007).

Delivery of mRNA precipitated on gold particles to the dermis via gene gun is yet another approach for mRNA-based vaccination.

By transfecting dermal DCs in mice, Qui et al. reported for the first time generation of antibodies against human alpha-1 antitrypsin protein after gene gun bombardment of mRNA-coated gold particles (Qiu et al. 1996). Another preclinical study provided evidence that both humoral and cellular immune responses as well as protective tumor immunity in a melanoma model can be generated using gene gun-based administration of tyrosine-related protein 2 (TRP2)-coding mRNA (Steitz et al. 2006). Although delivery of mRNA via gene gun is a promising tool for cancer immunotherapy, the clinical translation of this approach has not been achieved.

Direct utilization of naked mRNA for vaccination obviates the need for laborious generation of DCs in vitro, preparation of complex liposome/peptide-mRNA formulations, as well as use of expensive gold beads on which mRNAs are coated. Indeed, vaccination with naked mRNA was one of the earliest application strategies among mRNA-based vaccination studies such that those mice that received intramuscular injection of naked globin UTR-stabilized mRNA coding for carcinoembryonic antigen (CEA) were able to generate anti-CEA antibodies (Conry et al. 1995).

Direct injection of naked globin UTR-stabilized ß-galactosidase-encoding mRNA into the ear pinna of mice resulted in induction of antigen-specific antibody and CD8+ T-cell responses (Hoerr et al. 2000). The study suggested that by intradermal application of naked mRNA alone, a Th2-skewed antigen-specific immune response is induced. By co-application of adjuvants such as GM-CSF (Carralot et al. 2004) or by co-delivery of a protamine-complexed mRNA (Scheel et al. 2005), a strong shift toward Th1 was accomplished. Protamine-complexed mRNA as well as antigen-encoding mRNA combined with GM-CSF was tested in early clinical trials proving feasibility, safety, and immunogenicity in a portion of vaccinated patients (Weide et al. 2008; Rittig et al. 2011). The biotechnology company CureVac (Tübingen/Germany) further improved that approach developing a pharmaceutical grade two component vaccine which consists of a free antigen-encoding mRNA plus protamine-complexed mRNA. This mRNA vaccine showed potent activity in preclinical studies (Fotin-Mleczek et al. 2011). Interim results of ongoing clinical trials in patients with prostate cancer and non-small cell lung cancer proved high immunogenicity of this vaccine.

Our group has followed another delivery strategy. Following classical pharmacological principles and acknowledging the pivotal role of dendritic cells in priming of immune responses, we combined pharmacological optimization of mRNA with delivery routes allowing in situ transfection of dendritic cells in vivo. Our aims were to improve mRNA translation and stability and to enhance presentation of the mRNA-encoded antigen on MHC class I and II molecules of murine and human DC (Holtkamp et al. 2006; Kreiter et al. 2007, 2008; Diken et al. 2013; Kuhn et al. 2010). We tested various application routes for these optimized mRNA vaccines. Direct injection of naked mRNA into lymph nodes resulted in unmatched strong antigen-specific $CD4^+$ and $CD8^+$ T-cell responses. We showed that after intranodal injection mRNA molecules are selectively and effectively taken up by lymph node resident DCs. Moreover, the inherent adjuvant activity of the mRNA mediated a maturation of the respective DC via TLR7 signaling and induced the secretion of various inflammatory cytokines and T-cell-attracting chemokines. The immune-stimulatory intralymphatic milieu created thereby promoted strong antigen-specific Th1 type T-cell responses and effective antitumoral immunity in preclinical animal models. Interestingly, we found that mRNA was internalized by macropinocytosis, a special kind of endocytosis restricted to immature DCs (Kreiter et al. 2010, 2011a; Diken et al. 2011). The potency of intranodal vaccination could be further improved by combination with FLT3L, a molecular adjuvant facilitating the increase of DCs in lymph nodes (Kreiter et al. 2011a) or by co-delivery of mRNA-encoded immunomodulators as demonstrated by Thielemans and colleagues (2012). The intranodal delivery approach is being pharmaceutically developed by the biotechnology company BioNTech (Mainz/Germany), which recently started first-in-human testing in melanoma patients (NCT01684241).

Personalized mRNA Cancer Vaccines: Toward Bedside

Since cancer is a disease caused by mutations, it is tempting to also harvest this central characteristic in immunotherapeutic approaches. Mutations in cancer can lead to changes in the protein sequence (e.g., non-synonymous single-nucleotide variations (SNV), indels, fusions, splice site mutations) and thereby generate neoantigens. These are not encoded in the germline and therefore confer the advantage of lacking development of central immunological tolerance. Due to the tumor specificity of the encoded neoantigens, autoimmune toxicity against healthy tissues is not expected from T cells evoked against those neoantigens. As in general the number of mutations varies (Alexandrov et al. 2013), also the number of non-synonymous mutations varies between 10s and 100 s in different tumor entities (Greenman et al. 2007; Stratton 2011a; Ding et al. 2010; Parsons et al. 2008; Jones et al. 2008; Totoki et al. 2011; Sjoblom et al. 2006; Wood et al. 2007; Pleasance et al. 2010a, b; Wei et al. 2011; Lee et al. 2010). Further complexity is added to the task by the fact that more than 95 % of these mutations are unique to a given tumor (Stratton 2011b). Therefore, any strategy aiming for a broad usage of mutation-encoded neoantigens in immunotherapy has to incorporate measures to enable active personalization with acceptable effort (Britten et al. 2013). Importantly, it was shown already by Lennerz et al. that natural antitumoral T-cell reactivity in a melanoma patient is dominated by T-cell responses against neoantigens (Wolfel et al. 2008). Also in adoptive T-cell transfer therapies, it was shown that patient-derived T-cell lines from three clinically responding patients recognized mutated antigens (Robbins et al. 2013). The recent observation that a melanoma patient clinically responding to anti-CTLA4 treatment developed a strong neoantigen-specific T-cell response further supports the hypothesis that those T cells can be of therapeutical value (van Rooij et al. 2013). With respect to clinical translation, only a small number of vaccination studies have been performed so far (Carbone et al. 2005; Rahma et al. 2010).

The option of clinical development of a personalized cancer vaccines was opened up by the development of affordable deep sequencing technology (Koboldt et al. 2013). Further progress made regarding standardized sequencing methods, epitope prediction, and mutation evaluation as well as prioritization paved the way (Scholtalbers et al. 2013; Lower et al. 2012; Lundegaard et al. 2008; Cibulskis et al. 2013).

Entering hitherto unknown territories, we systematically investigated the immunogenicity of cancer mutations. For that aim we combined high-coverage deep sequencing of the B16F10 melanoma exome with systematic immunogenicity analyses of identified mutations. Our study showed that 20–30 % of identified non-synonymous mutations are immunogenic and can be used for induction of T cells specific for mutation-encoded neoantigens (Castle et al. 2012). Vaccination with mutated epitopes results in tumor growth retardation even though B16F10 has a very low MHC expression (Boegel et al. 2013). Further studies demonstrated that mRNA-encoded neoantigens identified in different murine tumor models can be used to induce T-cell responses that elicit antitumoral effects (unpublished results).

In another study, Schreiber and coworkers showed that a mutated epitope can be lost in tumors by immunoediting (Matsushita et al. 2012). In order to minimize the risk of such escape mutants, it seems desirable to induce T cells against more than one neoepitope. This importance of the issue is supported by our observation that many of the immunogenic epitopes in B16F10 seem to be derived from passenger-type mutations (Castle et al. 2012).

A rapidly determined patient-specific tumor mutation pattern combined with a flexible mutation-targeting drug platform could generate a mutation-targeting individualized therapy from which each single patient would benefit.

Among the different antigen formats for vaccination, antigen-coding messenger RNA (mRNA) is a particularly attractive option. In vitro-transcribed mRNA is a favorable drug format for actively personalized cancer vaccines. It can be produced also under GMP conditions

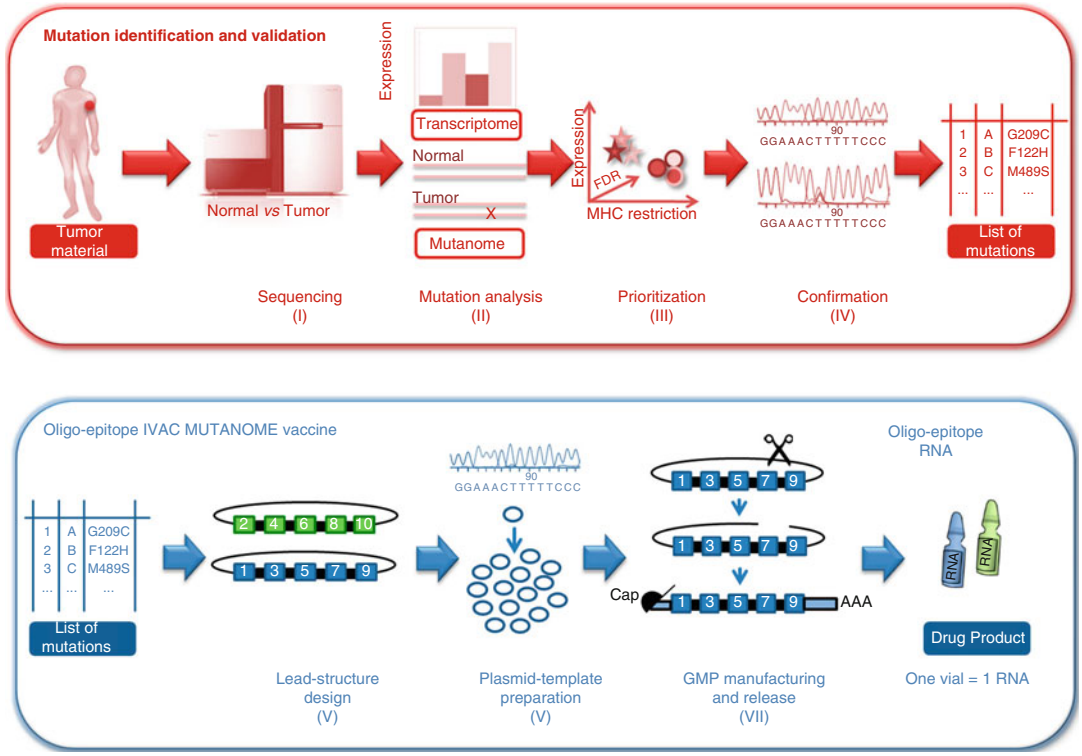

Fig. 2 From tumor to peronalized therapy: The process for production of a mutanome directed personalized RNA vaccine

in a short time range allowing a fast return to the patient. Since mRNA itself only transports the information for an antigen, its chemical features will not differ dramatically between batches for different patients obviating the necessity for deep-characterizing studies of every single patient-specific vaccine batch. Utilizing in vitro transcription allows generating polytopic mRNA vaccines targeting multiple neoantigens, and it does not require knowledge of the minimal epitope (Fig. 2).

Accordingly, we started the development of actively personalized mRNA-based cancer vaccines by launching in 2010 the "Individualized Vaccines for Cancer (IVAC)" project aiming for treatment of melanoma patients supported by the German Federal Ministry for Education and Research. The project is driven by a public-private partnership consortium that pursues the development, production, clinical testing, and commercialization of individualized mRNA vaccines (http://tron-mainz.de/news-archiv-2012/ivac-consortium-wins-bmbf-grant/). The IVAC phase I study (NCT02035956), initiated in December 2013, will allow treatment of patients from up to ten mRNA-encoded mutated epitopes, and in the future potentially even larger numbers of neoepitopes could be utilized in parallel or sequential treatment protocols.

References

Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, Bignell GR, Bolli N, Borg A, Borresen-Dale AL, Boyault S, Burkhardt B, Butler AP, Caldas C, Davies HR, Desmedt C, Eils R, Eyfjord JE, Foekens JA, Greaves M, Hosoda F, Hutter B, Ilicic T, Imbeaud S, Imielinski M, Jager N, Jones DT, Jones D, Knappskog S, Kool M, Lakhani SR, Lopez-Otin C, Martin S, Munshi NC, Nakamura H, Northcott PA, Pajic M, Papaemmanuil E, Paradiso A, Pearson JV, Puente XS, Raine K, Ramakrishna M, Richardson AL, Richter J, Rosenstiel P, Schlesner M, Schumacher TN,

Span PN, Teague JW, Totoki Y, Tutt AN, Valdes-Mas R, van Buuren MM, van't Veer L, Vincent-Salomon A, Waddell N, Yates LR, Zucman-Rossi J, Futreal PA, McDermott U, Lichter P, Meyerson M, Grimmond SM, Siebert R, Campo E, Shibata T, Pfister SM, Campbell PJ, Stratton MR (2013) Signatures of mutational processes in human cancer. Nature 500:415–421

Baer BW, Kornberg RD (1980) Repeating structure of cytoplasmic poly(A)-ribonucleoprotein. Proc Natl Acad Sci U S A 77:1890–1892

Balagopal V, Fluch L, Nissan T (2012) Ways and means of eukaryotic mRNA decay. Biochim Biophys Acta 1819:593–603

Bergman N, Moraes KC, Anderson JR, Zaric B, Kambach C, Schneider RJ, Wilusz CJ, Wilusz J (2007) Lsm proteins bind and stabilize RNAs containing 5′ poly(a) tracts. Nat Struct Mol Biol 14:824–831

Bernstein P, Peltz SW, Ross J (1989) The poly(A)-poly(A)-binding protein complex is a major determinant of mRNA stability in vitro. Mol Cell Biol 9:659–670

Boczkowski D, Nair SK, Snyder D, Gilboa E (1996) Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo. J Exp Med 184:465–472

Boegel S, Lower M, Schafer M, Bukur T, de Graaf J, Boisguerin V, Tureci O, Tureci O, Diken M, Castle JC, Sahin U (2013) HLA typing from RNA-Seq sequence reads. Genome Med 4:102

Britten CM, Singh-Jasuja H, Flamion B, Hoos A, Huber C, Kallen KJ, Khleif SN, Kreiter S, Nielsen M, Rammensee HG, Sahin U, Hinz T, Kalinke U (2013) The regulatory landscape for actively personalized cancer immunotherapies. Nat Biotechnol 31:880–882

Carbone DP, Ciernik IF, Kelley MJ, Smith MC, Nadaf S, Kavanaugh D, Maher VE, Stipanov M, Contois D, Johnson BE, Pendleton CD, Seifert B, Carter C, Read EJ, Greenblatt J, Top LE, Kelsey MI, Minna JD, Berzofsky JA (2005) Immunization with mutant p53- and K-ras-derived peptides in cancer patients: immune response and clinical outcome. J Clin Oncol 23:5099–5107

Carralot JP, Probst J, Hoerr I, Scheel B, Teufel R, Jung G, Rammensee HG, Pascolo S (2004) Polarization of immunity induced by direct injection of naked sequence-stabilized mRNA vaccines. Cell Mol Life Sci 61:2418–2424

Castle JC, Kreiter S, Diekmann J, Lower M, van de Roemer N, de Graaf J, Selmi A, Diken M, Boegel S, Paret C, Koslowski M, Kuhn AN, Britten CM, Huber C, Tureci O, Sahin U (2012) Exploiting the mutanome for tumor vaccination. Cancer Res 72:1081–1091

Chang JH, Xiang S, Xiang K, Manley JL, Tong L (2011) Structural and biochemical studies of the 5′→3′ exoribonuclease Xrn1. Nat Struct Mol Biol 18:270–276

Chiu WW, Kinney RM, Dreher TW (2005) Control of translation by the 5′- and 3′-terminal regions of the dengue virus genome. J Virol 79:8303–8315

Cibulskis K, Lawrence MS, Carter SL, Sivachenko A, Jaffe D, Sougnez C, Gabriel S, Meyerson M, Lander ES, Getz G (2013) Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. Nat Biotechnol 31:213–219

Conry RM, LoBuglio AF, Wright M, Sumerel L, Pike MJ, Johanning F, Benjamin R, Lu D, Curiel DT (1995) Characterization of a messenger RNA polynucleotide vaccine vector. Cancer Res 55:1397–1400

Diken M, Kreiter S, Selmi A, Britten CM, Huber C, Tureci O, Sahin U (2011) Selective uptake of naked vaccine RNA by dendritic cells is driven by macropinocytosis and abrogated upon DC maturation. Gene Ther 18:702–708

Diken M, Kreiter S, Selmi A, Tureci O, Sahin U (2013) Antitumor vaccination with synthetic mRNA: strategies for in vitro and in vivo preclinical studies. Methods Mol Biol 969:235–246

Ding L, Ellis MJ, Li S, Larson DE, Chen K, Wallis JW, Harris CC, McLellan MD, Fulton RS, Fulton LL, Abbott RM, Hoog J, Dooling DJ, Koboldt DC, Schmidt H, Kalicki J, Zhang Q, Chen L, Lin L, Wendl MC, McMichael JF, Magrini VJ, Cook L, McGrath SD, Vickery TL, Appelbaum E, Deschryver K, Davies S, Guintoli T, Lin L, Crowder R, Tao Y, Snider JE, Smith SM, Dukes AF, Sanderson GE, Pohl CS, Delehaunty KD, Fronick CC, Pape KA, Reed JS, Robinson JS, Hodges JS, Schierding W, Dees ND, Shen D, Locke DP, Wiechert ME, Eldred JM, Peck JB, Oberkfell BJ, Lolofie JT, Du F, Hawkins AE, O'Laughlin MD, Bernard KE, Cunningham M, Elliott G, Mason MD, Thompson DM Jr, Ivanovich JL, Goodfellow PJ, Perou CM, Weinstock GM, Aft R, Watson M, Ley TJ, Wilson RK, Mardis ER (2010) Genome remodelling in a basal-like breast cancer metastasis and xenograft. Nature 464:999–1005

Duan J, Antezana MA (2003) Mammalian mutation pressure, synonymous codon choice, and mRNA degradation. J Mol Evol 57:694–701

Fotin-Mleczek M, Duchardt KM, Lorenz C, Pfeiffer R, Ojkic-Zrna S, Probst J, Kallen KJ (2011) Messenger RNA-based vaccines with dual activity induce balanced TLR-7 dependent adaptive immune responses and provide antitumor activity. J Immunother 34:1–15

Garneau NL, Wilusz J, Wilusz CJ (2007) The highways and byways of mRNA decay. Nat Rev Mol Cell Biol 8:113–126

Garneau NL, Sokoloski KJ, Opyrchal M, Neff CP, Wilusz CJ, Wilusz J (2008) The 3′ untranslated region of sindbis virus represses deadenylation of viral transcripts in mosquito and mammalian cells. J Virol 82:880–892

Grantham R, Gautier C, Gouy M, Mercier R, Pave A (1980) Codon catalog usage and the genome hypothesis. Nucleic Acids Res 8:r49–r62

Greenman C, Stephens P, Smith R, Dalgliesh GL, Hunter C, Bignell G, Davies H, Teague J, Butler A, Stevens C, Edkins S, O'Meara S, Vastrik I, Schmidt EE, Avis T, Barthorpe S, Bhamra G, Buck G, Choudhury B, Clements J, Cole J, Dicks E, Forbes S, Gray K, Halliday K, Harrison R, Hills K, Hinton J, Jenkinson A, Jones D, Menzies A, Mironenko T, Perry J, Raine K, Richardson D, Shepherd R, Small A, Tofts C,

Varian J, Webb T, West S, Widaa S, Yates A, Cahill DP, Louis DN, Goldstraw P, Nicholson AG, Brasseur F, Looijenga L, Weber BL, Chiew YE, DeFazio A, Greaves MF, Green AR, Campbell P, Birney E, Easton DF, Chenevix-Trench G, Tan MH, Khoo SK, Teh BT, Yuen ST, Leung SY, Wooster R, Futreal PA, Stratton MR (2007) Patterns of somatic mutation in human cancer genomes. Nature 446:153–158

Grudzien-Nogalska E, Jemielity J, Kowalska J, Darzynkiewicz E, Rhoads RE (2007) Phosphorothioate cap analogs stabilize mRNA and increase translational efficiency in mammalian cells. RNA 13:1745–1755

Grudzien-Nogalska E, Kowalska J, Su W, Kuhn AN, Slepenkov SV, Darzynkiewicz E, Sahin U, Jemielity J, Rhoads RE (2013) Synthetic mRNAs with superior translation and stability properties. Methods Mol Biol 969:55–72

Hoerr I, Obst R, Rammensee HG, Jung G (2000) In vivo application of RNA leads to induction of specific cytotoxic T lymphocytes and antibodies. Eur J Immunol 30:1–7

Holtkamp S, Kreiter S, Selmi A, Simon P, Koslowski M, Huber C, Tureci O, Sahin U (2006) Modification of antigen-encoding RNA increases stability, translational efficacy, and T-cell stimulatory capacity of dendritic cells. Blood 108:4009–4017

Houseley J, Tollervey D (2009) The many pathways of RNA degradation. Cell 136:763–776

Inaba K, Metlay JP, Crowley MT, Steinman RM (1990) Dendritic cells pulsed with protein antigens in vitro can prime antigen-specific, MHC-restricted T cells in situ. J Exp Med 172:631–640

Jacobson A, Peltz SW (1996) Interrelationships of the pathways of mRNA decay and translation in eukaryotic cells. Annu Rev Biochem 65:693–739

Jemielity J, Fowler T, Zuberek J, Stepinski J, Lewdorowicz M, Niedzwiecka A, Stolarski R, Darzynkiewicz E, Rhoads RE (2003) Novel "anti-reverse" cap analogs with superior translational properties. RNA 9:1108–1122

Jinek M, Coyle SM, Doudna JA (2011) Coupled 5′ nucleotide recognition and processivity in Xrn1-mediated mRNA decay. Mol Cell 41:600–608

Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Kamiyama H, Jimeno A, Hong SM, Fu B, Lin MT, Calhoun ES, Kamiyama M, Walter K, Nikolskaya T, Nikolsky Y, Hartigan J, Smith DR, Hidalgo M, Leach SD, Klein AP, Jaffee EM, Goggins M, Maitra A, Iacobuzio-Donahue C, Eshleman JR, Kern SE, Hruban RH, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW (2008) Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science 321:1801–1806

Khabar KS (2005) The AU-rich transcriptome: more than interferons and cytokines, and its role in disease. J Interferon Cytokine Res 25:1–10

Koboldt DC, Steinberg KM, Larson DE, Wilson RK, Mardis ER (2013) The next-generation sequencing revolution and its impact on genomics. Cell 155:27–38

Kowalska J, Lewdorowicz M, Zuberek J, Grudzien-Nogalska E, Bojarska E, Stepinski J, Rhoads RE, Darzynkiewicz E, Davis RE, Jemielity J (2008) Synthesis and characterization of mRNA cap analogs containing phosphorothioate substitutions that bind tightly to eIF4E and are resistant to the decapping pyrophosphatase DcpS. RNA 14:1119–1131

Kreiter S, Konrad T, Sester M, Huber C, Tureci O, Sahin U (2007) Simultaneous ex vivo quantification of antigen-specific CD4+ and CD8+ T cell responses using in vitro transcribed RNA. Cancer Immunol Immunother 56:1577–1587

Kreiter S, Selmi A, Diken M, Sebastian M, Osterloh P, Schild H, Huber C, Tureci O, Sahin U (2008) Increased antigen presentation efficiency by coupling antigens to MHC class I trafficking signals. J Immunol 180:309–318

Kreiter S, Selmi A, Diken M, Koslowski M, Britten CM, Huber C, Tureci O, Sahin U (2010) Intranodal vaccination with naked antigen-encoding RNA elicits potent prophylactic and therapeutic antitumoral immunity. Cancer Res 70:9031–9040

Kreiter S, Diken M, Selmi A, Diekmann J, Attig S, Husemann Y, Koslowski M, Huber C, Tureci O, Sahin U (2011a) FLT3 Ligand enhances the cancer therapeutic potency of naked RNA vaccines. Cancer Res 71:6132–6142

Kreiter S, Diken M, Selmi A, Tureci O, Sahin U (2011b) Tumor vaccination using messenger RNA: prospects of a future therapy. Curr Opin Immunol 23:399–406

Kuhn AN, Diken M, Kreiter S, Selmi A, Kowalska J, Jemielity J, Darzynkiewicz E, Huber C, Tureci O, Sahin U (2010) Phosphorothioate cap analogs increase stability and translational efficiency of RNA vaccines in immature dendritic cells and induce superior immune responses in vivo. Gene Ther 17:961–971

Kuhn AN, Diken M, Kreiter S, Vallazza B, Tureci O, Sahin U (2011) Determinants of intracellular RNA pharmacokinetics: implications for RNA-based immunotherapeutics. RNA Biol 8:35–43

Kuhn AN, Beibetaert T, Simon P, Vallazza B, Buck J, Davies BP, Tureci O, Sahin U (2012) MRNA as a versatile tool for exogenous protein expression. Curr Gene Ther 12:347–361

Lee W, Jiang Z, Liu J, Haverty PM, Guan Y, Stinson J, Yue P, Zhang Y, Pant KP, Bhatt D, Ha C, Johnson S, Kennemer MI, Mohan S, Nazarenko I, Watanabe C, Sparks AB, Shames DS, Gentleman R, de Sauvage FJ, Stern H, Pandita A, Ballinger DG, Drmanac R, Modrusan Z, Seshagiri S, Zhang Z (2010) The mutation spectrum revealed by paired genome sequences from a lung cancer patient. Nature 465:473–477

Lower M, Renard BY, de Graaf J, Wagner M, Paret C, Kneip C, Tureci O, Diken M, Britten C, Kreiter S, Koslowski M, Castle JC, Sahin U (2012) Confidence-based somatic mutation evaluation and prioritization. PLoS Comput Biol 8:e1002714

Lundegaard C, Lamberth K, Harndahl M, Buus S, Lund O, Nielsen M (2008) NetMHC-3.0: accurate web accessible predictions of human, mouse and monkey

MHC class I affinities for peptides of length 8–11. Nucleic Acids Res 36:W509–W512

Lykke-Andersen S, Tomecki R, Jensen TH, Dziembowski A (2011) The eukaryotic RNA exosome: same scaffold but variable catalytic subunits. RNA Biol 8:61–66

Martinon F, Krishnan S, Lenzen G, Magne R, Gomard E, Guillet JG, Levy JP, Meulien P (1993) Induction of virus-specific cytotoxic T lymphocytes in vivo by liposome-entrapped mRNA. Eur J Immunol 23:1719–1722

Matsushita H, Vesely MD, Koboldt DC, Rickert CG, Uppaluri R, Magrini VJ, Arthur CD, White JM, Chen YS, Shea LK, Hundal J, Wendl MC, Demeter R, Wylie T, Allison JP, Smyth MJ, Old LJ, Mardis ER, Schreiber RD (2012) Cancer exome analysis reveals a T-cell-dependent mechanism of cancer immunoediting. Nature 482:400–404

Meyer S, Temme C, Wahle E (2004) Messenger RNA turnover in eukaryotes: pathways and enzymes. Crit Rev Biochem Mol Biol 39:197–216

Mockey M, Bourseau E, Chandrashekhar V, Chaudhuri A, Lafosse S, Le CE, Quesniaux VF, Ryffel B, Pichon C, Midoux P (2007) mRNA-based cancer vaccine: prevention of B16 melanoma progression and metastasis by systemic injection of MART1 mRNA histidylated lipopolyplexes. Cancer Gene Ther 14:802–814

Morse MA, Lyerly HK, Gilboa E, Thomas E, Nair SK (1998) Optimization of the sequence of antigen loading and CD40-ligand-induced maturation of dendritic cells. Cancer Res 58:2965–2968

Moura GR, Pinheiro M, Freitas A, Oliveira JL, Frommlet JC, Carreto L, Soares AR, Bezerra AR, Santos MA (2011) Species-specific codon context rules unveil non-neutrality effects of synonymous mutations. PLoS One 6:e26817

Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Siu IM, Gallia GL, Olivi A, McLendon R, Rasheed BA, Keir S, Nikolskaya T, Nikolsky Y, Busam DA, Tekleab H, Diaz LA Jr, Hartigan J, Smith DR, Strausberg RL, Marie SK, Shinjo SM, Yan H, Riggins GJ, Bigner DD, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW (2008) An integrated genomic analysis of human glioblastoma multiforme. Science 321:1807–1812

Pascolo S (2008) Vaccination with messenger RNA (mRNA). Handb Exp Pharmacol (183):221–235

Pasquinelli AE, Dahlberg JE, Lund E (1995) Reverse 5′ caps in RNAs made in vitro by phage RNA polymerases. RNA 1:957–967

Peng ZH, Sharma V, Singleton SF, Gershon PD (2002) Synthesis and application of a chain-terminating dinucleotide mRNA cap analog. Org Lett 4:161–164

Perche F, Benvegnu T, Berchel M, Lebegue L, Pichon C, Jaffres PA, Midoux P (2011) Enhancement of dendritic cells transfection in vivo and of vaccination against B16F10 melanoma with mannosylated histidylated lipopolyplexes loaded with tumor antigen messenger RNA. Nanomedicine 7:445–453

Pleasance ED, Cheetham RK, Stephens PJ, McBride DJ, Humphray SJ, Greenman CD, Varela I, Lin ML, Ordonez GR, Bignell GR, Ye K, Alipaz J, Bauer MJ, Beare D, Butler A, Carter RJ, Chen L, Cox AJ, Edkins S, Kokko-Gonzales PI, Gormley NA, Grocock RJ, Haudenschild CD, Hims MM, James T, Jia M, Kingsbury Z, Leroy C, Marshall J, Menzies A, Mudie LJ, Ning Z, Royce T, Schulz-Trieglaff OB, Spiridou A, Stebbings LA, Szajkowski L, Teague J, Williamson D, Chin L, Ross MT, Campbell PJ, Bentley DR, Futreal PA, Stratton MR (2010a) A comprehensive catalogue of somatic mutations from a human cancer genome. Nature 463:191–196

Pleasance ED, Stephens PJ, O'Meara S, McBride DJ, Meynert A, Jones D, Lin ML, Beare D, Lau KW, Greenman C, Varela I, Nik-Zainal S, Davies HR, Ordonez GR, Mudie LJ, Latimer C, Edkins S, Stebbings L, Chen L, Jia M, Leroy C, Marshall J, Menzies A, Butler A, Teague JW, Mangion J, Sun YA, McLaughlin SF, Peckham HE, Tsung EF, Costa GL, Lee CC, Minna JD, Gazdar A, Birney E, Rhodes MD, McKernan KJ, Stratton MR, Futreal PA, Campbell PJ (2010b) A small-cell lung cancer genome with complex signatures of tobacco exposure. Nature 463:184–190

Pogue GP, Cao XQ, Singh NK, Nakhasi HL (1993) 5′ sequences of rubella virus RNA stimulate translation of chimeric RNAs and specifically interact with two host-encoded proteins. J Virol 67:7106–7117

Ponsaerts P, Van den Bosch G, Cools N, Van DA, Nijs G, Lenjou M, Lardon F, Van BC, Van Bockstaele DR, Berneman ZN, Van Tendeloo VF (2002) Messenger RNA electroporation of human monocytes, followed by rapid in vitro differentiation, leads to highly stimulatory antigen-loaded mature dendritic cells. J Immunol 169:1669–1675

Qiu P, Ziegelhoffer P, Sun J, Yang NS (1996) Gene gun delivery of mRNA in situ results in efficient transgene expression and genetic immunization. Gene Ther 3:262–268

Rahma OE, Ashtar E, Ibrahim R, Toubaji A, Gause B, Herrin VE, Linehan WM, Steinberg SM, Grollman F, Grimes G, Bernstein SA, Berzofsky JA, Khleif SN (2010) A pilot clinical trial testing mutant von Hippel-Lindau peptide as a novel immune therapy in metastatic renal cell carcinoma. J Transl Med 8:8

Rains N, Cannan RJ, Chen W, Stubbs RS (2001) Development of a dendritic cell (DC)-based vaccine for patients with advanced colorectal cancer. Hepatogastroenterology 48:347–351

Rittig SM, Haentschel M, Weimer KJ, Heine A, Muller MR, Brugger W, Horger MS, Maksimovic O, Stenzl A, Hoerr I, Rammensee HG, Holderried TA, Kanz L, Pascolo S, Brossart P (2011) Intradermal vaccinations with RNA coding for TAA generate CD8+ and CD4+ immune responses and induce clinical benefit in vaccinated patients. Mol Ther 19:990–999

Robbins PF, Lu YC, el-Gamil M, Li YF, Gross C, Gartner J, Lin JC, Teer JK, Cliften P, Tycksen E, Samuels Y, Rosenberg SA (2013) Mining exomic sequencing

data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. Nat Med 19:747–752

Rock KL, Gamble S, Rothstein L (1990) Presentation of exogenous antigen with class I major histocompatibility complex molecules. Science 249:918–921

Rydzik AM, Kulis M, Lukaszewicz M, Kowalska J, Zuberek J, Darzynkiewicz ZM, Darzynkiewicz E, Jemielity J (2012) Synthesis and properties of mRNA cap analogs containing imidodiphosphate moiety–fairly mimicking natural cap structure, yet resistant to enzymatic hydrolysis. Bioorg Med Chem 20:1699–1710

Sachs AB, Davis RW, Kornberg RD (1987) A single domain of yeast poly(A)-binding protein is necessary and sufficient for RNA binding and cell viability. Mol Cell Biol 7:3268–3276

Sahin U, Tureci O, Schmitt H, Cochlovius B, Johannes T, Schmits R, Stenner F, Luo G, Schobert I, Pfreundschuh M (1995) Human neoplasms elicit multiple specific immune responses in the autologous host. Proc Natl Acad Sci U S A 92:11810–11813

Scheel B, Teufel R, Probst J, Carralot JP, Geginat J, Radsak M, Jarrossay D, Wagner H, Jung G, Rammensee HG, Hoerr I, Pascolo S (2005) Toll-like receptor-dependent activation of several human blood cell types by protamine-condensed mRNA. Eur J Immunol 35:1557–1566

Scholtalbers J, Rossler J, Sorn P, de Graaf J, Boisguerin V, Castle J, Sahin U (2013) Galaxy LIMS for next-generation sequencing. Bioinformatics 29:1233–1234

Sjoberg EM, Suomalainen M, Garoff H (1994) A significantly improved Semliki Forest virus expression system based on translation enhancer segments from the viral capsid gene. Biotechnology (N Y) 12:1127–1131

Sjoblom T, Jones S, Wood LD, Parsons DW, Lin J, Barber TD, Mandelker D, Leary RJ, Ptak J, Silliman N, Szabo S, Buckhaults P, Farrell C, Meeh P, Markowitz SD, Willis J, Dawson D, Willson JK, Gazdar AF, Hartigan J, Wu L, Liu C, Parmigiani G, Park BH, Bachman KE, Papadopoulos N, Vogelstein B, Kinzler KW, Velculescu VE (2006) The consensus coding sequences of human breast and colorectal cancers. Science 314:268–274

Song MG, Li Y, Kiledjian M (2010) Multiple mRNA decapping enzymes in mammalian cells. Mol Cell 40:423–432

Steitz J, Britten CM, Wolfel T, Tuting T (2006) Effective induction of anti-melanoma immunity following genetic vaccination with synthetic mRNA coding for the fusion protein EGFP.TRP2. Cancer Immunol Immunother 55:246–253

Stepinski J, Waddell C, Stolarski R, Darzynkiewicz E, Rhoads RE (2001) Synthesis and properties of mRNAs containing the novel "anti-reverse" cap analogs 7-methyl(3′–O-methyl)GpppG and 7-methyl (3′-deoxy)GpppG. RNA 7:1486–1495

Stratton MR (2011) Exploring the genomes of cancer cells: progress and promise. Science 331:1553–1558

Totoki Y, Tatsuno K, Yamamoto S, Arai Y, Hosoda F, Ishikawa S, Tsutsumi S, Sonoda K, Totsuka H, Shirakihara T, Sakamoto H, Wang L, Ojima H, Shimada K, Kosuge T, Okusaka T, Kato K, Kusuda J, Yoshida T, Aburatani H, Shibata T (2011) High-resolution characterization of a hepatocellular carcinoma genome. Nat Genet 43:464–469

van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T (1991) A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 254:1643–1647

van der Werf S, Bradley J, Wimmer E, Studier FW, Dunn JJ (1986) Synthesis of infectious poliovirus RNA by purified T7 RNA polymerase. Proc Natl Acad Sci U S A 83:2330–2334

Van Lint S, Goyvaerts C, Maenhout S, Goethals L, Disy A, Benteyn D, Pen J, Bonehill A, Heirman C, Breckpot K, Thielemans K (2012) Preclinical evaluation of TriMix and antigen mRNA-based antitumor therapy. Cancer Res 72:1661–1671

Van Lint S, Heirman C, Thielemans K, Breckpot K (2013) mRNA: from a chemical blueprint for protein production to an off-the-shelf therapeutic. Hum Vaccin Immunother 9:265–274

van Rooij N, van Buuren MM, Philips D, Velds A, Toebes M, Heemskerk B, van Dijk LJ, Behjati S, Hilkmann H, El Atmioui D, Nieuwland M, Stratton MR, Kerkhoven RM, Kesmir C, Haanen JB, Kvistborg P, Schumacher TN (2013) Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. J Clin Oncol 31:e439–e442

Van Tendeloo VF, Ponsaerts P, Lardon F, Nijs G, Lenjou M, Van BC, Van Bockstaele DR, Berneman ZN (2001) Highly efficient gene delivery by mRNA electroporation in human hematopoietic cells: superiority to lipofection and passive pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells. Blood 98:49–56

Waggoner SA, Liebhaber SA (2003) Identification of mRNAs associated with alphaCP2-containing RNP complexes. Mol Cell Biol 23:7055–7067

Warminski M, Kowalska J, Buck J, Zuberek J, Lukaszewicz M, Nicola C, Kuhn AN, Sahin U, Darzynkiewicz E, Jemielity J (2013) The synthesis of isopropylidene mRNA cap analogs modified with phosphorothioate moiety and their evaluation as promoters of mRNA translation. Bioorg Med Chem Lett 23:3753–3758

Wei X, Walia V, Lin JC, Teer JK, Prickett TD, Gartner J, Davis S, Stemke-Hale K, Davies MA, Gershenwald JE, Robinson W, Robinson S, Rosenberg SA, Samuels Y (2011) Exome sequencing identifies GRIN2A as frequently mutated in melanoma. Nat Genet 43: 442–446

Weide B, Carralot JP, Reese A, Scheel B, Eigentler TK, Hoerr I, Rammensee HG, Garbe C, Pascolo S (2008) Results of the first phase I/II clinical vaccination trial with direct injection of mRNA. J Immunother 31:180–188

Wolfel C, Lennerz V, Lindemann E, Hess G, Derigs HG, Huber C, Herr W, Wolfel T (2008) Dissection and molecular analysis of alloreactive CD8+ T cell responses in allogeneic haematopoietic stem cell transplantation. Cancer Immunol Immunother 57:849–857

Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, Felgner PL (1990) Direct gene transfer into mouse muscle in vivo. Science 247:1465–1468

Wood LD, Parsons DW, Jones S, Lin J, Sjoblom T, Leary RJ, Shen D, Boca SM, Barber T, Ptak J, Silliman N, Szabo S, Dezso Z, Ustyanksky V, Nikolskaya T, Nikolsky Y, Karchin R, Wilson PA, Kaminker JS, Zhang Z, Croshaw R, Willis J, Dawson D, Shipitsin M, Willson JK, Sukumar S, Polyak K, Park BH, Pethiyagoda CL, Pant PV, Ballinger DG, Sparks AB, Hartigan J, Smith DR, Suh E, Papadopoulos N, Buckhaults P, Markowitz SD, Parmigiani G, Kinzler KW, Velculescu VE, Vogelstein B (2007) The genomic landscapes of human breast and colorectal cancers. Science 318:1108–1113

Ying H, Zaks TZ, Wang RF, Irvine KR, Kammula US, Marincola FM, Leitner WW, Restifo NP (1999) Cancer therapy using a self-replicating RNA vaccine. Nat Med 5:823–827

Yu J, Russell JE (2001) Structural and functional analysis of an mRNP complex that mediates the high stability of human beta-globin mRNA. Mol Cell Biol 21: 5879–5888

Zhou X, Berglund P, Rhodes G, Parker SE, Jondal M, Liljestrom P (1994) Self-replicating Semliki Forest virus RNA as recombinant vaccine. Vaccine 12: 1510–1514

Zhou WZ, Hoon DS, Huang SK, Fujii S, Hashimoto K, Morishita R, Kaneda Y (1999) RNA melanoma vaccine: induction of antitumor immunity by human glycoprotein 100 mRNA immunization. Hum Gene Ther 10:2719–2724

Antibody Therapy in Oncology

Martin Glennie, Özlem Türeci, and
Peter W.M. Johnson

Therapeutic Monoclonal Antibodies in Oncology: A Short History

During the three decades since monoclonal antibodies (mAbs) first became widely available, they have seen remarkable progress in their application to cancer treatment. In the early days the focus was on finding targets which would allow recruitment of the body's innate immunity, in the form of Fc receptor-expressing cellular effectors and complement, against the unwanted cancer (Scott et al. 2012). Following some initial encouraging results from the use of anti-idiotype mAbs to treat lymphoma, this technology proved too cumbersome for wide application, and fully murine reagents failed to yield convincing responses in the clinic. Significant progress was not made until major advances in molecular biology allowed antibody engineering and the production of human/mouse chimeric or humanised products. This resulted in drugs which target tumours directly for destruction by the body's natural effectors as originally conceived, as with rituximab, or which also had the capacity to block the oncogenic properties of their target protein, as with the blocking of Her2 by trastuzumab. Antibodies were still relatively ineffective at treating solid tumours, partly because of a lack of good tumour-restricted targets but also because epithelial tumours are generally resistant to the cytotoxic activity of natural effectors. Throughout this period, the ability of mAbs to manipulate the immune system itself was being investigated in murine models, with different antibody formats either blocking or stimulating immune receptors and able to regulate conventional acquired Ab and T-cell immunity. This immunomodulatory activity appears critical for the latest, and perhaps most promising, application of mAb.

Even though early attempts to use mAb to treat cancer were considered largely unsuccessful, they were critical in defining many of the obstacles to their use as therapeutics and understanding their mechanism of action (MoA). The successes were sufficient to sustain mAb development through the difficult periods of the 1980s and early 1990s. They also established that mAbs were generally much more cytolytic against haematopoietic malignancies as compared to epithelial tumours, which is an observation that remains true even after mAb function is optimised to increase potency. This resistance of solid tumours is only overcome when mAbs are used in a completely different way, not to attack the tumour directly, but to target the blood supply or to activate endogenous anticancer immunity (below).

M. Glennie (✉)
Antibody and Vaccine Group, Cancer Sciences Unit, Faculty of Medicine, Southampton General Hospital, University of Southampton, Southampton, UK
e-mail: m.j.glennie@soton.ac.uk

Ö. Türeci
Ganymed Pharmaceutical AG, Mainz, Germany

P.W.M. Johnson
Southampton Cancer Research UK Centre, Southampton General Hospital, University of Southampton, Southampton, UK
e-mail: johnsonp@soton.ac.uk

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_10,

Early attempts to use mAbs against solid tumours focused on 17.1A, which recognised the anti-epithelial cell adhesion molecule (EPCAM) (Göttlinger et al. 1986) and was one of the many thousands tested that showed good specificity for colon cancer. While modest success was reported, with some stabilisation of disease and occasional objective responses, 17.1A lacked potency and promoted strong human anti-mouse antibody (HAMA) responses. Interestingly, careful analysis of the results showed a possible correlation between outcome and HAMA response, hinting at a therapeutic effect from recruitment of an anti-idiotype network, although this remains a controversial idea. The lack of efficacy for EPCAM was later confirmed in a phase III clinical trial using a humanised version of the mAb.

Work targeting lymphoid tumours fared much better: firstly, because of the ease with which tumour-specific (idiotype) or cell-restricted (CD52, CD20) mAbs could be raised and, secondly, because of the greater sensitivity of the target cells to lysis by natural effector systems and finally because patients with lymphoid tumours proved less likely to raise HAMA responses. The lack of HAMA responses was attributed to a compromised immune system by previous chemotherapy and to the immunosuppressive nature of the disease and was critical for allowing multiple administrations of rodent mAb. Work by the Stevenson in Southampton and by Levy at Stanford showed that while the idiotope of the B-cell receptor (BCR) provided a highly specific target for individual lymphoid tumours, tailor-making these reagents for each patient was beyond the capacity of either academic or industrial partners. However, although they would never be drugs on a large scale, it was this experience which demonstrated the potential efficacy of immunoglobulin (Ig)G mAbs and their lack of overt toxicity. A corollary of this work was the demonstration that when mAbs bound and cross-linked a membrane receptor (in this case, the BCR), they could generate transmembrane signals which mimicked those elicited by a natural ligand, triggering cellular responses including antigenic modulation (internalisation) of the BCR, a rapid rise in intracellular signalling molecules such as cAMP, and various inhibition or stimulation of cell growth. This cross-linking activity of mAb, often promoted by Fc receptors (FcRs), would emerge as a vital property of therapeutic mAb, exploited in many subsequent developments.

The 1980s saw the exploration of a family of molecules based on the Campath mAb, recognising a dodecameric peptide, CDw52, expressed at high levels on most leucocytes. Waldmann and colleagues raised the Campath 1 M IgM mAb which was highly effective in activating human complement and lysing target cells (Hale et al. 1990). However, this activity, while useful for clearing cells from the circulation of patients with leukaemia, was unable to resolve more bulky disease in lymphomatous nodes. This was only achieved when the IgM mAb was converted to a rat IgG2b isotype, which was notably more active. This important observation pointed the way for future mAb design by identifying cellular effectors, including the FcR on myeloid and natural killer (NK) cells, as the primary mechanism mediating therapeutic activity, at least against lymphoid tumours. The Campath molecule was one of the first used to develop human/mouse chimeric reagents, in which the specificity of the rodent mAb was genetically grafted onto human constant regions. This technology made mAbs into effective drugs. Reagents were converted either into chimeric mAbs with entire heavy- and light-chain variable domains from the required specificity genetically spliced onto human constant region genes or using a more sophisticated process of humanisation using just the mAb complementarity-determining regions (CDRs) grafted into a suitable human framework. This latter process resulted on only 5 % of the mAb molecule being of rodent origin, and hence in practical terms it could be considered human. However, a cost of this elegant engineering was a slight but consistently observed reduction in binding affinity. Later technology would overtake the need for genetic engineering, allowing the isolation of fully human mAb either from phage libraries of human V regions or from transgenic mice, in which the mouse V regions had been replaced with most of the human V regions.

Genetic engineering of this type was used in the first widely successful anticancer mAbs rituximab (anti-CD20, chimeric) and trastuzumab (anti-Her2/neu, humanised) and provided three important features required for clinical efficacy. Firstly, it greatly increased their ability to engage natural effectors, particularly using human IgG1 for recruiting via one of the activatory FcR, such as FcRIIIa on NK cells. Secondly, it reduced immunogenicity, allowing multiple doses of most mAbs to be administered without HAMA responses. Thirdly, it greatly extended the biological survival of the reagents by their ability to bind to the so-called Brambell receptor, or FcRn, which rescues human IgG from degradation in endothelial cells and recycles it back to the plasma. This leads to a half-life for most subclasses of human IgG of over 20 days, compared with around 18 h for mouse mAb. Despite these improvements, it still proved hard to find mAb which would tackle the difficult epithelial cancers. The success of reagents such as trastuzumab and cetuximab probably relate in large part to the ability of mAb to block the growth-promoting activity of their respective targets as much as to the recruitment of natural effectors, although the relative contribution of cytotoxic FcR-expressing effector cells versus mAb-blocking activity remains a contentious issue. It remains to be seen whether naked mAbs, directed at "inert" targets on solid tumours where the antigen is not a growth receptor, such as MUC1 or CEA, will become effective therapies with the capacity to control cancer. While these targets have failed to date, strategies to promote the efficacy of the mAb or to augment the potency of effector cells may change this.

Types of Monoclonal Antibody Therapeutics and Their Mechanisms of Action

During three decades of clinical investigation, mAbs have been developed to exert their antitumour effect through different MoA, either naked or conjugated with radionuclides or toxins (Table 1, Fig. 1) (Sliwkowski and Mellman 2013).

For naked mAbs, one possible MoA is to perturb tumour-cell signalling. The mAbs acting in this way can block important signalling pathways essential for tumour-cell survival. They may target either membrane-bound receptors such as Her2 (trastuzumab) or epidermal growth factor receptor (EGFR) (cetuximab) or soluble growth factors such as vascular epithelial growth factor A (VEGF-A) (bevacizumab). However, clinically validated functional targets are scarce, which may limit the development of such blocking agents.

Upon binding to tumour cells, mAbs can also act through their Fc region and recruit intrinsic immune mechanisms, such as complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC; Fig. 1a). In CDC, the complement cascade is activated to form a membrane attack complex which results in direct tumour-cell lysis, a mechanism which may be important for mAbs like rituximab and ofatumumab. In addition, the complement cascade protein C5a is a powerful inflammatory mediator with chemotactic activity especially for neutrophils but also for monocytes and macrophages (Savola et al. 2011). These cells are important contributors to antitumour activity through phagocytosis. A further important MoA of mAbs is ADCC, which follows the "cross-linking" of the tumour with an NK cell. The mAb interacts through its Fc region with the FcγRIIIA present on NK cells, which release cytokines such as interferon (IFN) and cytotoxic granules containing perforin and granzymes, triggering apoptosis and tumour-cell death. Recent research has focused on increasing the moderate affinity between the mAb and the FcγRIIIA. It has been shown that the absence or low presence of a fucose residue on the sugar moiety of the mAb is associated with better binding to the FcγRIIIA and with increased ADCC activity (Niwa et al. 2004). The most advanced glyco-engineered mAb, obinutuzumab, targets CD20 and has recently been approved for the treatment of chronic lymphocytic leukaemia.

Recent results with immune-stimulating mAbs have revitalised the belief that the immune system not only has the potential to control solid

Table 1 Antibody-based therapeutics in oncology

Antibody-based therapeutic	Target	Mechanisms of action	Targeted cancers
Naked mAbs			
Rituximab (Rituxan)	CD20	ADCC, CDC, apoptosis induction	NHL, relapsed CLL
Ofatumumab (Arzerra)	CD20	ADCC, CDC	CLL refractory to fludarabine and alemtuzumab
Obinutuzumab (Gazyva)	CD20	Apoptosis, ADCC	Recurrent CLL
Alemtuzumab (Campath)	CD52	ADCC, CDC, apoptosis induction	B-cell CLL
Trastuzumab (Herceptin)	Her2/neu	Inhibition of ERBB/Her2/neu signalling –blockade of cell proliferation	Early and advanced breast cancer, advanced gastric or gastro-oesophageal carcinoma
Pertuzumab (Perjeta)	Her2/neu	Inhibition of ERBB/Her2/neu signalling –blockade of cell proliferation	Neo-adjuvant treatment of $Her2^+$ breast cancer, metastatic $Her2^+$ breast cancer both in combination with trastuzumab and docetaxel
Bevacizumab (Avastin)	VEGF-A	Angiogenesis inhibition by deceleration of tumour vascularisation	Metastatic colorectal, breast, non-small-cell lung and renal cell cancer; recurrent glioblastoma multiforme; advanced ovarian cancer
Cetuximab (Erbitux)	EGFR	EGFR signalling inhibition, ADCC	Locally advanced or recurrent/metastatic head and neck cancer of squamous histology, in combination with chemotherapy: wild-type *KRAS* metastatic colorectal cancer
Panitumumab (Vectibix)	EGFR	EGFR signalling inhibition	Wild-type *KRAS* metastatic colorectal cancer
Ipilimumab (Yervoy)	CTLA-4	Potentiation of T-cell activation by blocking CTLA-4	Unresectable or metastatic melanoma (second-line treatment)
Chemo-labelled ADCs			
Brentuximab vedotin (MMAE; Adcetris)	CD30	Cytotoxicity due to chemotherapeutics delivery to target cells; cytoskeletal disruption	Relapsed/refractory $CD30^+$ Hodgkin lymphoma
Ado-trastuzumab emtansine (DM1; Kadcyla)	ERBB2/Her2/neu	Cytotoxicity due to chemotherapeutics delivery to target cells; cytoskeletal disruption	Refractory $Her2^+$ breast cancer
Gemtuzumab ozogamicin (Mylotarg)	CD33	Cytotoxicity due to chemotherapeutics delivery to target cells; DNA binding	Elderly patients with advanced AML (2000–2010; drug withdrawn from market due to lack of efficacy benefit in post-marketing phase III trial)
Radio-labelled ADCs			
^{90}Y-labelled ibritumomab tiuxetan (Zevalin)	CD20	Cytotoxicity due to radiation delivery to target cells	Relapsed follicular lymphoma, or as consolidation treatment in follicular lymphoma
^{131}I-labelled tositumomab (Bexxar)	CD20	Cytotoxicity due to radiation delivery to target cells	NHL

ADC antibody-drug conjugate, *ADCC* antibody-dependent cytotoxicity, *AML* acute myeloid leukaemia, *CD* cluster of differentiation, *CDC* complement-dependent cytotoxicity, *CLL* chronic lymphocytic leukaemia, *CTLA-4* cytotoxic T-lymphocyte antigen 4, *DM1* mertansine, *EGFR* endothelial growth factor receptor, *ERBB* avian erythroblastosis oncogene B, *KRAS* Kirsten rat sarcoma viral oncogene homolog, *MMAE* monomethyl auristatin E, *NHL* non-Hodgkin lymphoma, *VEGF* vascular endothelial growth factor

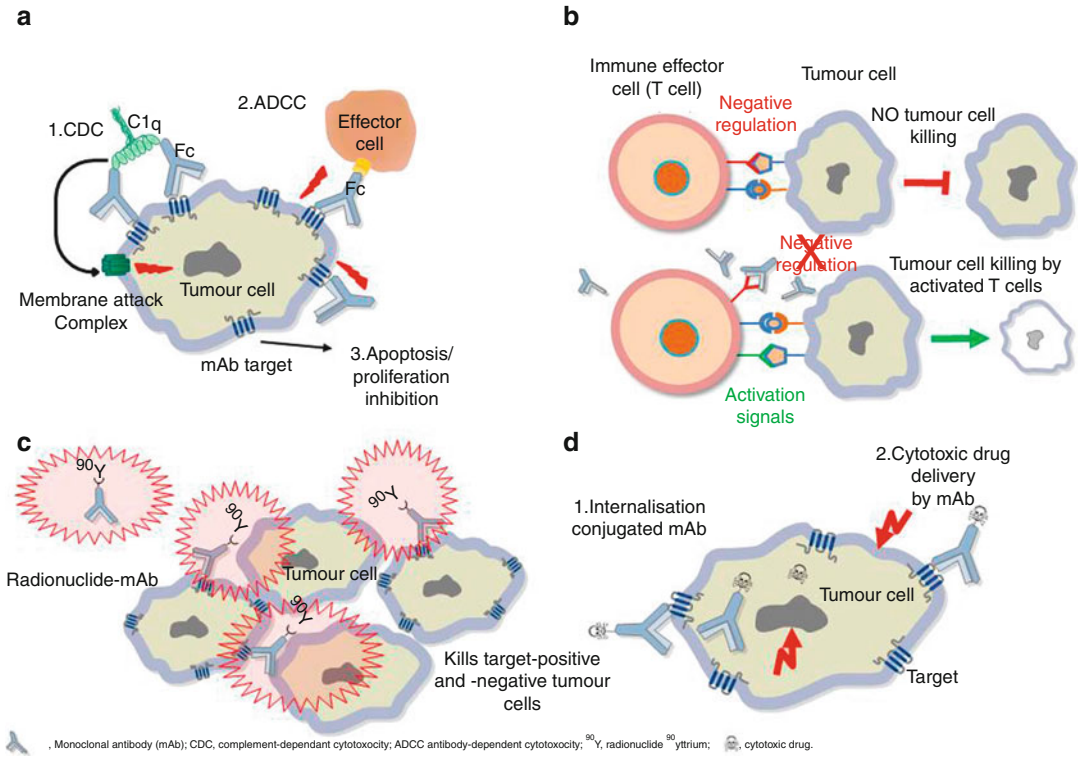

Fig. 1 Mechanism of action of antibody-based tumour therapies: (**a**) naked mAbs, (**b**) immune-stimulating mAbs, (**c**) radionuclide-conjugate mAbs, (**d**) Ab-drug conjugate

tumour growth but that it can also be upregulated by appropriate intervention to deliver clinically meaningful responses (Lee et al. 2013). The current leading candidates in this exciting field are anti-cytotoxic T-lymphocyte antigen (CTLA)-4 (ipilimumab) and anti-programmed death (PD)-1/PD-ligand (L)1, which have delivered impressive clinical results both alone and in combination. The original concept for these immunomodulatory mAbs, sometimes called checkpoint blockers, was that they could block the negative feedback signals provided to effector T cells via CTLA-4 and PD-1 (Fig. 1b). Such an interpretation was supported by considerable in vitro data showing that adding a CTLA-4-blocking mAb promoted effector T-cell responses. This interpretation was also supported by growing evidence showing that T-cell infiltrates in a range of tumour types were anergic and showed signs of exhaustion including high expression of CTLA-4, PD-1 and other inhibitory receptors such as TIM3 and LAG3. Hence, blocking the function of inhibitory receptors seemed a logical step towards reversing the inactive state of immune effector cells. More recently, however, growing evidence demonstrated, at least for anti-CTLA-4 in mice, that the isotype of the therapeutic mAb is important and that one with cytotoxic activity, such as IgG2a, is more active than an IgG which is unable to recruit cytotoxic cellular effectors. This is inconsistent with a blocking function alone, and recently convincing data from elegant animal models has suggested that anti-CTLA-4, at least in part, may work through its ability to delete regulatory T cells (Tregs) in the tumour which, unlike effector cells, express CTLA-4 at high levels. The observation that sufficient levels necessary to mediate cell killing of the most active Fc receptor, FcRIV, on macrophages are only available in the tumour itself supports the earlier explanation and may explain why not all Tregs are deleted. It is not clear whether

anti-PD-1 mAb might have a similar ability to differentially deplete Tregs, but it is interesting to speculate that the success of ipilimumab, a cytotoxic human IgG1, was not matched by a similar anti-CTLA-4 reagent (tremelimumab), which was a human IgG2 with significantly less activity in ADCC.

The two approved radioimmunotherapeutic mAbs are ^{90}Y-labelled ibritumomab tiuxetan and ^{131}I-labelled tositumomab, both targeting CD20. The lethal beta radiation emitted by the radionuclides induces cellular damage by the formation of free radicals in the targeted tumour cells as well as in healthy neighbouring cells (Fig. 1c). Thus, the long beta emission, in millimetres, limits their therapeutic index. There is an interest in exploiting short-range, high-energy α-particle-emitting nuclides for the eradication of minimal residual cancerous disease.

The recently emerging technology based on mAbs is the antibody-drug conjugate (ADC). ADCs are mAbs targeting a cytotoxic drug directly and selectively to tumour cells (Fig. 1d). The major drawback with nontargeted chemotherapy is the systemic damage to all dividing cells which limits its potential use. In ADCs, the cytotoxic drug is conjugated to the mAb by a chemical linker. In order to be effective, the mAb has to bind to a membrane receptor that is internalised. Subsequently, the drug should be cleaved off the mAb by intracellular enzymes and be released in its functional cytotoxic form into the cytoplasm of the tumour cell. Gemtuzumab ozogamicin was the first ADC approved by the FDA in 2000. However, it was withdrawn from the market in 2010 because, in a post-marketing follow-up clinical trial, it failed to meet prospective efficacy targets that were required for its accelerated approval by the FDA. During recent years a lot of research focussed on the improvement of the linker and drug technology to optimise the release of the active drugs into the cytoplasm. Recently, two ADCs, trastuzumab emtansine and brentuximab vedotin, have been approved by the FDA. Due to the experiences hitherto, nearly 20 additional ADCs have been constructed and are in early-stage clinical development in several cancer types.

Thus, mAbs are multipurpose drugs, which combine highly specific targeting with a diversity of modes of action, each of which can be engineered to be highly efficient in its own right to induce antitumour activity.

The Clinical Impact of Antibody Therapies for Cancer

The introduction of mAbs into some areas of oncology was transformative. Although the results of treatment with murine anti-idiotype antibodies were striking in a few cases of B-cell lymphoma, it was only with the development of generic "tumour-selective" reagents with human Fc regions that the true potential of Kohler and Milstein's invention became apparent (Köhler and Milstein 1975). The advanced state of knowledge of the membrane of B lymphocytes was responsible for the early identification of B-cell antigens as targets, and the development of the chimeric rituximab molecule for treating lymphoma boosted the field in a way that the anti-CD52 antibodies had failed to do. Although anti-CD52 (alemtuzumab) had shown some efficacy against diffuse bone marrow infiltrates in low-grade lymphoid malignancies, it was disappointing in the treatment of more substantial nodal disease, and its development was further hampered by the profound immunosuppression caused by targeting both T and B cells, leading to reactivation of lymphotropic infections. It was however this capacity for immunosuppression which led to its successful use for the control of graft-versus-host responses in allogeneic transplantation, and it is licensed for the treatment of chronic lymphocytic leukaemia.

The licensing of rituximab was the turning point for this field, following the pivotal study in which it evoked a response rate of nearly 50 % in recurrent and refractory low-grade lymphoma. It was rapidly incorporated into combination schedules with chemotherapy, where it has increased response rates and duration of remission in every type of B-cell lymphoma in which it has been formally tested, including follicular, marginal zone, mantle cell and diffuse large B cell. It was initially

difficult to demonstrate survival advantages in low-grade lymphomas where several lines of salvage treatment were available, and many patients crossed over in randomised trials following progression on the control arm. However, following the demonstration of surprisingly good results with rituximab alone in diffuse large B-cell lymphoma, a further breakthrough came with the demonstration that combining it with CHOP chemotherapy substantially increased survival in this lymphoma, where progress had previously been stalled for 20 years. R-CHOP as standard treatment for diffuse large B-cell lymphoma has been shown to drive up cure rates by around 20 %. Further advances have come from the use of prolonged maintenance therapy for low-grade lymphomas, an approach which had rarely been successful with chemotherapy. The use of maintenance rituximab has shown significant survival advantages for several types of indolent lymphoma and has become a standard approach to care. It is possible to track increasing lymphoma survival figures across the population as a whole back to the introduction of rituximab, a very striking achievement for a single drug. A new generation of anti-CD20 antibodies is now in development, and the first type 2 reagent, obinutuzumab, has been licensed for the treatment of CLL. This has the theoretical advantage of avoiding modulation off the cell surface and more effectively inducing apoptosis, although the results of direct comparisons with rituximab are not yet available. Another extension of anti-CD20 targeting was the development of radioimmunotherapy by direct conjugation of radionuclides via chelating linkers. Although lymphoma is highly radiosensitive and there was evidence of improved progression-free survival from the use of ^{90}Y-labelled ibritumomab tiuxetan consolidation in a randomised trial in follicular lymphoma, this approach has not been widely adopted, principally owing to the logistic difficulties of preparing and managing radioactive treatments and also from concerns over the potential for bone marrow dysplasia resulting from irradiation due to the presence there of subclinical deposits of lymphoma.

The extension of Ab therapy to solid tumours has been most successfully explored by targeting the Her2 receptor in breast cancer (e.g. trastuzumab), following the demonstration that dysregulation of this molecule was in part responsible for the uncontrolled proliferation of the tumours over-expressing it. mAbs raised against the Her2 extracellular domain are capable of preventing its dimerisation and constitutive signalling, although it is still not clear whether the dominant therapeutic effect stems from targeting the tumour cells for host-effector mechanisms or from signal blockade. It is interesting that small-molecule inhibitors of the Her2 kinase domain such as lapatinib are capable of producing responses in tumours which have progressed during treatment with trastuzumab, suggesting that escape from immune effector mechanisms may play a role, while the tumour remains partly sensitive to blockade of the Her2 signal. In any case, initial studies with trastuzumab in advanced metastatic disease demonstrated a modest but clear improvement in progression-free survival, an effect which was more pronounced in combination with chemotherapy, and which translated into a significant survival advantage when applied in combination with adjuvant chemotherapy in early-stage Her2-positive disease. Another antibody, pertuzumab, has been developed which recognises a distinct part of the Her2 extracellular domain and which appears to have an additive effect when given in combination with trastuzumab and chemotherapy, probably by preventing heterodimerisation with other members of the EGFR family. It is also possible that double antibody binding is responsible for a more effective mobilisation of effector mechanisms. A different extension of this approach has been in the development of an immunotoxin, ado-trastuzumab emtansine, capable of targeting a maytansinoid spindle antagonist linked to anti-Her2 antibody, which has shown impressive results in comparison to a conventional chemotherapy regimen. Another immunotoxin, brentuximab vedotin, targeting an auristatin spindle toxin to the CD30 molecule in Hodgkin lymphoma and anaplastic large-cell lymphoma has similarly shown very impressive response rates in phase II studies of relapsed disease and is being investigated in first-line treatment in combination with chemotherapy.

Despite the successes of rituximab and trastuzumab, other antibodies targeting tumour-cell surface antigens in epithelial malignancies have been generally less impressive in the magnitude of their effects, although it is not clear whether this is attributable to low drug levels or modulation of antigen expression or resistance of the tumour cells to complement and ADCC. Cetuximab targeting over-expressed EGFR-1 on colorectal cancer has a modest single-agent activity and an additive effect in combination with irinotecan chemotherapy for metastatic disease, but there is continuing controversy over its wider role, and it is not effective in those tumours with mutant KRAS driving proliferation. It is moderately active in head and neck cancer, where it improves the results in patients undergoing radiotherapy or chemotherapy with cisplatin and 5-fluorouracil. Another anti-EGFR-1, panitumumab, has the IgG2 isotype and in theory should be less active in triggering ADCC. It is not clear whether this impairs its effect, as it has never been compared directly to the IgG1 mAb cetuximab. In contrast to the findings with Her2 targeting, small-molecule inhibitors of EGFR-1 appear to have a quite different spectrum of activity. If anything, the results of combined antibody and small-molecule blockade of EGFR-1 seem to show antagonistic effects. Other antibodies targeting tumour-surface molecules such as EPCAM or the insulin-like growth factor 1 receptor have shown limited efficacy, and it seems likely that simple targeting of host-effector mechanisms may not be sufficient for strong therapeutic effects in many epithelial cancers: a specific biologic effect in the tumour such as induction of apoptosis or interruption of signalling is usually needed.

The approach of targeting the tumour microenvironment rather than tumour cells themselves has given rise to several interesting antibody therapies. At the simplest level, bevacizumab was developed to target the VEGF-A protein, with the intention of inhibiting tumour angiogenesis. This has produced moderate improvements in response rates and progression-free survival in colorectal cancer, and it also appears active in renal cell carcinoma, glioma and non-small-cell lung cancer in combination with chemotherapy. Overall, however, the absolute benefits reported for bevacizumab in any of these tumour types are relatively limited, and it has not been demonstrated to produce long-term cures in any of them. It seems likely that tumours are capable of circumventing the suppression of angiogenesis quite readily. A more compelling development has been the targeting of the adaptive immune response to the tumour by the use of mAbs targeting signalling molecules of the immune system. The demonstration that blockade of CTLA-4 by ipilimumab was capable of producing significant response rates and a small but clear proportion of long-term remissions when used as a single agent in melanoma has opened up a new field of enquiry. It has now been shown to improve the results of treatment in combination with dacarbazine in metastatic melanoma, and very substantial response rates are observed in combination with another checkpoint blocking antibody, nivolimumab, which targets the PD-1 receptor. A large number of antibody-as-ligand molecules, both activating and blocking, are now in clinical testing and hoping that these may be especially active in combination either with each other or with other immune-directed approaches such as vaccination or stimulation of innate immunity or with conventional chemotherapy and radiation.

When antibodies were first in clinical development, two important objections were raised to their use: the possibility that previous anticancer treatment or the illness itself would have depleted the capacity for host-effector responses via complement or ADCC and concerns over potential toxicity. In practice, it appears that even patients treated with quite extensive chemotherapy for lymphoma retain sufficient effector capability for rituximab to be effective, so this concern has been largely allayed. mAb therapies entail some toxicity, but this is generally specific to their MoA and less severe than that observed with cytotoxic treatment. Extended treatment with anti-CD20 can result in reduced Ig levels and impaired mucosal immunity, although this is rarely of clinical significance. There have been a number of cases of progressive multifocal leukoencephalopathy reported with anti-CD20 and

other immunosuppressive mAbs, but these usually are quite uncommon and relate to the overall extent of treatment. Trastuzumab carries the risk of additive cardiotoxicity with anthracycline treatments, and this effect was more pronounced with bevacizumab, probably as a result of an impaired tissue repair response: neither antibody should be given in combination with other cardiotoxic drugs. The immune modulating drugs such as ipilimumab and nivolimumab, which are effectively non-specific immunostimulators, have recognised patterns of autoimmune toxicity which bear some resemblance to graft-versus-host disease such as colitis, dermatitis, pneumonitis and liver toxicity but also distinct effects such as endocrinopathy involving the pituitary and thyroid glands in particular. These may require control by steroid therapy or sometimes more powerful immunosuppressive drugs.

Future Prospects

The prospects for cancer treatment with mAb are increasingly exciting. In addition to the many reagents that target tumours directly, the checkpoint blockers are producing impressive results and are likely to dominate the field for the next few years, a success which is forcing many cancer specialists to look again at immune intervention. It is also clear that CTLA-4 and PD-1 are only the beginning for this class of reagents, and soon clinical data will be available for VISTA, TIM3 and LAG3 either as single agents or in combination with ipilimumab and anti-PD-1. It is encouraging that combination immunomodulatory mAb therapy is already showing significant response rates in non-small-cell lung cancer, a tumour not previously regarded as especially immunogenic.

The other class of immunomodulatory mAbs which are gaining traction are the immunostimulatory molecules, such as anti-CD40 and anti-4-1BB, which target TNFR superfamily members and promote activation of effector cells or antigen-presenting cells. There is still considerable work needed to define their exact MoA and the optimal antibody format as well as to understand which FcR is optimal for clinical activity. Current results suggest that immunostimulatory mAbs require FcR, not for recruiting cytotoxic effectors but to hyper-cross-link the mAb and promote sufficient multimeriation to deliver effective transmembrane signalling and immune activation. For this function the inhibitory FcR, FcRIIb, appears exceptionally potent, especially when given its wide distribution on both normal cells and tumour cells themselves (White et al. 2013; Li and Ravetch 2013). Two examples in particular that look exciting in this respect are the early studies in pancreatic cancer where Vonderheide (Beatty et al. 2013) has shown that an anti-CD40 mAb might be useful for activating myeloid cells, particularly in the presence of cytotoxic chemotherapy. Another approach uses anti-4-1BB, which is effective at activating NK cells to promote ADCC activity. Early results suggest that a combination of anti-4-1BB to promote effector activation together with a mAb which targets tumour cells directly, such as anti-CD20 or anti-Her2, is a potent combination. This approach has the advantage that the anti-4-1BB mAb might also promote a broader T-cell response against the tumour (Chen et al. 2000), allowing the short-term gain in direct killing of the tumour to be enhanced by a long-term immune stimulation. Such epitope spreading seems to occur in patients responding to checkpoint blocking drugs.

In summary, the last 30 years have seen antibodies move from a hypothetical “silver bullet” to a real form of treatment, which has transformed the outlook for some types of malignancy. The emerging evidence suggests that there is considerable scope for further progress in the future, as combinatorial approaches enhance the recruitment of host immune defences against tumours that have hitherto resisted systemic attack.

References

Beatty GL, Torigian DA, Chiorean EG, Saboury B, Brothers A, Alavi A, Troxel AB, Sun W, Teitelbaum UR, Vonderheide RH, O’Dwyer P (2013) A phase I study of an agonist CD40 monoclonal antibody

(CP-870,893) in combination with gemcitabine in patients with advanced pancreatic ductal adenocarcinoma. Clin Cancer Res 19(22):6286–95

Chen SH, Pham-Nguyen KB, Martinet O, Huang Y, Yang W, Thung SN, Chen L, Mittler R, Woo SL (2000) Rejection of disseminated metastases of colon carcinoma by synergism of IL-12 gene therapy and 4-1BB costimulation. Mol Ther 2(1):39–46

Göttlinger H, Johnson J, Riethmüller G (1986) Biochemical and epitope analysis of the 17-1A membrane antigen. Hybridoma 5(Suppl 1):S29–S37

Hale G, Xia MQ, Tighe HP, Dyer MJ, Waldmann H (1990) The CAMPATH-1 antigen (CDw52). Tissue Antigens 35(3):118–127

Köhler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256(5517):495–497

Lee CS, Cragg M, Glennie M, Johnson P (2013) Novel antibodies targeting immune regulatory checkpoints for cancer therapy. Br J Clin Pharmacol 76(2):233–247

Li F, Ravetch JV (2013) Antitumor activities of agonistic anti-TNFR antibodies require differential FcγRIIB coengagement in vivo. Proc Natl Acad Sci U S A 110(48):19501–19506

Niwa R, Hatanaka S, Shoji-Hosaka E, Sakurada M, Kobayashi Y, Uehara A, Yokoi H, Nakamura K, Shitara K (2004) Enhancement of the antibody-dependent cellular cytotoxicity of low-fucose IgG1 is independent of FcgammaRIIIa functional polymorphism. Clin Cancer Res 10(18 Pt 1):6248–6255

Savola S, Klami A, Myllykangas S, Manara C, Scotlandi K, Picci P, Knuutila S, Vakkila J (2011) High Expression of Complement component 5 (C5) at Tumor Site Associates with Superior Survival in Ewing's Sarcoma Family of Tumour Patients. ISRN Oncol 2011:168712

Scott AM, Wolchok JD, Old LJ (2012) Antibody therapy of cancer. Nat Rev Cancer 12(4):278–287

Sliwkowski MX, Mellman I (2013) Antibody therapeutics in cancer. Science 341(6151):1192–1198

White AL, Chan HT, French RRC, Beers SA, Cragg MS, Johnson PWM, Glennie MJ (2013) FcγRIIB controls the potency of agonistic anti-TNFR mAbs. Cancer Immunol Immunother 62(5):941–948

Bispecific Antibodies for the Treatment of Cancer: The Challenges of Translation

Gundram Jung and Ludger Große-Hovest

Introduction

The most attractive feature of bispecific antibodies is that they can do what conventional monospecific antibodies cannot do: recruit T cells with their superior effector potential. To some it may come as a surprise that the foundations of this approach have been laid already in the 1980s. However, it was not until recently that the good news about blinatumomab (Bargou et al. 2008; Topp et al. 2011, 2012; Bassan 2012), a bispecific single-chain (bssc) antibody with CD19 X CD3 specificity, culminated in a commentary in *Blood* entitled "Toward victory in ALL. Blinatumomab joins in" (Bassan 2012). There is hardly anyone in the field who does not appreciate the remarkable achievement that gave rise to these publications. Our appreciation however comes with an unpleasant question: "Why did it take so long?" In fact, it is our conviction that the current state of affairs could have been reached earlier, if the translational process for bispecific antibodies would have been more efficient, and that we shall improve on this process in the future only if we draw lessons from the past.

G. Jung (✉)
Department of Immunology,
University of Tübingen, Tübingen, Germany

German Cancer Consortium (DKTK),
Heidelberg, Germany
e-mail: gundram.jung@uni-tuebingen.de

L. Große-Hovest
Synimmune GmbH, Tübingen, Germany

Historical Remarks

The field was established by three papers which appeared in 1985. They demonstrated that mouse (Staerz et al. 1985) or human (Liu et al. 1985; Perez et al. 1985) T cells can be focused to cancer cells using bispecific antibodies directed to target antigens on these cells and to constant epitopes of the T cell receptor (TCR/CD3) complex, thus bypassing the antigen specificity of this receptor. These results have been obtained using chemically conjugated intact antibodies and cloned or pre-activated T cells as effector cells.

In our own initial experiments, we noticed that bispecific antibody-mediated killing by resting human T cells was ineffective, and thus we concentrated our efforts on the generation of lytic capability as part of the T cell activation process. To this end, we stimulated peripheral blood mononuclear cells (PBMC) with CD3 antibodies for various times and then focused the activated cells with bispecific antibodies (Jung et al. 1986). We found that the lytic activity of the cells reached a maximum after 2–3 days of stimulation and declined thereafter. It was well established at that time that activation of PBMC by anti-CD3 antibodies requires binding of the antibodies to Fc receptor (FcR)-carrying cells, such as monocytes, that multimerize the antibodies and in addition provide costimuli for T cell activation (Williams et al. 1985). For bispecific antibodies, binding to FcR-expressing cells is not desirable for two reasons: (1) FcR-dependent T cell activation might lead to off-target

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_11,

activation in vivo. (2) By binding bispecific antibodies FcR+ cells might become targets for stimulated T cells. We therefore suggested the use of Fc-free or Fc-attenuated antibodies to allow for a strictly target cell-dependent rather than an Fc-induced T cell activation (Jung et al. 1988, 1991). Moreover we found that with some target antigens, such as a melanoma-associated chondroitin sulfate proteoglycan (CSPG), a combination of two bispecific antibodies with CSPG X CD3 and CSPG X CD28 specificity, triggering the costimulatory CD28 molecule in addition to the TCR/CD3 complex, is required to achieve efficient target cell-dependent T cell activation (Jung et al. 1988, 1991).

The Challenges of Translation: Scientific Problems

Based on the findings outlined above, several clinical trials were performed during the 1990s, most of them with Fc-free bispecific CD3 antibodies either produced by chemical hybridization of Fab fragments or by fragmentation of antibodies derived from hybrid hybridomas (quadromas) (Nitta et al. 1990; Kroesen et al. 1994; Canevari et al. 1995; Tibben et al. 1996; Jung et al. 2001). In one approach Fc-containing antibodies, generated by chimeric rat/mouse quadromas, have been used (Lindhofer et al. 1995; Heiss et al. 2005). All of these studies were initiated by academic institutions. The industry, at that time, did not seem to be interested in bispecific antibodies although some of the early trials revealed remarkable antitumor effects after local application of these novel therapeutic compounds (Nitta et al. 1990; Canevari et al. 1995; Jung et al. 2001). Following systemic administration, however, remarkable toxicity was noted due to pronounced release of cytokines by activated T cells (Kroesen et al. 1994; Tibben et al. 1996): in fact, the safely applicable doses (100–200 μg per patient and day) were orders of magnitude lower than those used during treatment with conventional monospecific antibodies. It is quite obvious that such doses are not sufficient to achieve optimal biologic activity in vivo, in particular, if solid tumors are to be targeted. In principle, this "dosing problem" might be caused by several different mechanisms:

1. Unspecific binding of the antibodies to FcR+ cells (if they are not completely devoid of active Fc parts) or to normal cells carrying the respective target antigen. This problem calls for strictly Fc-free antibodies targeting highly specific antigens.
2. Specific binding of antibodies, if the target cells are systemically distributed. In this case side effects obviously depend upon the number of target cells and are avoidable only if this number does not exceed a certain threshold level.
3. T cell activation after monomeric binding of the CD3 antibodies contained in bispecific molecules. The role of this phenomenon is not clear yet. It has been postulated that it causes transmigration of lymphocytes through the endothelial layer of blood vessels (Molema et al. 2000), thereby inducing a pronounced lymphopenia shortly after the start of the treatment (Kroesen et al. 1994; Tibben et al. 1996). In our view, the impact of this phenomenon has been underestimated. It might be avoidable, at least to some extent, by the choice of the particular TCR/CD3 antibody used.

In addition to the dosing problem, the field suffered enormously over the years from laborious production procedures. At this point help came again from recombinant antibody technology allowing, among others, the generation and large-scale production of bispecific single-chain (bssc) antibodies. This will be dealt with in the following section. Whereas gene technology largely facilitated the development of blinatumomab, the prototypic bssc antibody, it did not solve the dosing problem: Applicable blinatumomab doses do not exceed 100 μg per patient and day resulting in steady-state serum concentrations below 1 ng/ml upon continuous i.v. infusion (Klinger et al. 2012). The success of this antibody in the treatment of B cell-derived leukemia and lymphoma is all the more remarkable (Bargou et al. 2008; Topp et al. 2011, 2012; Bassan 2012).

In Search for the Optimal Format: The View of an Antibody Engineer

Since the groundbreaking work of Köhler and Milstein, allowing the generation of monoclonal antibodies of predefined specificity, these reagents have proven to be an excellent paradigm for the design of high-affinity, protein-based binding reagents. To convert mouse monoclonal antibodies into successful therapeutics, the most immediate task for recombinant antibody technology was humanization[1] in order to minimize immunogenicity and improve Fc-dependent effector functions. In fact, most "second-generation" antibodies currently approved for the treatment of cancer as well as infectious and inflammatory diseases are humanized[1] by genetic engineering. The next stage of this development was the construction of human Fc parts employed with markedly increased affinity to Fc receptors by defined modification of either amino acid residues (Lazar et al. 2006; Hofmann et al. 2012) or glycosylation patterns (Salles et al. 2012). At present, the first of these "third-generation" antibodies is evaluated in clinical trials (Salles et al. 2012), others are in late preclinical development (Hofmann et al. 2012).

With humanization, however, ambitions of antibody engineers were not satisfied. Already in the late 1980s, they started dissecting antibodies into minimal binding fragments. It was demonstrated that VH and VL regions, expressed separately in *E. coli*, spontaneously reassemble to form small but functional antigen-binding Fv units (Skerra et al. 1988). Shortly thereafter the Fv structure was stabilized by linkers between the two V regions resulting in single-chain Fv fragments (Bird et al. 1988). This structure became kind of the central "Lego brick" module for the modular construction system depicted in Fig. 1. With this building block at hand and with the ease of prokaryotic production, a plethora of scFv-based derivatives were generated including the development of diabodies (Holliger et al. 1993). Moreover, strategies to further stabilize the scFv fragment with disulfide bonds were introduced (Brinkmann et al. 1993). It was at this stage that scFv molecules were used to construct bispecific bssc antibodies, again first produced in *E. coli* (Mallender et al. 1994; Gruber et al. 1994; Kurucz et al. 1995). However the limitations of bacterial expression of the more complicated bispecific molecules soon became apparent. Whereas eukaryotic expression, first described by Mack et al. (1995), allows the production of functional bssc molecules in most cases, the bssc format as such suffers from additional weaknesses: production rates in cell culture are moderate, the molecular weight (approx. 50 kDa) is low resulting in a short serum half-life, and the tendency to form large molecular-weight aggregates (Worn et al. 2001) complicates production and purification.

Curiously, with parts of our work, we first benefited from these potential disadvantages: we found that a spontaneously formed, bispecific single-chain dimer with CSPGXCD28 specificity is capable of inducing target cell-restricted T cell activation by supra-agonistic stimulation of CD28 (Grosse-Hovest et al. 2003). In an attempt to address the problem of low production rates in eukaryotic cell culture, we produced the protein in the milk and the serum of cloned transgenic cows demonstrating at that time that "gene farming" might be a viable alternative for large-scale production of bispecific antibodies and other complex proteins (Grosse-Hovest et al. 2004). However, recent progress in the propagation of eukaryotic high-producer cell lines has led us to follow a more conservative approach not only with respect to the mode of production but also by considering antibody formats more closely resembling the physiological antibody structure. At present, our favorite bispecific format is the Fabsc-like molecule depicted in Fig. 1E. It consists of a Fab fragment and a C-terminal single-chain linked by an Fc-attenuated CH2 domain. Up to date, we have constructed numerous bispecific antibodies using this format. One of them (with FLT3 X CD3-specificity) we have compared side by side with a bssc molecule with identical specificities. As expected the Fabsc was clearly superior to the bssc molecule with respect to the

[1] Since humanization is not the major focus of this article, we use this expression here as a collective term for chimeric, CDR-grafted, and entirely human antibodies.

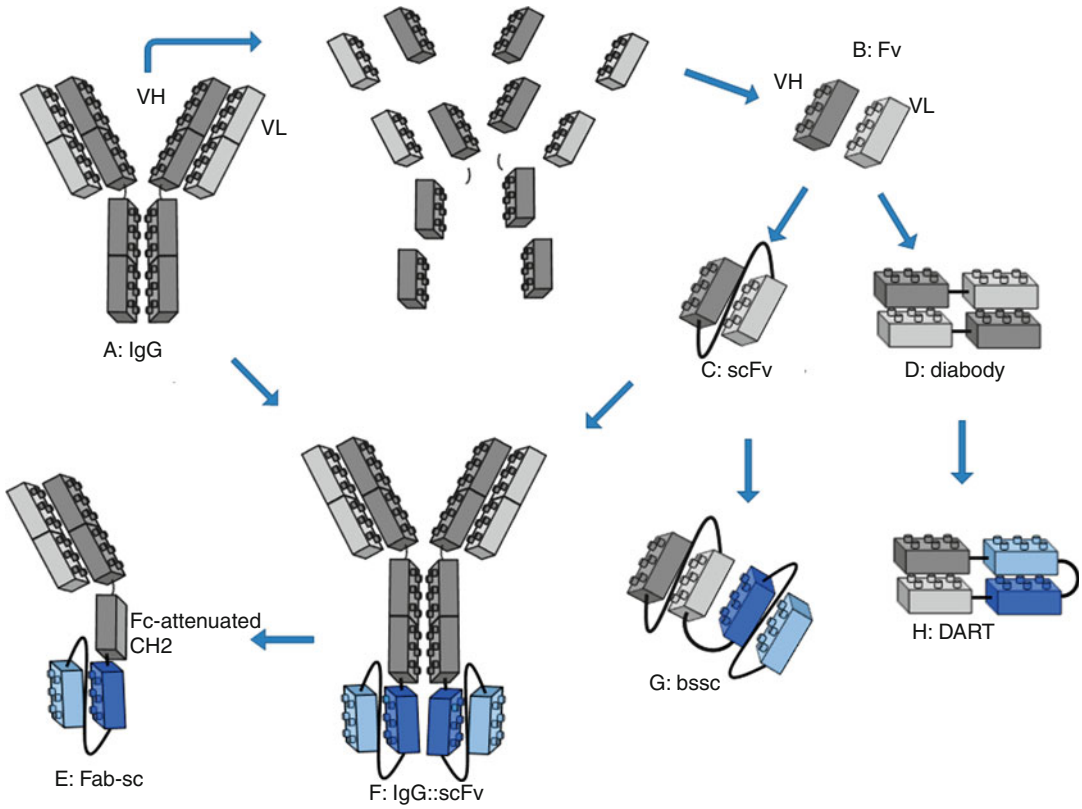

Fig. 1 An antibody engineer building block system. Starting from scFv molecules (C), various formats of recombinant mono- and bispecific antibody constructs can be generated (upper and lower half, respectively). For T cell recruiting molecules, we favor the Fabsc format (E). It consists of a Fab and an scFv part linked by an Fc-attenuated CH2 domain to ensure target cell-induced rather than Fc-mediated T cell activation

critical parameters mentioned above: serum half-life, production rate, and the tendency to aggregate (Durben et al. 2014). Thus, to us, it looks as if some antibody constructs live up to the expectations of their creators at last.

The Challenges of Translation: Nonscientific Hurdles

As already hinted above, it has been not only scientific problems preventing rapid progress. When one of us (GJ) tried at the beginning of the 1990s to generate some interest in bispecific antibodies among the pharmaceutical industry, he was repeatedly told by company officials that CD3 is a "problematic molecule" with respect to intellectual property issues. Obviously, the fact that the CD3 molecule was covered by an industrial patent at that time prevented other companies from working with this protein for at least as long as patent protection was valid.

Headwind for bispecific antibodies and other investigator-driven drugs did not only come from patent offices but also from regulatory authorities imposing an increasing pressure on both the process of antibody manufacturing (GMP regulations) and the execution of clinical trials (GCP regulations) (Hemminki et al. 2006). As a result of these restrictions, development of innovative drugs, including bispecific antibodies, is nowadays completely in the hands of the pharmaceutical industry "where many factors other than strictly scientific/clinical ones influence the decision to develop a particular product" (Hale et al. 2000).

Lessons for Translation

In our personal view, the lessons from the cumbersome translational process for bispecific antibodies and other innovative biologicals are clear-cut and may be summarized as follows:

- Special programs should enable academic medical institutions to substantially participate in the process of drug development. This should include the capability to produce GMP-compliant material for clinical pilot studies.
- Both GMP and GCP regulations for such studies should be changed to practical and meaningful ones. For selected patients with a life-threatening disease and no satisfactory conventional treatment option available, it should be possible, for example, to produce biological drugs for initial clinical studies (phase I/II) following the principle of vigorous end-product rather than in-process controls.
- Patent legislation should not allow protection of naturally occurring genes or proteins.
- For the construction of bispecific antibodies, improved recombinant formats should be used.

We believe that, if such measures are implemented, it will take less than another 25 years for a new generation of T cell recruiting reagents, such as optimized bispecific antibodies, to reach the bedside.

References

Bargou R et al (2008) Tumor regression in cancer patients by very low doses of a T cell-engaging antibody. Science 321:974–977

Bassan R (2012) Toward victory in adult ALL: blinatumomab joins in. Blood 120:5094–5095

Bird RE et al (1988) Single-chain antigen-binding proteins. Science 242:423–426

Brinkmann U et al (1993) A recombinant immunotoxin containing a disulfide-stabilized Fv fragment. Proc Natl Acad Sci U S A 90:7538–7542

Canevari S et al (1995) Regression of advanced ovarian carcinoma by intraperitoneal treatment with autologous T lymphocytes retargeted by a bispecific monoclonal antibody. J Natl Cancer Inst 87:1463–1469

Durben M et al (2014) Selection and characterization of a bispecific FLT3 x CD3 antibody in an improved, recombinant format for the treatment of myeloid leukemia (in press)

Grosse-Hovest L et al (2003) A recombinant bispecific single-chain antibody induces targeted, supra-agonistic CD28-stimulation and tumor cell killing. Eur J Immunol 33:1334–1340

Grosse-Hovest L et al (2004) Cloned transgenic farm animals produce a bispecific antibody for T cell-mediated tumor cell killing. Proc Natl Acad Sci U S A 101:6858–6863

Gruber M et al (1994) Efficient tumor cell lysis mediated by a bispecific single chain antibody expressed in *Escherichia coli*. J Immunol 152:5368–5374

Hale G et al (2000) From laboratory to clinic: the story of CAMPATH-1. Methods Mol Med 40:243–266

Heiss MM et al (2005) Immunotherapy of malignant ascites with trifunctional antibodies. Int J Cancer 117:435–443

Hemminki A et al (2006) Harmful impact of EU clinical trials directive. BMJ 332:501–502

Hofmann M et al (2012) Generation, selection and preclinical characterization of an Fc-optimized FLT3 antibody for the treatment of myeloid leukemia. Leukemia 26:1228–1237

Holliger P et al (1993) "Diabodies": small bivalent and bispecific antibody fragments. Proc Natl Acad Sci U S A 90:6444–6448

Jung G et al (1986) Activation of human peripheral blood mononuclear cells by anti-T3: killing of tumor target cells coated with anti-target-anti-T3 conjugates. Proc Natl Acad Sci U S A 83:4479–4483

Jung G et al (1988) An in-vitro model for tumor immunotherapy with antibody heteroconjugates. Immunol Today 9:257–260

Jung G et al (1991) Target cell-induced T cell activation with bi- and trispecific antibody fragments. Eur J Immunol 21:2431–2435

Jung G et al (2001) Local immunotherapy of glioma patients with a combination of 2 bispecific antibody fragments and resting autologous lymphocytes: evidence for in situ t-cell activation and therapeutic efficacy. Int J Cancer 91:225–230

Klinger M et al (2012) Immunopharmacologic response of patients with B-lineage acute lymphoblastic leukemia to continuous infusion of T cell-engaging CD19/CD3-bispecific BiTE antibody blinatumomab. Blood 119:6226–6233

Kroesen BJ et al (1994) Phase I study of intravenously applied bispecific antibody in renal cell cancer patients receiving subcutaneous interleukin 2. Br J Cancer 70:652–661

Kurucz I et al (1995) Retargeting of CTL by an efficiently refolded bispecific single-chain Fv dimer produced in bacteria. J Immunol 154:4576–4582

Lazar GA et al (2006) Engineered antibody Fc variants with enhanced effector function. Proc Natl Acad Sci U S A 103:4005–4010

Lindhofer H et al (1995) Preferential species-restricted heavy/light chain pairing in rat/mouse quadromas. Implications for a single-step purification of bispecific antibodies. J Immunol 155:219–225

Liu MA et al (1985) Heteroantibody duplexes target cells for lysis by cytotoxic T lymphocytes. Proc Natl Acad Sci U S A 82:8648–8652

Mack M et al (1995) A small bispecific antibody construct expressed as a functional single-chain molecule with high tumor cell cytotoxicity. Proc Natl Acad Sci U S A 92:7021–7025

Mallender WD et al (1994) Construction, expression, and activity of a bivalent bispecific single-chain antibody. J Biol Chem 269:199–206

Molema G et al (2000) CD3 directed bispecific antibodies induce increased lymphocyte-endothelial cell interactions in vitro. Br J Cancer 82:472–479

Nitta T et al (1990) Preliminary trial of specific targeting therapy against malignant glioma. Lancet 335:368–371

Perez P et al (1985) Specific targeting of cytotoxic T cells by anti-T3 linked to anti-target cell antibody. Nature 316:354–356

Salles G et al (2012) Phase 1 study results of the type II glycoengineered humanized anti-CD20 monoclonal antibody obinutuzumab (GA101) in B-cell lymphoma patients. Blood 119:5126–5132

Skerra A et al (1988) Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. Science 240: 1038–1041

Staerz UD et al (1985) Hybrid antibodies can target sites for attack by T cells. Nature 314:628–631

Tibben JG et al (1996) Pharmacokinetics, biodistribution and biological effects of intravenously administered bispecific monoclonal antibody OC/TR F(ab′)2 in ovarian carcinoma patients. Int J Cancer 66: 477–483

Topp MS et al (2011) Targeted therapy with the T-cell-engaging antibody blinatumomab of chemotherapy-refractory minimal residual disease in B-lineage acute lymphoblastic leukemia patients results in high response rate and prolonged leukemia-free survival. J Clin Oncol 29:2493–2498

Topp MS et al (2012) Long-term follow-up of hematologic relapse-free survival in a phase 2 study of blinatumomab in patients with MRD in B-lineage ALL. Blood 120:5185–5187

Williams JM et al (1985) The events of primary T cell activation can be staged by use of Sepharose-bound anti-T3 (64.1) monoclonal antibody and purified interleukin 1. J Immunol 135:2249–2255

Worn A et al (2001) Stability engineering of antibody single-chain Fv fragments. J Mol Biol 305: 989–1010

The ABCs of T Cell Receptor Gene Therapy

Dolores J. Schendel and Philip D. Greenberg

Adoptive T Cell Therapy

It has long been observed that T cells with the capacity to recognize and destroy tumor cells in vitro can be isolated from some cancer patients. Mostly, however, the endogenous T cell response to the tumor has been subjected to negative regulation, limiting the effective control of cancer in vivo (Schreiber et al. 2011). This hindrance has been successfully bypassed in some patients by isolating tumor-infiltrating lymphocytes (TIL) from the tumor microenvironment, activating them in vitro, and then expanding them to large numbers before reinfusing them back into the patients (Besser et al. 2013; Dudley et al. 2013; Rosenberg and Dudley 2009). Clinical benefit has been reported, particularly for treatment of melanoma, but only a fraction of patients respond. Furthermore, technical hurdles in preparing specific cells have limited the wide application of TIL therapy to date.

Currently, TIL therapy is experiencing a revival based on the better understanding that TIL populations may include T cells with specificity for unique peptides derived from mutant proteins expressed in tumor cells that are missing from normal cells (Lennerz et al. 2005; Robbins et al. 2013; van Rooij et al. 2013). This is particularly true in melanomas, which have accumulated a very large number of mutations before becoming clinically evident (Alexandrov et al. 2013). These T cells are predicted to express TCRs of higher affinity since they have not been exposed to negative selection. Thus, such T cells can recognize tumor cells more efficiently, while at the same time providing ideal specificity for distinction of malignant and healthy cells.

Nevertheless, the development of TIL-based immunotherapy remains limited to particular tumor types, with melanoma being the primary indication, and only for those patients from whom adequate numbers of T cells can be isolated and expanded from accessible tumor samples. Furthermore, efficient technologies essential to ascertain whether relevant T cells with mutant-peptide specificity are present among expanded TIL populations are still not broadly available, which impedes development as well as in vitro and in vivo evaluation of potentially effective TIL populations.

The power of adoptively transferred lymphocytes to fully eradicate leukemia has been clearly demonstrated in the setting of allogeneic

D.J. Schendel (✉)
Institute of Molecular Immunology,
Helmholtz Zentrum München, National Research Center for Environmental Health, Munich, Germany
e-mail: schendel@helmholtz-muenchen.de

P.D. Greenberg
Department of Medicine and Immunology,
University of Washington, South Lake Union,
Bldg E, 750 Republican Street,
Seattle, WA 98109, USA

Program in Immunology, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave North,
Seattle, WA 98109-1024, USA
e-mail: pgreen@u.washington.edu

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_12,

hematopoietic cell transplantation (HCT). In fact, the early discovery of graft-versus-leukemia (GVL) effects occurring during HCT established the principle that T cell-mediated immune responses could cure leukemia.

Unfortunately, GVL is often accompanied by toxic graft-versus-host disease (GVHD), as unselected donor T cells can recognize antigens expressed not only by leukemic cells but also by normal host tissues. Thus, many efforts have been directed at finding suitable target antigens that can elicit T cell responses capable of mediating GVL in the absence of GVHD (Bleakley and Riddell 2011; Falkenburg and Warren 2011; Kolb 2008; Spierings et al. 2013). One such approach has been to search for genes expressed uniquely in cells of the hematopoietic lineage that are encoded by alleles with different (and immunogenic) sequences in the host and donor, as the only residual host hematopoietic cells that donor cells with specificity for such minor histocompatibility antigens should be capable of targeting after an HCT are persistent leukemia cells. As such donor T cells recognize foreign host peptides, it should be possible to isolate T cells that express TCRs of high affinity, and the transfer of T cells with such specificity should provide effective GVL in the absence of GVHD. These efforts are now yielding promising outcomes in clinical studies (Burdach and Kolb 2013; Spierings et al. 2013; Warren et al. 2010). They also point the way to understanding the nature of T cells that contribute to successful eradication of tumor cells in vivo. As with TIL, the generation of T cells specific for minor histocompatibility antigens capable of mediating GVL is laborious and time consuming and limited by the success in obtaining specific T cells for each patient. Consequently, broad application of this strategy to treatment of patients is greatly restricted at this time.

TCR Gene Therapy

Many more patients could be treated with specific adoptive T cell therapy by engineering their own peripheral blood T cells to express recombinant TCRs that immediately imbue the transduced cells with effective antitumor specificity, an approach that is known as TCR gene therapy. To date, most strategies for TCR gene therapy are based on isolation and characterization of T cells, and subsequently the corresponding TCRs, which are specific for non-mutated tumor-associated antigens (TAAs). These TAAs are more widely detected and often overexpressed in tumors, allowing the same TCRs to be used in multiple patients.

A major problem encountered in targeting TAAs is the low affinity of TCRs on T cells derived from the natural repertoire, since the T cells with high-affinity TCRs for these antigens have undergone negative selection during in vivo development in the patients or healthy donors from whom they were isolated. These low-affinity TCRs have reduced capacity for effective tumor cell recognition. Fortunately, several approaches are available to improve TCR affinity through directed mutagenesis or by alteration of TCR glycosylation (Chervin et al. 2008; Kuball et al. 2009; Liddy et al. 2012).

As an alternative, TCRs of natural high affinity can be isolated from non-tolerant repertoires. For example, allogeneic donors can be used for generation of T cells specific for antigenic peptides restricted by a foreign MHC molecule not encountered during negative selection, in a manner learned from MHC-mismatched HCT (Amir et al. 2011; Morris et al. 2006; Wilde et al. 2012). TCRs can also be isolated from mice expressing a human MHC molecule and responding to foreign peptides from a human protein (Kuball et al. 2005; Parkhurst et al. 2011). Such xenogeneic TCRs, however, have the potential to be recognized as foreign antigens in patients, initiating immune responses that can limit expansion and long-term persistence of T cells in vivo, both of which seem to be critical for clinical efficacy. Alternatively, TCRs can be obtained from recombinant mice that have been developed to express the full repertoire of human TCRs (Li et al. 2010), as in vivo priming in these mice can induce T cells specific for human peptides that express fully human TCR sequences of high affinity.

On- and Off-Target Toxicity Associated with TCR Gene Therapy

Initial patient studies using higher-affinity receptors for TCR gene therapy indicate that they can provide better clinical efficacy, but their use can also increase on- and off-target toxicity. One study of 20 patients given higher-avidity MART-1-specific TCR-engineered T cells resulted in no patient deaths. However, some patients suffered from reversible hearing loss or vision impairment and most experienced skin melanocyte destruction (Johnson et al. 2009). These side effects represented clear cases of on-target toxicity since all of the healthy tissues that suffered unwanted attack were known to express the MART-1 antigen. In a second trial using a different high-affinity MART-1-specific TCR, a patient died of undetermined causes associated with the TCR gene therapy (van den Berg 2013). It remains unknown whether this patient death was related to on- or off-target toxicity. Therefore, it is unclear whether the lethal toxicity observed only in the second study represents a consequence of the poor health of the patient, differences in TCR affinities, or differences in TCR-engineered recipient lymphocytes, since the target pMHC ligand for both TCRs was identical in the two studies.

Cancer/testis (CT) antigens have long been considered as excellent targets for TCR gene therapy to reduce on-target toxicity, based on the limited normal tissue expression to germ cells. In fact, TCRs with NY-ESO-1 specificity demonstrated good clinical efficacy (Robbins et al. 2011) with no apparent toxicity, in comparison to the toxicity noted to date with non-CT antigens as targets of high-affinity TCRs (Johnson et al. 2009; Parkhurst et al. 2011; van den Berg 2013).

However, unexpected risks associated with CT targets have also been illustrated by the death of patients in two trials using MAGE-A3-specific TCRs modified to achieve higher affinity. In the first trial, cross-reactive recognition of a small fraction of neurons expressing MAGE-A12, which contains an epitope that exhibits partial sequence sharing with MAGE-A3 and could be recognized by the higher-affinity TCR specific for MAGE-A3, was postulated to have caused lethal neurotoxicity and patient death (Morgan et al. 2013). This toxicity was unpredicted, as the initial screen for expression of MAGE-A3 and MAGE-A12 failed to identify expression in the CNS. In the second instance, two patients died of cardiac impairment, presumably due to off-target recognition of the heart muscle protein, titin. This cross-recognition was also based on partial sequence sharing with MAGE-A3 (Cameron et al. 2013; Linette et al. 2013), with recognition likely facilitated by the way in which this TCR was affinity enhanced, which included modifications to the CDR2 region that usually results in enhanced binding of the TCR to the MHC backbone that can be independent of the peptide epitope.

Finally, lethal GVHD has been associated with TCR gene therapy in animal models, due to mispairing of endogenous and transgenic TCR chains with each other creating new TCRs of unknown specificities (Bendle et al. 2010; Ferrara et al. 2010). This has not yet been observed in patient studies, and strategies to limit mispairing and facilitate proper pairing, such as by introduction of a point mutation into each of the inserted chains to create an interchain disulfide bond (Kuball et al. 2007), are now routinely employed. However, such countermeasures do not completely abrogate TCR-chain mispairing, and, as the numbers of patients who are treated with TCR gene therapy expand, it will be essential to monitor patients for this toxicity to determine if more absolute strategies, such as excision of the endogenous TCR genes (Provasi et al. 2012), are needed.

These examples of lethal toxicity caused by TCR-engineered lymphocytes clearly show that new and better approaches are needed to fully understand the specificity of high-affinity TCRs and to help predict in advance their potential on- and off-target toxicities. Ideas to address these issues are emerging from the field and new tools are being created to aid safety assessment of TCRs and their target ligands. Better means to judge pMHC-ligand expression and the consequences or recognition of normal tissues are of particular importance with respect to on-target toxicity for target antigens known to be expressed at low levels in healthy tissues.

It should be stressed, however, that even high-affinity TCRs directed against mutant peptides that are only expressed in tumors and thus lack on-target toxicity for normal tissues are not risk-free with respect to off-target cross-recognition of other pMHC ligands of unknown origin. It is a particular challenge to find strategies to analyze off-target toxicity. The retrospective analysis of the cross-recognition of titin in patients treated with MAGE-A3 TCR gene therapy used more elaborate cell culture methods to assess off-target tissue recognition; this provides one new approach that could be moved up front in preclinical studies (Cameron et al. 2013; Linette et al. 2013). Additionally, greater use of in silico tools could provide better insight into tissue expression of potentially cross-reactive target antigens that could be prescreened and might thereby help to reduce off-target recognition (Stone and Kranz 2013).

Our efforts in Seattle and Munich have focused most recently on developing TCR gene therapy for AML. The clinical experience with GVL effects from donor lymphocytes in HCT has demonstrated that T cell-mediated long-lasting remissions and even potential cures can be achieved in AML patients, but many patients still do not benefit from such GVL activity and/or exhibit toxicity from accompanying GVHD. Thus, there is still a need to improve AML outcomes, and TCR gene therapy offers such a possibility. The selection of target antigens that are primarily expressed by cells of the hematopoietic system, particularly those with prominent expression on AML blasts and leukemic stem cells (LSCs), may allow for effective utilization of higher-affinity TCRs with lower potential for serious on-target toxicity. Using the setting of HCT for developing and testing TCR gene therapy rests on the long-standing experience of adoptive lymphocyte transfer to mobilize T cells that proliferate and persist to provide effective and long-term control of leukemia.

WT1-Based Adoptive Cell Therapy for Relapsed and High-Risk AML

WT1 is a transcription factor that has very limited expression in normal adult tissues, but is detected at very high levels in most leukemic cells and many other malignancies, and contributes to cancer phenotypes by promoting proliferation and oncogenicity (Sugiyama 2010). $CD8^+$ T cell responses specific for WT1 have been detected following immunization in a variety of vaccine trials and have encouragingly exhibited some therapeutic responses with no evidence of toxicity to normal tissues (Keilholz et al. 2009; Oka et al. 2008). Based in part on these observations, we have explored the use of WT1-specific T cells for adoptive therapy in AML.

We have recently reported the results of a clinical trial in which HLA-A*02:01-restricted WT1-specific $CD8^+$ cytotoxic T cell clones were generated from cells obtained from the peripheral blood of the HLA-matched donor of an allogeneic HCT and then administered to 11 patients who relapsed or were at high risk of relapse from leukemia after HCT and observed no evidence of on-target toxicity in any of these patients (Chapuis et al. 2013). Evidence of antileukemic activity was directly demonstrable in two patients: one patient with advanced progressive disease who had a dramatic but transient response with elimination of circulating blasts, and a second patient with minimal residual disease (MRD) detectable after HCT who achieved a prolonged complete remission. Moreover, there were three patients treated after HCT who had no detectable leukemia at the time of T cell therapy, but who were at very high risk for relapse with projected median survival times of <1 year, and all three are surviving now for >4 years without relapse, GVHD, or additional antileukemic treatment. These studies have affirmed the potential benefit and safety of targeting WT1, but have also identified obstacles to attaining broad efficacy, including failure to achieve in most patients high-avidity T cell responses of large magnitude that persist to eradicate the tumor (Chapuis et al. 2013).

To better address these obstacles, we have designed and have now initiated a clinical trial in which patients with poor prognosis AML that has either relapsed or is at high risk of relapse after allogeneic HCT are receiving donor $CD8^+$ T cells genetically modified with a high-affinity WT1-specific TCR. The TCR was selected after screening thousands of WT1-specific $CD8^+$ T cell clones generated from ~100 normal HLA-A2 donors and identifying the highest avidity clone

as the TCR source. The TCR from this clone has a higher affinity than the TCR expressed in any of the clones administered in our previous trial, and T cells transduced with this TCR recognize leukemic cells better than any of the administered clones. The use of TCR-transduced cells also makes it possible to generate cells for transfer that have undergone less in vitro proliferation than T cell clones and thus should have greater proliferative potential after transfer. It also allows for selection of cells for transduction that have desired properties. For example, in our current trial, we are transducing EBV-specific donor $CD8^+$ T cells, which are highly enriched for central memory cells, a subset that has been shown to be capable of self-renewing and persisting better in vivo after transfer (Berger et al. 2008; Buchholz et al. 2013; Gattinoni et al. 2012).

Cyclin-A1 Represents a New Target for TCR Gene Therapy of AML

We have also searched for additional target antigens in AML, as having the ability to target more than a single antigen would both increase the breadth of patients that can be treated and reduce the risk of outgrowth of antigen-loss variants. Analysis with microarray hybridization of differential gene expression in purified LSCs compared to hematopoietic stem cells (HSCs) and peripheral tissues revealed that *CCNA1*, which encodes the protein cyclin-A1, is a candidate gene (Ochsenreither et al. 2012).

Normal expression of cyclin-A1 is limited to germ cells, where it is essential for meiosis, whereas the related family member, *CCNA2* encoding cyclin-A2, is ubiquitously expressed and essential for mitosis (Wolgemuth et al. 2004). *CCNA1* apparently has been co-opted by leukemic cells, as well as other malignancies, to promote cell proliferation and survival. Enforced expression of cyclin-A1 in hematopoietic cells that develop in transgenic mice has been shown to be leukemogenic (Liao et al. 2001). T cells specific for several cyclin-A1 oligopeptides were readily generated following stimulation of peripheral $CD8^+$ T cells from normal donors, and such T cells efficiently lysed primary AML cells, consistent with the expression of high-affinity TCRs as a consequence of minimal negative selection of T cells specific for cancer/testis antigens (Ochsenreither et al. 2012). Thus, cyclin-A1 appears to be a prototypic leukemia-testis-antigen expressed in AML LSCs, and we are currently isolating TCRs to be used for targeting this antigen in TCR gene therapy trials.

HMMR-Directed TCR Gene Therapy for AML in HLA-Mismatched HCT

Hyaluronan-mediated motility receptor (HMMR/Rhamm) is overexpressed in numerous tumor types, including AML. It is also expressed in AML LSCs (Snauwaert et al. 2012). Prolonged survival of AML patients has been shown to correlate with the prevalence of HMMR-specific T cells (Greiner et al. 2006, 2008). Peptide vaccination induced strong HMMR-specific immune responses without apparent toxicity, but still failed to cure disease (Schmitt et al. 2008; Greiner et al. 2010). Based on these clinical observations, we reasoned that adoptive transfer of T cells expressing TCRs specific for HMMR might provide a more potent response and improve clinical outcomes in AML.

We previously developed methods for isolation of natural high-affinity TCRs from healthy individuals, bypassing the need for mutation to improve affinity. This approach is based on DC priming of naïve T cells in vitro using antigen presentation by allogeneic MHC molecules to enable T cell responses to be generated from a non-tolerant responding T cell repertoire (Wilde et al. 2009). Allo-restricted TCRs are selected to be strictly peptide specific, as determined by extensive in vitro characterization. By this approach, we obtained HMMR-specific, HLA-A2*02:01-allo-restricted T cell clones (Spranger et al. 2012). After characterization of numerous clones, clone 150 and its corresponding TCR150 were selected for further assessment as a potential therapeutic reagent for treatment of leukemia. TCR150 showed high expression and specificity after transfer into recipient T cells.

T cells transduced with TCR150 recognized freshly isolated human $HLA\text{-}A2^+$ AML leukemic cells in vitro. Immune-deficient Nod/SCID/$IL\text{-}2Rg^{-/-}$ (NSG) mice transplanted with the $HLA\text{-}A2^+$ THP1 human AML cell line were mon-

itored for leukemia dissemination and outgrowth after transfer of T cells transduced to express TCR150, and a substantial delay of leukemia development was achieved in vivo (Spranger et al. 2012). However, we also found that the low levels of HMMR expression in human CD34$^+$ HSC were sufficient to allow in vitro recognition of HLA-A2$^+$ HSC by cells expressing TCR150. Studies in HHD (HLA-A2$^+$ transgenic) mice, carrying the identical HMMR epitope, confirmed TCR150 recognition of HSCs, as murine lineage-negative bone marrow cells preexposed to TCR150-engineered mouse T cells were not able to reconstitute lethally irradiated mice.

Thus, TCR150-based gene therapy would be limited to use in patients undergoing MHC-mismatched HCT (Schendel and Frankenberger 2013). In this setting, lymphocytes from an HLA-A2$^-$ donor could be engineered with TCR150 and infused into HLA-A2$^+$ hosts undergoing HCT for relapsed AML. This TCR gene therapy should allow for elimination of residual host AML, while donor HSCs would be spared because they lack HLA-A2 expression. However, it remains to be determined if any host somatic tissue might be a target of T cells recognizing HMMR.

MHC-Restricted Fratricide Can Limit TCR Transgene Expression

Survivin is an anti-apoptosis protein present in most AMLs. It was found to be one of the four top transcripts among 3.5 million human transcriptomes uniformly upregulated in cancer tissues but not in normal tissues (Sugahara et al. 2004). Therefore, it could be a versatile tumor antigen for treatment of many different malignancies. Importantly, it is also expressed in AML LSCs (Carter et al. 2012), which must be eliminated to achieve cure of AML.

We compared self-restricted and allo-restricted DC priming to isolate survivin-specific T cell clones. Interestingly, self-MHC-restricted T cell clones demonstrated early signs of proliferation but failed to expand to large numbers, while allo-restricted clones were abundant and expanded well. The TCRs of four HLA-A*02:01-allo-restricted clones were isolated and demonstrated to have a range of affinities for peptide. These TCRs were used to engineer recipient T cells from HLA-A2$^+$ donors, reflecting how they would be applied clinically. For comparison, recipient T cells of HLA-A2$^-$ donors were also studied. As observed with the original survivin-reactive T cells derived from HLA-A2$^+$ donors, TCR-engineered recipient HLA-A2$^+$ T cells died in culture, while the HLA-A2$^-$ T cells expressing the same survivin-specific TCRs proliferated well. Further studies established that cell death in the HLA-A2$^+$ recipient T cells was caused by MHC-restricted fratricide due to expression of survivin in the T cells, making them direct target cells for the HLA-A2-restricted survivin-specific TCRs (Leisegang et al. 2010).

These findings hinder further development of these TCRs for gene therapy because the MHC-restricted fratricide of HLA-A2$^+$ patient lymphocytes would limit preparation of adequate numbers of TCR-engineered lymphocytes for therapeutic use. However, it would still be possible to use these TCRs in an HLA-mismatched setting, as proposed above for TCR150.

Conclusions

The field of TCR gene therapy has moved from a vision to an emerging clinical reality. The potential curative treatments for cancer patients that might be achieved using high-affinity TCR gene therapy, juxtaposed to the possible dangers of lethal toxicity, have now moved the emphasis in the field from developing technologies to acquire efficacious TCRs to identifying those TCRs with clinical efficacy and safety profiles that will allow their use in larger numbers of patients. The development of TCR-based therapeutics offers significant promise but still has critical challenges from safety and feasibility perspectives and must also overcome the enormous regulatory hurdles before it will be possible to bring individualized TCR-based therapies forward in pharmaceutical development.

Currently, most TCRs used in clinical studies have originated from patient samples or

xenogeneic murine sources. Some of these TCRs have additionally been mutated to improve affinity with the objective of achieving better clinical efficacy. An increase in affinity does appear to increase therapeutic activity, but side effects from high-affinity and mutated TCRs have become apparent, dramatically shifting the risk-benefit analysis of TCR gene therapy. Therefore, new technologies and strategies are needed to make sound decisions on which TCRs, among a pool of many candidates, are most appropriate to be selected for early clinical studies. New methods to directly assess on- and off-target toxicity are a critical need. The scientific community is actively pooling ideas, information, and resources to meet this challenge. The inspiration is given to solve these problems, because the evidence from early clinical studies has demonstrated that TCR gene therapy indeed has the capacity to provide major clinical benefit to patients who otherwise would have no suitable treatment options to prolong their lives and potentially eradicate their cancers.

References

Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, Bignell GR, Bolli N, Borg A, Børresen-Dale AL, Boyault S, Burkhardt B, Butler AP, Caldas C, Davies HR, Desmedt C, Eils R, Eyfjörd JE, Foekens JA, Greaves M, Hosoda F, Hutter B, Ilicic T, Imbeaud S, Imielinski M, Jäger N, Jones DT, Jones D, Knappskog S, Kool M, Lakhani SR, López-Otín C, Martin S, Munshi NC, Nakamura H, Northcott PA, Pajic M, Papaemmanuil E, Paradiso A, Pearson JV, Puente XS, Raine K, Ramakrishna M, Richardson AL, Richter J, Rosenstiel P, Schlesner M, Schumacher TN, Span PN, Teague JW, Totoki Y, Tutt AN, Valdés-Mas R, van Buuren MM, van 't Veer L, Vincent-Salomon A, Waddell N, Yates LR; Australian Pancreatic Cancer Genome Initiative; ICGC Breast Cancer Consortium; ICGC MMML-Seq Consortium; ICGC PedBrain, Zucman-Rossi J, Futreal PA, McDermott U, Lichter P, Meyerson M, Grimmond SM, Siebert R, Campo E, Shibata T, Pfister SM, Campbell PJ, Stratton MR (2013) Signatures of mutational processes in human cancer. Nature 500(7463):415–421

Amir AL, van der Steen DM, van Loenen MM, Hagedoorn RS, de Boer R, Kester MD, de Ru AH, Lugthart GJ, van Kooten C, Hiemstra PS, Jedema I, Griffioen M, van Veelen PA, Falkenburg JH, Heemskerk MH (2011) PRAME-specific Allo-HLA-restricted T cells with potent antitumor reactivity useful for therapeutic T-cell receptor gene transfer. Clin Cancer Res 17(17): 5615–5625

Bendle GM, Linnemann C, Hooijkaas AI, Bies L, de Witte MA, Jorritsma A, Kaiser AD, Pouw N, Debets R, Kieback E, Uckert W, Song JY, Haanen JB, Schumacher TN (2010) Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy. Nat Med 16(5):565–570

Berger C, Jensen MC, Lansdorp PM, Gough M, Elliott C, Riddell SR (2008) Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. J Clin Invest 118(1):294–305

Besser MJ, Shapira-Frommer R, Itzhaki O, Treves AJ, Zippel DB, Levy D, Kubi A, Shoshani N, Zikich D, Ohayon Y, Ohayon D, Shalmon B, Markel G, Yerushalmi R, Apter S, Ben-Nun A, Ben-Ami E, Shimoni A, Nagler A, Schachter J (2013) Adoptive transfer of tumor-infiltrating lymphocytes in patients with metastatic melanoma: intent-to-treat analysis and efficacy after failure to prior immunotherapies. Clin Cancer Res 19(17):4792–4800

Bleakley M, Riddell SR (2011) Exploiting T cells specific for human minor histocompatibility antigens for therapy of leukemia. Immunol Cell Biol 89(3):396–407

Buchholz VR, Flossdorf M, Hensel I, Kretschmer L, Weissbrich B, Gräf P, Verschoor A, Schiemann M, Höfer T, Busch DH (2013) Disparate individual fates compose robust CD8+ T cell immunity. Science 340(6132):630–635

Burdach S, Kolb HJ (2013) The vigor of defense against non-self: potential superiority of allorestricted T cells in immunotherapy of cancer? Front Oncol 3:100

Cameron BJ, Gerry AB, Dukes J, Harper JV, Kannan V, Bianchi FC, Grand F, Brewer JE, Gupta M, Plesa G, Bossi G, Vuidepot A, Powlesland AS, Legg A, Adams KJ, Bennett AD, Pumphrey NJ, Williams DD, Binder-Scholl G, Kulikovskaya I, Levine BL, Riley JL, Varela-Rohena A, Stadtmauer EA, Rapoport AP, Linette GP, June CH, Hassan NJ, Kalos M, Jakobsen BK (2013) Identification of a Titin-derived HLA-A1-presented peptide as a cross-reactive target for engineered MAGE A3-directed T cells. Sci Transl Med 5(197):197ra103

Carter BZ, Qiu Y, Huang X, Diao L, Zhang N, Coombes KR, Mak DH, Konopleva M, Cortes J, Kantarjian HM, Mills GB, Andreeff M, Kornblau SM (2012) Survivin is highly expressed in CD34(+)38(−) leukemic stem/progenitor cells and predicts poor clinical outcomes in AML. Blood 120(1):173–180

Chapuis AG, Ragnarsson GB, Nguyen HN, Chaney CN, Pufnock JS, Schmitt TM, Duerkopp N, Roberts IM, Pogosov GL, Ho WY, Ochsenreither S, Wölfl M, Bar M, Radich JP, Yee C, Greenberg PD (2013) Transferred WT1-reactive CD8+ T cells can mediate antileukemic activity and persist in post-transplant patients. Sci Transl Med 5(174):174ra27

Chervin AS, Aggen DH, Raseman JM, Kranz DM (2008) Engineering higher affinity T cell receptors using a T cell display system. J Immunol Methods 339(2):175–184

Dudley ME, Gross CA, Somerville RP, Hong Y, Schaub NP, Rosati SF, White DE, Nathan D, Restifo NP, Steinberg SM, Wunderlich JR, Kammula US, Sherry RM, Yang JC, Phan GQ, Hughes MS, Laurencot CM, Rosenberg SA (2013) Randomized selection design trial evaluating CD8+-enriched versus unselected tumor-infiltrating lymphocytes for adoptive cell therapy for patients with melanoma. J Clin Oncol 31(17):2152–2159

Falkenburg JH, Warren EH (2011) Graft versus leukemia reactivity after allogeneic stem cell transplantation. Biol Blood Marrow Transplant 17(1 Suppl):S33–S38

Ferrara J, Reddy P, Paczesny S (2010) Immunotherapy through T-cell receptor gene transfer induces severe graft-versus-host disease. Immunotherapy 2(6):791–794

Gattinoni L, Klebanoff CA, Restifo NP (2012) Paths to stemness: building the ultimate antitumour T cell. Nat Rev Cancer 12(10):671–684

Greiner J, Schmitt M, Li L, Giannopoulos K, Bosch K, Schmitt A, Dohner K, Schlenk RF, Pollack JR, Dohner H, Bullinger L (2006) Expression of tumor-associated antigens in acute myeloid leukemia: Implications for specific immunotherapeutic approaches. Blood 108(13):4109–4117

Greiner J, Bullinger L, Guinn BA, Döhner H, Schmitt M (2008) Leukemia-associated antigens are critical for the proliferation of acute myeloid leukemia cells. Clin Cancer Res 14(22):7161–7166

Greiner J, Schmitt A, Giannopoulos K, Rojewski MT, Götz M, Funk I, Ringhoffer M, Bunjes D, Hofmann S, Ritter G, Döhner H, Schmitt M (2010) High-dose RHAMM-R3 peptide vaccination for patients with acute myeloid leukemia, myelodysplastic syndrome and multiple myeloma. Haematologica 95(7):1191–1197

Johnson LA, Morgan RA, Dudley ME, Cassard L, Yang JC, Hughes MS, Kammula US, Royal RE, Sherry RM, Wunderlich JR, Lee CCR, Restifo NP, Schwarz SL, Cogdill AP, Bishop RJ, Kim H, Brewer CC, Rudy SF, VanWaes C, Davis JL, Mathur A, Ripley RT, Nathan DA, Laurencot CM, Rosenberg SA (2009) Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. Blood 114(3):535–546

Keilholz U, Letsch A, Busse A, Asemissen AM, Bauer S, Blau IW, Hofmann WK, Uharek L, Thiel E, Scheibenbogen C (2009) A clinical and immunologic phase 2 trial of Wilms tumor gene product 1 (WT1) peptide vaccination in patients with AML and MDS. Blood 113(26):6541–6548

Kolb HJ (2008) Graft-versus-leukemia effects of transplantation and donor lymphocytes. Blood 112(12): 4371–4383

Kuball J, Schmitz FW, Voss RH, Ferreira EA, Engel R, Guillaume P, Strand S, Romero P, Huber C, Sherman LA, Theobald M (2005) Cooperation of human tumor-reactive CD4+ and CD8+ T cells after redirection of their specificity by a high-affinity p53A2.1-specific TCR. Immunity 22(1):117–129

Kuball J, Dossett ML, Wolfl M, Ho WY, Voss RH, Fowler C, Greenberg PD (2007) Facilitating matched pairing and expression of TCR chains introduced into human T cells. Blood 109(6):2331–2338

Kuball J, Hauptrock B, Malina V, Antunes E, Voss RH, Wolfl M, Strong R, Theobald M, Greenberg PD (2009) Increasing functional avidity of TCR-redirected T cells by removing defined N-glycosylation sites in the TCR constant domain. J Exp Med 206(2):463–475

Leisegang M, Wilde S, Spranger S, Milosevic S, Frankenberger B, Uckert W, Schendel DJ (2010) MHC-restricted fratricide of human lymphocytes expressing survivin-specific transgenic T cell receptors. J Clin Invest 120(11):3869–3877

Lennerz V, Fatho M, Gentilini C, Frye RA, Lifke A, Ferel D, Wölfel C, Huber C, Wölfel T (2005) The response of autologous T cells to a human melanoma is dominated by mutated neoantigens. Proc Natl Acad Sci U S A 102(44):16013–16018

Li LP, Lampert JC, Chen X, Leitao C, Popović J, Müller W, Blankenstein T (2010) Transgenic mice with a diverse human T cell antigen receptor repertoire. Nat Med 16(9):1029–1034

Liao C, Wang XY, Wei HQ, Li SQ, Merghoub T, Pandolfi PP, Wolgemuth DJ (2001) Altered myelopoiesis and the development of acute myeloid leukemia in transgenic mice overexpressing cyclin A1. Proc Natl Acad Sci U S A 98(12):6853–6858

Liddy N, Bossi G, Adams KJ, Lissina A, Mahon TM, Hassan NJ, Gavarret J, Bianchi FC, Pumphrey NJ, Ladell K, Gostick E, Sewell AK, Lissin NM, Harwood NE, Molloy PE, Li Y, Cameron BJ, Sami M, Baston EE, Todorov PT, Paston SJ, Dennis RE, Harper JV, Dunn SM, Ashfield R, Johnson A, McGrath Y, Plesa G, June CH, Kalos M, Price DA, Vuidepot A, Williams DD, Sutton DH, Jakobsen BK (2012) Monoclonal TCR-redirected tumor cell killing. Nat Med 18(6):980–987

Linette GP, Stadtmauer EA, Maus MV, Rapoport AP, Levine BL, Emery L, Litzky L, Bagg A, Carreno BM, Cimino PJ, Binder-Scholl GK, Smethurst DP, Gerry AB, Pumphrey NJ, Bennett AD, Brewer JE, Dukes J, Harper J, Tayton-Martin HK, Jakobsen BK, Hassan NJ, Kalos M, June CH (2013) Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. Blood 122:863–871

Morgan RA, Chinnasamy N, Abate-Daga D, Gros A, Robbins PF, Zheng Z, Dudley ME, Feldman SA, Yang JC, Sherry RM, Phan GQ, Hughes MS, Kammula US, Miller AD, Hessman CJ, Stewart AA, Restifo NP, Quezado MM, Alimchandani M, Rosenberg AZ, Nath A, Wang T, Bielekova B, Wuest SC, Akula N, McMahon FJ, Wilde S, Mosetter B, Schendel DJ, Laurencot CM, Rosenberg SA (2013) Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy. J Immunother 36(2):133–151

Morris E, Hart D, Gao L, Tsallios A, Xue SA, Stauss H (2006) Generation of tumor-specific T-cell therapies. Blood Rev 20(2):61–69

Ochsenreither S, Majeti R, Schmitt T, Stirewalt D, Keilholz U, Loeb KR, Wood B, Choi YE, Bleakley M, Warren EH, Hudecek M, Akatsuka Y, Weissman IL, Greenberg PD (2012) Cyclin-A1 represents a new immunogenic targetable antigen expressed in acute myeloid leukemia stem cells with characteristics of a cancer-testis antigen. Blood 119(23):5492–5501

Oka Y, Tsuboi A, Oji Y, Kawase I, Sugiyama H (2008) WT1 peptide vaccine for the treatment of cancer. Curr Opin Immunol 20(2):211–220

Parkhurst MR, Yang JC, Langan RC, Dudley ME, Nathan DA, Feldman SA, Davis JL, Morgan RA, Merino MJ, Sherry RM, Hughes MS, Kammula US, Phan GQ, Lim RM, Wank SA, Restifo NP, Robbins PF, Laurencot CM, Rosenberg SA (2011) T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. Mol Ther 19(3):620–626

Provasi E, Genovese P, Lombardo A, Magnani Z, Liu PQ, Reik A, Chu V, Paschon DE, Zhang L, Kuball J, Camisa B, Bondanza A, Casorati G, Ponzoni M, Ciceri F, Bordignon C, Greenberg PD, Holmes MC, Gregory PD, Naldini L, Bonini C (2012) Editing T cell specificity towards leukemia by zinc finger nucleases and lentiviral gene transfer. Nat Med 18(5):807–815

Robbins PF, Morgan RA, Feldman SA, Yang JC, Sherry RM, Dudley ME, Wunderlich JR, Nahvi AV, Helman LJ, Mackall CL, Kammula US, Hughes MS, Restifo NP, Raffeld M, Lee CC, Levy CL, Li YF, El-Gamil M, Schwarz SL, Laurencot C, Rosenberg SA (2011) Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. J Clin Oncol 29(7):917–924

Robbins PF, Lu YC, El-Gamil M, Li YF, Gross C, Gartner J, Lin JC, Teer JK, Cliften P, Tycksen E, Samuels Y, Rosenberg SA (2013) Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. Nat Med 19(6): 747–752

Rosenberg SA, Dudley ME (2009) Adoptive cell therapy for the treatment of patients with metastatic melanoma. Curr Opin Immunol 21(2):233–240

Schendel DJ, Frankenberger B (2013) Limitations for TCR gene therapy by MHC-restricted fratricide and TCR-mediated hematopoietic stem cell toxicity. Oncoimmunology 2(1):e22410

Schmitt M, Schmitt A, Rojewski MT, Chen J, Giannopoulos K, Fei F, Yu Y, Götz M, Heyduk M, Ritter G, Speiser DE, Gnjatic S, Guillaume P, Ringhoffer M, Schlenk RF, Liebisch P, Bunjes D, Shiku H, Dohner H, Greiner J (2008) RHAMM-R3 peptide vaccination in patients with acute myeloid leukemia, myelodysplastic syndrome, and multiple myeloma elicits immunologic and clinical responses. Blood 111(3):1357–1365

Schreiber RD, Old LJ, Smyth MJ (2011) Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science 331(6024): 1565–1570

Snauwaert S, Vanhee S, Goetgeluk G, Verstichel G, Van Caeneghem Y, Velghe I, Philippé J, Berneman ZN, Plum J, Taghon T, Leclercq G, Thielemans K, Kerre T, Vandekerckhove B (2012) RHAMM/HMMR (CD168) is not an ideal target antigen for immunotherapy of acute myeloid leukemia. Haematologica 97(10): 1539–1547

Spierings E, Kim YH, Hendriks M, Borst E, Sergeant R, Canossi A, Oudshoorn M, Loiseau P, Dolstra H, Markiewicz M, Leffell MS, Pereira N, Kircher B, Turpeinen H, Eliaou JF, Gervais T, Laurin D, Enczmann J, Martinetti M, Thomson J, Oguz F, Santarone S, Partanen J, Siekiera U, Alessandrino EP, Kalayoglu S, Brand R, Goulmy E (2013) Multicenter analyses demonstrate significant clinical effects of minor histocompatibility antigens on GvHD and GvL after HLA-matched related and unrelated hematopoietic stem cell transplantation. Biol Blood Marrow Transplant 19(8):1244–1253

Spranger S, Jeremias I, Wilde S, Leisegang M, Stärck L, Mosetter B, Uckert W, Heemskerk MH, Schendel DJ, Frankenberger B (2012) TCR-transgenic lymphocytes specific for HMMR/Rhamm limit tumor outgrowth in vivo. Blood 119(15):3440–3449

Stone JD, Kranz DM (2013) Role of T cell receptor affinity in the efficacy and specificity of adoptive T cell therapies. Front Immunol 4:244

Sugahara K, Uemura A, Harasawa H, Nagai H, Hirakata Y, Tomonaga M, Murata K, Sohda H, Nakagoe T, Shibasaki S, Yamada Y, Kamihira S (2004) Clinical relevance of survivin as a biomarker in neoplasms, especially in adult T-cell leukemias and acute leukemias. Int J Hematol 80(1):52–58

Sugiyama H (2010) WT1 (Wilms' tumor gene 1): biology and cancer immunotherapy. Jpn J Clin Oncol 40(5): 377–387

van den Berg JH (Ton Schumacher) (2013) ATTACK cellular therapy of cancer symposium, London

van Rooij N, van Buuren MM, Philips D, Velds A, Toebes M, Heemskerk B, van Dijk LJ, Behjati S, Hilkmann H, El Atmioui D, Nieuwland M, Stratton MR, Kerkhoven RM, Kesmir C, Haanen JB, Kvistborg P, Schumacher TN (2013) Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. J Clin Oncol 31(32):e439–e442

Warren EH, Fujii N, Akatsuka Y, Chaney CN, Mito JK, Loeb KR, Gooley TA, Brown ML, Koo KKW, Rosinski KW, Ogawa S, Matsubara A, Appelbaum FR, Riddell SR (2010) Therapy of relapsed leukemia after allogeneic hematopoietic cell transplantation with T cells specific for minor histocompatibility antigens. Blood 115:3869–3878

Wilde S, Sommermeyer D, Frankenberger B, Schiemann M, Milosevic S, Spranger S, Pohla H, Uckert W, Busch DH, Schendel DJ (2009) Dendritic cells pulsed with RNA

encoding allogeneic MHC and antigen induce T cells with superior antitumor activity and higher TCR functional avidity. Blood 114(10):2131–2139

Wilde S, Geiger C, Milosevic S, Mosetter B, Eichenlaub S, Schendel DJ (2012) Generation of allo-restricted peptide-specific T cells using RNA-pulsed dendritic cells: A three phase experimental procedure. Oncoimmunology 1(2):129–140

Wolgemuth DJ, Lele KM, Jobanputra V, Salazar G (2004) The A-type cyclins and the meiotic cell cycle in mammalian male germ cells. Int J Androl 27(4): 192–199

The Express Drivers: Chimeric Antigen Receptor-Redirected T Cells Make It to the Clinic

Hinrich Abken, Winfried S. Wels, and Klaus Kühlcke

The "T-Body" Strategy: Chimeric Antigen Receptor (CAR)-Redirected T Cells

Adoptive cell therapy with tumor-infiltrating lymphocytes showed some encouraging efficacy in the therapy of melanoma (Kalos et al. 2011; Porter et al. 2011; Grupp et al. 2013). However, technical difficulties in obtaining sufficient T-cell numbers and the rarity of these cells in other malignancies stimulated the development of alternative procedures to obtain engineered T cells with predefined specificity. In this situation, Zelig Eshhar, Weizmann Institute, proposed to redirect T cells by a recombinant receptor molecule, a chimeric antigen receptor (CAR) nicknamed "T-body," which confers targeting specificity to T cells and initiates T-cell activation upon target engagement (Eshhar 2008). In contrast to the T-cell receptor (TCR), the archetypal CAR is composed of one polypeptide chain and consists in the extracellular part of a single-chain fragment of variable binding (scFv) antibody and in the intracellular part of a TCR-derived activation domain, preferentially derived from the CD3ζ chain of the TCR/CD3 complex (Fig. 1). CAR binding to cognate targets on the tumor cell surface produces receptor clustering and finally phosphorylation of the immunoreceptor tyrosine activation motifs (ITAMs) which initiates a downstream signaling cascade. As a consequence, T cells amplify, secrete a panel of proinflammatory cytokines, and produce cytolytic activity toward the cognate target cell.

Here we briefly review the current knowledge on the optimized composition of a CAR, the consequences of CAR expression in T cells as well as other cytotoxic leukocytes such as natural killer (NK) cells, the clinical-grade production of CAR-modified T cells, the most significant progress recently made in early-stage clinical trials, and perspectives on future developments to treat cancer by CAR T-cell therapy.

The Targeting Specificity Matters: Advantages in Antibody-Mediated Targeting by a CAR

By using an antibody-derived binding domain, CAR T cells overcome some limitations of the TCR-based recognition in that CAR T cells bind target cells in an MHC-independent fashion,

H. Abken (✉)
Center for Molecular Medicine Cologne, University of Cologne, Robert-Koch-Str. 21, D-50931 Cologne, Germany

Department I for Internal Medicine, University Hospital Cologne, Cologne, Germany
e-mail: hinrich.abken@uk-koeln.de

W.S. Wels
Chemotherapeutisches Forschungsinstitut Georg-Speyer-Haus, Institut für Tumorbiologie und experimentelle Therapie, Frankfurt am Main, Germany

K. Kühlcke
EUFETS GmbH, Idar-Oberstein, Germany

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_13,

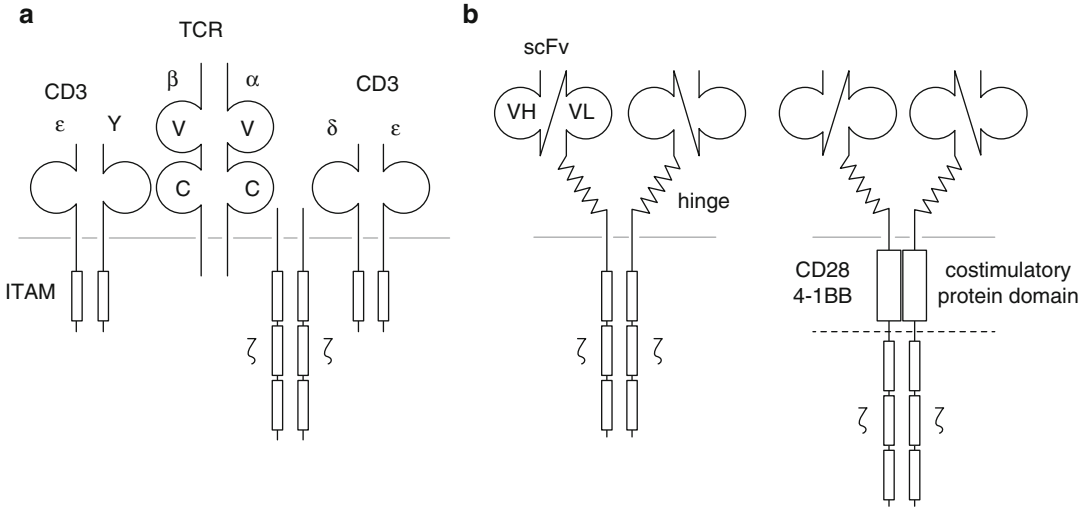

Fig. 1 (**a**) T-cell receptor (TCR) complex. The TCR consists of the α/β dimer-recognizing antigenic peptides presented by MHC molecules. Complexed with the TCR are the invariant chains of the CD3 complex CD3γ, CD3δ, CD3ε, and CD3ζ. The invariant chains couple the TCR to intracellular signaling molecules which bind to phosphorylated immunoreceptor tyrosine-based activation motifs (ITAM). (**b**) Chimeric antigen receptor (CAR). The prototype CAR consists of a target-specific scFv antibody fragment comprising antibody heavy- (VH) and light-chain (VL) variable domains fused via a flexible hinge region to transmembrane and intracellular regions of the TCR-associated ζ chain (first-generation CAR; *left*). Alternative CAR designs include in addition one or more protein domains derived from costimulatory receptors such CD28 or 4-1BB (second- and third-generation CAR; *right*)

bind with higher affinity than the TCR, and form synapses independently of the TCR. An additional advantage is that T cells can be redirected toward a plethora of antigens of different composition as long as a suitable antibody is available. For instance, CARs were engineered which target T cells toward the carbohydrate antigens CA19-9 or TAG72 (Mezzanzanica et al. 1998; Hombach et al. 1997; Westwood et al. 2005). The TCR, in contrast, is restricted to the recognition of specific peptides presented by the particular MHC. Instead of antibodies, other binding domains like growth factors were also successfully used to redirect T cells by such type of chimeric receptor (Altenschmidt et al. 1996).

By using an antibody, various epitopes of the same antigen can be targeted as long as the epitope is accessible on the surface of the target cell; however, each epitope may not be equally efficient in activating T cells. The accessibility of the epitope for binding impacts the efficiency in CAR-mediated T-cell activation; the membrane-distal epitope, which is thought to be more accessible than the more proximal epitope, is superior in binding but less capable in mediating CAR activation as exemplarily shown for targeting carcinoembryonic antigen (CEA) expressed on gastrointestinal carcinoma cells (Hombach and Abken 2007). The same observation was made when targeting B-cell lymphoma-associated CD22 by CAR T cells (James et al. 2008; Till et al. 2012). Taken together, the best suitable target epitope and binding affinity for optimal CAR T-cell activation remains to be empirically evaluated in each case.

In addition, the binding affinity substantially impacts the efficiency in CAR-mediated T-cell targeting. While increase in binding affinity increases T-cell activation, there is a threshold in binding affinity above which furthermore increase does not improve T-cell activation (Chmielewski et al. 2004). Such "affinity ceiling" for antibody-derived T-cell targeting was recently also observed for TCR-mediated T-cell targeting (Zhong et al. 2013) and adds to the ongoing discussion whether a redirected T-cell attack can be furthermore improved by increasing binding affinity.

Combined Costimulation Matters: Recent Progress Toward an Optimized CAR Design

First-generation CARs provide exclusively the CD3ζ signal, while second-generation CARs additionally incorporate a costimulatory domain, mostly CD28, to prevent engineered T cells from activation-induced cell death and anergy. CD28 is the prototype of a family of costimulatory molecules that is physiologically engaged on T cells by binding to the respective agonistic ligands B7.1 (CD80) and B7.2 (CD86) on antigen-presenting cells (APCs). By linking the costimulatory domain to CD3ζ in one polypeptide chain, both signals are simultaneously provided to T cells independently of APCs. This is of some relevance since tumor cells frequently lack these molecules with the consequence that CD3ζ CAR T cells upon binding to cancer cells do not gain the costimulation required for full activation. Other costimulatory molecules including 4-1BB (CD137) and OX40 (CD134) can also be integrated into the CAR, with each costimulatory signal modulating the effector functions in each T-cell subset in a different fashion (Hombach et al. 2007).

The CD28 or 4-1BB endodomain is integrated into most currently used CARs because this type of costimulation sustains survival, increases proinflammatory cytokine secretion, and prolongs polyclonal expansion of engineered T cells (Hombach and Abken 2013a). CD28 co-signaling induces IL-2 that is used in an autocrine fashion by redirected T cells to increase their amplification (Beecham et al. 2000). CD28 costimulation, however, does not increase sensitivity toward target cells with intermediate or low densities of the respective target antigen. Also the "affinity ceiling" mentioned above is not altered by CD28 costimulation (Chmielewski et al. 2011a). As a consequence, low-affinity CAR interactions still require substantial amounts of target antigen to induce T-cell activation even in the presence of CD28 costimulation which potentially protects cells with low target antigen load from a CAR T-cell attack.

Costimulation moreover has profound impact on the efficacy of the individual T-cell compartments in executing an antitumor attack. While young T cells, in particular cells with central memory phenotype, persisted longer and were superior in mediating an antitumor response in preclinical models, effector memory or terminally differentiated T cells were less effective and disappeared rapidly from the circulation (Klebanoff et al. 2005). Repetitive T-cell activation, however, promotes T-cell maturation producing cells with altered functional properties and dramatic changes in phenotype, i.e., CAR T cells with a naive phenotype convert to a $CCR7^{-}$ $CD62L^{low}$ $CD57^{+}$ $KLRG1^{+}$ effector memory phenotype with $CD45RO^{high}$ $CD45RA^{low}$ and $CD27^{low}$ $CD28^{low}$ expression in a mouse tumor model (Hombach and Abken 2013b). One consequence is that due to loss of CCR7, those cells have a diminished capacity to reenter the lymph and to recirculate. When redirected by a CD28ζ CAR, the antitumor response of $CCR7^{-}$ CAR T cells is less efficient than that of $CCR7^{+}$ T cells, although those cells secrete higher amounts of proinflammatory cytokines and harbor higher levels of perforin and granzymes. The mechanistic basis is that $CCR7^{-}$ T cells are prone to spontaneous and activation-induced cell death, which is insufficiently prevented by CAR-mediated CD28 costimulation. The deficit can be counteracted by a so-called third-generation CD28-OX40 CAR which combines CD28 "early" with OX40 "late" costimulation. Similar results were obtained with cytokine-induced killer (CIK) cells (Hombach et al. 2013b). The particular combination of costimulatory signals achieves the effect since OX40 alone does not provide a benefit in this context, whereas CD28 costimulation alone, which prevents young T-cell apoptosis, does not reduce the number of apoptotic cells in late differentiation stages. As a consequence, a combined CD28-OX40 CAR will be of benefit for adoptive cell therapy since T cells of any stage will progress in maturation during activation and need to be protected from apoptosis when converting into late stages of differentiation.

CAR-Engineered Natural Killer (NK) Cells

In their pioneering work, Eshhar and colleagues demonstrated that the "T-body" strategy is not only applicable to T cells but can also be

employed to provide cells of the innate immune system such as mast and NK cells with tumor specificity (Bach et al. 1994, 1995). In contrast to T cells, the natural cytotoxicity of NK cells can be rapidly activated and is regulated by a complex balance of signals from germ line-encoded activating and inhibitory cell surface receptors (Lanier 2008). Due to their exquisite cytotoxic potential, donor-derived primary NK cells as well as continuously growing NK cell lines such as NK-92 can be used for adoptive cancer immunotherapy (Klingemann 2013). Established and primary NK cells endogenously express the CD3ζ chain which is involved in the signaling of natural cytotoxicity receptors. Hence, CARs based on CD3ζ chain in their signaling moiety readily link to the endogenous signaling pathways in NK cells and trigger cytolytic activities as demonstrated for CARs with specificity for the B-cell antigens CD19 and CD20 (Boissel et al. 2009; Müller et al. 2008), the multiple myeloma antigen CS1 (Chu et al. 2013), as well as solid tumor-associated antigens such as HER2/neu (ErbB2), EpCAM, and GD-2 (Uherek et al. 2002; Kruschinski et al. 2008; Tavri et al. 2009; Esser et al. 2012). Similar to T cells, the functionality of CAR-modified primary NK cells was improved by adding costimulatory protein domains derived from 4-1BB or 2B4 (CD244) to the CAR signaling moieties (Imai et al. 2005; Altvater et al. 2009). The lack of an endogenous TCR of unknown specificity and potential autoreactivity may be considered a safety feature of CAR-expressing NK cells. In contrast to T cells, however, NK cells cannot provide IL-2 required for amplification in an autocrine fashion. To bypass the need for adding cytokines, established NK cells were modified to co-express IL-15 together with the CAR which permitted continuous cell expansion and CAR-mediated cytotoxicity in the absence of IL-2 (Sahm et al. 2012).

Despite these advances during the last years, experience with CAR-engineered primary NK cells and their clinical development is still limited. Due to efficient antiviral defense mechanisms, gene transfer into NK cells with retro- and lentiviral vectors as well as physical transfection methods are less efficient than in T cells (Sutlu et al. 2012). This does not constitute a problem for clinically applicable NK cell lines such as NK-92, which allow isolation and expansion of CAR-expressing cells from a bulk of untransduced cells (Uherek et al. 2002). Early-phase clinical studies in cancer patients demonstrated the safety of infused unmodified NK-92 cells, which were irradiated prior to application to prevent permanent engraftment. Clinical responses were achieved in a subset of patients (Arai et al. 2008; Tonn et al. 2013). Likewise, CAR-engineered NK-92 cells may be developed as an allogeneic off-the-shelf cell therapeutic agent, in particular for application in cases where autologous or MHC-matched donor cells are not available or in geographic regions without the infrastructure for engineering of genetically modified cells on an individual basis.

Production of CAR-Engineered T Cells for Clinical Application

One of the key questions to answer for clinical production of CAR-engineered T cells is the vector used to genetically modify T cells. Table 1 depicts the systems currently used in clinical approaches and summarizes the advantages and disadvantages of the respective vector system.

To date, most clinical trials have been performed employing retroviral vectors. Recently, other vector systems have alternatively been utilized, and it is expected that these systems, likely with the exception of plasmid DNA, will be used in parallel in the near future.

Another crucial issue is the way of stimulating the T cells for amplification and modification. While this has been primarily achieved by stimulating the CD3 complex by the OKT3 antibody in addition to mitogenic stimulation by IL-2 (Kershaw et al. 2006), most researchers now use magnetic beads coated with antibodies against CD3 and CD28 to provide a more physiological stimulation (Porter et al. 2011; Brentjens et al. 2013). These reagents have been made available in GMP quality. Also cell lines engineered to express various costimulating molecules have been applied as “artificial APCs” (Suhoski et al. 2007). While these cells may provide even more

Table 1 Currently used vector systems to modify T cells with a CAR

Vector system	Features	Advantages	Disadvantages	Reference
Gamma retroviral	Stable integration	Scalable production from stable producer cells, long-lasting experience	Insertional mutagenesis in stem cells, silencing of transgene expression possible	Kershaw et al. (2006), Brentjens et al. (2013), Lamers et al. (2006)
Lentiviral	Stable integration	Less mutagenic insertion profile, produced to high titers, no known silencing	Limited production scales in transient production systems, expensive	Porter et al. (2011)
DNA (transposon)	Stable integration	Random insertion profile, lower cost of goods	Less clinical experience, two GMP reagents needed, i.e., transposase, transposon	Singh et al. (2013)
DNA (plasmid)	Stable integration	Random insertion profile, cheap	long in vitro selection for stable integration, integration as concatamer	Park et al. (2007)
RNA	Transient expression	No insertion, high safety profile	Short-time expression, multiple doses needed	Zhao et al. (2010)

physiological stimulation, they harbor additional GMP risks which makes it less likely that these cells will be used in more industrialized and large-scale approaches. Some researchers also replaced IL-2 by other cytokines such as IL-7 and IL-15 to obtain a T-cell population with a more naive and central memory phenotype (Kaneko et al. 2009). These cytokines have recently been made available in a quality suitable for GMP production.

The generation of clinically relevant numbers of engineered T cells with a favorable phenotype is a long-standing challenge. Traditionally, static culture systems in flasks or gas-permeable bags have been used for T-cell expansion. Because T cells optimally grow in rather low cell densities ($0.25–1 \times 10^6$/ml), high culture volumes are required which makes handling difficult and raises costs. More advanced systems like the Wave Bioreactor or the G-Rex device sustained T-cell expansion to much higher densities (Porter et al. 2011; Brentjens et al. 2013; Vera et al. 2010). In order to amplify CAR T cells for a large number of patients, it would be beneficial if the manufacturing process could be performed in a closed system allowing the production of multiple batches in parallel in the same clean room. While this has been achieved in certain parts of the process, to our best knowledge a fully closed system for all steps of the manufacturing process still needs to be set up.

CAR T Cells Are Making It to the Clinic

Adoptive cell therapy with CAR-engineered T cells is currently entering early-phase clinical trials. Basically, the patients' T cells are modified ex vivo by retro- or lentiviral gene transfer or by DNA transfection to express the respective CAR, amplified to therapeutically relevant numbers, and transfused to the patient by a single or by repetitive administrations. Trials targeting $CD19^+$ leukemia and lymphoma produced encouraging evidence of therapeutic efficacy with complete and lasting remission of refractory $CD19^+$ B-cell chronic lymphocytic leukemia (CLL) in the first three reported patients (Kalos et al. 2011; Porter et al. 2011). T cells were effective even at low dosage levels of about 1.5×10^5 cells per kg (Porter et al. 2011). This is likely due to the fact that, compared to the initial level, CAR T cells expanded more than 1,000-fold after administration and persisted in the peripheral blood and bone marrow for at least 6 months. A grade 3 tumor lysis

syndrome and a cytokine storm with an increase in the proinflammatory cytokines IFN-γ and IL-6 occurred which coincided with the elimination of leukemia cells from the bone marrow in those patients. Importantly, antibody-mediated neutralization of IL-6 substantially reduced the cytokine storm without affecting the clinical efficacy. The prolonged persistence of CAR T cells is probably due to the repetitive restimulation of CAR T cells by $CD19^+$ healthy B cells and their progenitors which are also targets for the anti-CD19 CAR T cells producing a lasting B-cell deficiency in those patients. The same anti-CD19 CAR is currently evaluated in the treatment of pediatric $CD19^+$ acute leukemia with success; however, a relapse of $CD19^-$ leukemia was also observed in one case (Grupp et al. 2013).

Despite recent success, also two fatal serious adverse events occurred after infusion of CAR-modified T cells, one of which has at least in part been attributed to the redirected specificity. In the NIH trial with T cells carrying a third-generation CAR, the adverse event occurred presumably due to "on-target off-organ" activation of the CAR T cells targeting HER2/neu (ErbB2), which is expressed at moderate levels on healthy tissues (Morgan et al. 2010). The other event at the Sloan Kettering Cancer Center was attributed to an extravasation of a latent bacterial infection subsequent to lymphodepletion (Brentjens et al. 2010).

Perspectives: What May Redirected Cell Therapy Look Like in the Long Term?

While first success in the therapy of leukemia is sustaining hope that adoptive therapy with redirected T cells may cure leukemia in the long term, a number of issues still need to be addressed when solid cancer lesions are targeted.

Effector T cells need to be tuned in a specific fashion to persist and execute redirected activities in solid tumor tissues over a prolonged period of time. T-cell expansion is mandatory to establish the transferred T cells in the long term; however, T cells finally convert to effector memory cells which require combined CD28-OX40 costimulation to rescue $CCR7^-$ cells when produced during an ongoing immune attack (Hombach et al. 2013a). Costimulation by 4-1BB may also provide benefit (Song et al. 2011). Such "muscle CARs" are designed to provide the required co-signals by the same CAR. Persistence of redirected T cells in the periphery may be improved by further engineering with the CCR2 receptor (Moon et al. 2011). An additional prerequisite for T-cell amplification and persistence is thought to be non-myeloablative lympho-depleting preconditioning followed by IL-2 administration to sustain T-cell expansion; other cytokines like IL-7 and IL-15 are currently also explored (Weber et al. 2011). Alternatively, CAR T cells can be engineered with a CAR-inducible expression of such cytokines which stimulate CAR T cells in an autocrine fashion or recruit and activate innate immune cells for an antitumor attack (Chmielewski et al. 2011b). Such "TRUCK" cells ("T cells redirected for unrestricted cytokine killing") may pave a novel way to deliver transgenic cell products to predefined, target lesions (Chmielewski et al. 2014). CAR T cells may be locally applied into the tumor lesion by puncture or endoscopy with only local diffusion within the following days (Parente-Pereira et al. 2011). This strategy is currently evaluated in the treatment of head and neck cancer (EudraCT 2012-001654-25, NCT01722149) and will be shortly applied to the treatment of cutaneous lymphoma (EudraCT 2011-003125-10).

An elegant solution of providing T-cell response in the long term is the use of virus-specific T cells which obtain survival and costimulatory signals when engaging virus-infected cells by their TCR. Current trials use EBV- or CMV-specific, autologous T cells engineered with a first- or second-generation CAR, for instance, directed against HER2/neu (ErbB2) (NCT01109095), CD30 (NCT01192464), CD19 (NCT00709033, NCT01475058, NCT01430390, NCT00840853, NCT01195480), or GD-2 (NCT00085930). Virus-specific T cells have a great capacity to amplify and are particularly applied in the context of allogeneic stem cell transplantation, where they protect from virus reactivation and tumor relapse while having low risk of inducing graft versus host disease.

Most T-cell compartments of cancer patients are underrepresented or have lost functional activities. Allogeneic T cells from healthy donors derived from a fully functional T-cell compartment and ex vivo engineered with a specific CAR may provide an alternative. This strategy would moreover have the advantage that the cell product can be produced in advance and stored until use, making "CAR T-cell banking" feasible. Although associated with much logistic efforts, such banked T cells would benefit cell therapy by providing a more standardized cell product.

Although a number of technical questions still need to be addressed, enormous progress has been made in the last years to translate the CAR strategy into clinical practice. In addition to targeting lymphoma/leukemia, adoptive CAR T-cell therapy needs to be evaluated for the treatment of solid cancer which is still associated with the majority of cancer deaths. CAR T cells are target specific, however, not always cancer selective. This may produce severe autoimmunity. These and other safety issues need to be explored for the variety of new CARs which are already or will become available in the near future. While recent progress with CAR-engineered T cells in the clinic has been spearheaded by groups based in the United States, groups in Europe continue to be actively engaged in bringing CAR T cells to clinical trials, both for the treatment of hematological malignancies and for the more challenging solid cancers. This has been fostered through EU-funded consortia, which allowed combining the expertise of otherwise competing groups and provide the critical mass needed for translational projects. However, a further commitment by public funding agencies and commercial entities will be required to provide sustainable structures and to establish standardized protocols for clinical application of CAR T cells and production facilities large enough to meet the expected demand for their production under GMP conditions on a national and European level.

Acknowledgments Work in the laboratory of HA was supported by the Deutsche Forschungsgemeinschaft, Else Kröner-Fresenius Stiftung, Wilhelm Sander-Stiftung, the Deutsche Krebshilfe, the European Union (European Regional Development Fund – Investing in your future), the German federal state North Rhine-Westphalia (NRW), and the Fortune program of the Medical Faculty of the University of Cologne. Work in the laboratory of WSW was supported by the German Federal Ministry of Education and Research, Deutsche Forschungsgemeinschaft, LOEWE Center for Cell and Gene Therapy Frankfurt, and by institutional funds of the Georg-Speyer-Haus, Frankfurt.

References

Altenschmidt U, Kahl R, Moritz D, Schnierle BS, Gerstmayer B, Wels W, Groner B (1996) Cytolysis of tumor cells expressing the Neu/erbB-2, erbB-3, and erbB-4 receptors by genetically targeted naive T lymphocytes. Clin Cancer Res 2(6):1001–1008

Altvater B, Landmeier S, Pscherer S, Temme J, Schweer K, Kailayangiri S, Campana D, Juergens H, Pule M, Rossig C (2009) 2B4 (CD244) signaling by recombinant antigen-specific chimeric receptors costimulates natural killer cell activation to leukemia and neuroblastoma cells. Clin Cancer Res 15(15):4857–4866

Arai S, Meagher R, Swearingen M, Myint H, Rich E, Martinson J, Klingemann H (2008) Infusion of the allogeneic cell line NK-92 in patients with advanced renal cell cancer or melanoma: a phase I trial. Cytotherapy 10(6):625–632

Bach NL, Waks T, Schindler DG, Eshhar Z (1994) Functional expression in mast cells of chimeric receptors with antibody specificity. Cell Biophys 24–25:229–236

Bach N, Waks T, Eshhar Z (1995) Specific lysis of tumor cells by an NK-like cell line transfected with chimeric receptor genes. Tumor Target 1:203–209

Beecham EJ, Ma Q, Ripley R, Junghans RP (2000) Coupling CD28 co-stimulation to immunoglobulin T-cell receptor molecules: the dynamics of T-cell proliferation and death. J Immunother 23(6):631–642

Boissel L, Betancur M, Wels WS, Tuncer H, Klingemann H (2009) Transfection with mRNA for CD19 specific chimeric antigen receptor restores NK cell mediated killing of CLL cells. Leuk Res 33(9):1255–1259

Brentjens R, Yeh R, Bernal Y, Riviere I, Sadelain M (2010) Treatment of chronic lymphocytic leukemia with genetically targeted autologous T cells: case report of an unforeseen adverse event in a phase I clinical trial. Mol Ther 18(4):666–668

Brentjens RJ, Davila ML, Riviere I, Park J, Wang X, Cowell LG, Bartido S, Stefanski J, Taylor C, Olszewska M, Borquez-Ojeda O, Qu J, Wasielewska T, He Q, Bernal Y, Rijo IV, Hedvat C, Kobos R, Curran K, Steinherz P, Jurcic J, Rosenblat T, Maslak P, Frattini M, Sadelain M (2013) CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. Sci Transl Med 5(177):177ra138

Chmielewski M, Hombach A, Heuser C, Adams GP, Abken H (2004) T cell activation by antibody-like immunoreceptors: increase in affinity of the single-chain fragment domain above threshold does not

increase T cell activation against antigen-positive target cells but decreases selectivity. J Immunol 173(12): 7647–7653

Chmielewski M, Hombach AA, Abken H (2011a) CD28 cosignalling does not affect the activation threshold in a chimeric antigen receptor-redirected T-cell attack. Gene Ther 18(1):62–72

Chmielewski M, Kopecky C, Hombach AA, Abken H (2011b) IL-12 release by engineered T cells expressing chimeric antigen receptors can effectively Muster an antigen-independent macrophage response on tumor cells that have shut down tumor antigen expression. Cancer Res 71(17):5697–5706

Chmielewski M, Hombach AA, Abken H (2014) Of CARs and TRUCKs: chimeric antigen receptor (CAR) T cells engineered with an inducible cytokine to modulate the tumor stroma. Immunol Rev 257:83–90

Chu J, Deng Y, Benson DM, Jr., He S, Hughes T, Zhang J, Peng Y, Mao H, Yi L, Ghoshal K, He X, Devine SM, Zhang X, Caligiuri MA, Hofmeister CC, Yu J (2013) CS1-specific chimeric antigen receptor (CAR)-engineered natural killer cells enhance In Vitro and In Vivo anti-tumor activity against human multiple myeloma. Leukemia. doi:10.1038/leu.2013.279 (in press)

Eshhar Z (2008) The T-body approach: redirecting T cells with antibody specificity. Handb Exp Pharmacol 181:329–342

Esser R, Müller T, Stefes D, Kloess S, Seidel D, Gillies SD, Aperlo-Iffland C, Huston JS, Uherek C, Schönfeld K, Tonn T, Huebener N, Lode HN, Koehl U, Wels WS (2012) NK cells engineered to express a GD2 -specific antigen receptor display built-in ADCC-like activity against tumour cells of neuroectodermal origin. J Cell Mol Med 16(3):569–581

Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR, Teachey DT, Chew A, Hauck B, Wright JF, Milone MC, Levine BL, June CH (2013) Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. N Engl J Med 368(16):1509–1518

Hombach A, Abken H (2007) Costimulation tunes tumor-specific activation of redirected T cells in adoptive immunotherapy. Cancer Immunol Immunother 56(5): 731–737

Hombach AA, Abken H (2013a) Of chimeric antigen receptors and antibodies: OX40 and 41BB costimulation sharpen up T cell-based immunotherapy of cancer. Immunotherapy 5(7):677–681

Hombach AA, Abken H (2013b) Young T cells age during a redirected anti-tumor attack: chimeric antigen receptor-provided dual costimulation is half the battle. Front Immunol 4:135

Hombach A, Heuser C, Sircar R, Tillmann T, Diehl V, Kruis W, Pohl C, Abken H (1997) T cell targeting of TAG72+ tumor cells by a chimeric receptor with antibody-like specificity for a carbohydrate epitope. Gastroenterology 113(4):1163–1170

Hombach AA, Schildgen V, Heuser C, Finnern R, Gilham DE, Abken H (2007) T cell activation by antibody-like immunoreceptors: the position of the binding epitope within the target molecule determines the efficiency of activation of redirected T cells. J Immunol 178(7): 4650–4657

Hombach AA, Chmielewski M, Rappl G, Abken H (2013a) Adoptive immunotherapy with redirected T cells produces CCR7- cells that are trapped in the periphery and benefit from combined CD28-OX40 costimulation. Hum Gene Ther 24(3):259–269

Hombach AA, Rappl G, Abken H (2013b) Arming cytokine-induced killer cells with chimeric antigen receptors: CD28 outperforms combined CD28-OX40 "super-stimulation". Mol Ther 21(12):2268–2277

Imai C, Iwamoto S, Campana D (2005) Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. Blood 106(1):376–383

James SE, Greenberg PD, Jensen MC, Lin Y, Wang J, Till BG, Raubitschek AA, Forman SJ, Press OW (2008) Antigen sensitivity of CD22-specific chimeric TCR is modulated by target epitope distance from the cell membrane. J Immunol 180(10):7028–7038

Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, June CH (2011) T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. Sci Transl Med 3(95):95ra73

Kaneko S, Mastaglio S, Bondanza A, Ponzoni M, Sanvito F, Aldrighetti L, Radrizzani M, La Seta-Catamancio S, Provasi E, Mondino A, Nagasawa T, Fleischhauer K, Russo V, Traversari C, Ciceri F, Bordignon C, Bonini C (2009) IL-7 and IL-15 allow the generation of suicide gene-modified alloreactive self-renewing central memory human T lymphocytes. Blood 113(5): 1006–1015

Kershaw MH, Westwood JA, Parker LL, Wang G, Eshhar Z, Mavroukakis SA, White DE, Wunderlich JR, Canevari S, Rogers-Freezer L, Chen CC, Yang JC, Rosenberg SA, Hwu P (2006) A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. Clin Cancer Res 12(20 Pt 1):6106–6115

Klebanoff CA, Gattinoni L, Torabi-Parizi P, Kerstann K, Cardones AR, Finkelstein SE, Palmer DC, Antony PA, Hwang ST, Rosenberg SA, Waldmann TA, Restifo NP (2005) Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. Proc Natl Acad Sci U S A 102(27):9571–9576

Klingemann HG (2013) Cellular therapy of cancer with natural killer cells-where do we stand? Cytotherapy 15(10):1185–1194

Kruschinski A, Moosmann A, Poschke I, Norell H, Chmielewski M, Seliger B, Kiessling R, Blankenstein T, Abken H, Charo J (2008) Engineering antigen-specific primary human NK cells against HER-2 positive carcinomas. Proc Natl Acad Sci U S A 105(45): 17481–17486

Lamers CH, Sleijfer S, Vulto AG, Kruit WH, Kliffen M, Debets R, Gratama JW, Stoter G, Oosterwijk E (2006) Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. J Clin Oncol 24(13):e20–e22

Lanier LL (2008) Up on the tightrope: natural killer cell activation and inhibition. Nat Immunol 9(5):495–502

Mezzanzanica D, Canevari S, Mazzoni A, Figini M, Colnaghi MI, Waks T, Schindler DG, Eshhar Z (1998) Transfer of chimeric receptor gene made of variable regions of tumor-specific antibody confers anticarbohydrate specificity on T cells. Cancer Gene Ther 5(6):401–407

Moon EK, Carpenito C, Sun J, Wang LC, Kapoor V, Predina J, Powell DJ Jr, Riley JL, June CH, Albelda SM (2011) Expression of a functional CCR2 receptor enhances tumor localization and tumor eradication by retargeted human T cells expressing a mesothelin-specific chimeric antibody receptor. Clin Cancer Res 17(14):4719–4730

Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA (2010) Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. Mol Ther 18(4):843–851

Müller T, Uherek C, Maki G, Chow KU, Schimpf A, Klingemann HG, Tonn T, Wels WS (2008) Expression of a CD20-specific chimeric antigen receptor enhances cytotoxic activity of NK cells and overcomes NK-resistance of lymphoma and leukemia cells. Cancer Immunol Immunother 57(3):411–423

Parente-Pereira AC, Burnet J, Ellison D, Foster J, Davies DM, van der Stegen S, Burbridge S, Chiapero-Stanke L, Wilkie S, Mather S, Maher J (2011) Trafficking of CAR-engineered human T cells following regional or systemic adoptive transfer in SCID beige mice. J Clin Immunol 31(4):710–718

Park JR, Digiusto DL, Slovak M, Wright C, Naranjo A, Wagner J, Meechoovet HB, Bautista C, Chang WC, Ostberg JR, Jensen MC (2007) Adoptive transfer of chimeric antigen receptor re-directed cytolytic T lymphocyte clones in patients with neuroblastoma. Mol Ther 15(4):825–833

Porter DL, Levine BL, Kalos M, Bagg A, June CH (2011) Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N Engl J Med 365(8):725–733

Sahm C, Schönfeld K, Wels WS (2012) Expression of IL-15 in NK cells results in rapid enrichment and selective cytotoxicity of gene-modified effectors that carry a tumor-specific antigen receptor. Cancer Immunol Immunother 61(9):1451–1461

Singh H, Figliola MJ, Dawson MJ, Olivares S, Zhang L, Yang G, Maiti S, Manuri P, Senyukov V, Jena B, Kebriaei P, Champlin RE, Huls H, Cooper LJ (2013) Manufacture of clinical-grade CD19-specific T cells stably expressing chimeric antigen receptor using Sleeping Beauty system and artificial antigen presenting cells. PLoS One 8(5):e64138

Song DG, Ye Q, Carpenito C, Poussin M, Wang LP, Ji C, Figini M, June CH, Coukos G, Powell DJ Jr (2011) In vivo persistence, tumor localization, and antitumor activity of CAR-engineered T cells is enhanced by costimulatory signaling through CD137 (4-1BB). Cancer Res 71(13):4617–4627

Suhoski MM, Golovina TN, Aqui NA, Tai VC, Varela-Rohena A, Milone MC, Carroll RG, Riley JL, June CH (2007) Engineering artificial antigen-presenting cells to express a diverse array of co-stimulatory molecules. Mol Ther 15(5):981–988

Sutlu T, Nystrom S, Gilljam M, Stellan B, Applequist SE, Alici E (2012) Inhibition of intracellular antiviral defense mechanisms augments lentiviral transduction of human natural killer cells: implications for gene therapy. Hum Gene Ther 23(10):1090–1100

Tavri S, Jha P, Meier R, Henning TD, Müller T, Hostetter D, Knopp C, Johansson M, Reinhart V, Boddington S, Sista A, Wels WS, Daldrup-Link HE (2009) Optical imaging of cellular immunotherapy against prostate cancer. Mol Imaging 8(1):15–26

Till BG, Jensen MC, Wang J, Qian X, Gopal AK, Maloney DG, Lindgren CG, Lin Y, Pagel JM, Budde LE, Raubitschek A, Forman SJ, Greenberg PD, Riddell SR, Press OW (2012) CD20-specific adoptive immunotherapy for lymphoma using a chimeric antigen receptor with both CD28 and 4-1BB domains: pilot clinical trial results. Blood 119(17):3940–3950

Tonn T, Schwabe D, Klingemann HG, Becker S, Esser R, Koehl U, Suttorp M, Seifried E, Ottmann OG, Bug G (2013) Treatment of patients with advanced cancer with the natural killer cell line NK-92. Cytotherapy 15(12):1563–1570

Uherek C, Tonn T, Uherek B, Becker S, Schnierle B, Klingemann HG, Wels W (2002) Retargeting of natural killer-cell cytolytic activity to ErbB2-expressing cancer cells results in efficient and selective tumor cell destruction. Blood 100(4):1265–1273

Vera JF, Brenner LJ, Gerdemann U, Ngo MC, Sili U, Liu H, Wilson J, Dotti G, Heslop HE, Leen AM, Rooney CM (2010) Accelerated production of antigen-specific T cells for preclinical and clinical applications using gas-permeable rapid expansion cultureware (G-Rex). J Immunother 33(3):305–315

Weber J, Atkins M, Hwu P, Radvanyi L, Sznol M, Yee C (2011) White paper on adoptive cell therapy for cancer with tumor-infiltrating lymphocytes: a report of the CTEP subcommittee on adoptive cell therapy. Clin Cancer Res 17(7):1664–1673

Westwood JA, Smyth MJ, Teng MW, Moeller M, Trapani JA, Scott AM, Smyth FE, Cartwright GA, Power BE, Honemann D, Prince HM, Darcy PK, Kershaw MH (2005) Adoptive transfer of T cells modified with a humanized chimeric receptor gene inhibits growth of Lewis-Y-expressing tumors in mice. Proc Natl Acad Sci U S A 102(52):19051–19056

Zhao Y, Moon E, Carpenito C, Paulos CM, Liu X, Brennan AL, Chew A, Carroll RG, Scholler J, Levine BL, Albelda SM, June CH (2010) Multiple injections of electroporated autologous T cells expressing a chimeric antigen receptor mediate regression of human disseminated tumor. Cancer Res 70(22):9053–9061

Zhong S, Malecek K, Johnson LA, Yu Z, Vega-Saenz de Miera E, Darvishian F, McGary K, Huang K, Boyer J, Corse E, Shao Y, Rosenberg SA, Restifo NP, Osman I, Krogsgaard M (2013) T-cell receptor affinity and avidity defines antitumor response and autoimmunity in T-cell immunotherapy. Proc Natl Acad Sci U S A 110(17):6973–6978

Part III

Cancer Entities

Immunotherapy of Malignant Melanoma

Cedrik Michael Britten, G. Schuler, and S. Grabbe

Introduction

For the field of cancer immunotherapy, malignant melanoma has a very special role. It has certainly critically influenced the past and the present of the field. Research performed in malignant melanoma has started to shape the field of cancer immunotherapy more than 20 years ago with the discovery of the first tumor-associated antigens documented to be recognized by antigen-specific T cells in patients. As discussed in the following sections, the privileged role of malignant melanoma has not been restricted to the pioneering phase of initial antigen discovery but has continued to exist through more than 20 years as documented by the clinical successes reported for immunotherapies such as cytokines, vaccines, and immunomodulatory antibodies and cellular therapies. In particular the successful clinical development of ipilimumab has been the birth of immuno-oncology as a novel specialty in medicine. At present cancer immunotherapy of malignant melanoma is surely one of the most vibrant areas of medicine which is reflected by that fact that the journal "Science" named cancer immunotherapy its 2013 breakthrough of the year (Couzin-Frankel 2013). More novel concepts and improved immunotherapies have reached clinical development and may soon lead to even more approved therapies.

C.M. Britten (✉)
Immunotherapy Development Center (IDC), TRON-Translational Oncology, University Medical Center, Johannes Gutenberg University, Mainz, Germany

BioNTech RNA Pharmaceuticals, Mainz, Germany
e-mail: cedrik.britten@tron-mainz.de

G. Schuler
Department of Dermatology, University Hospital Erlangen, Erlangen, Germany

S. Grabbe
Department of Dermatology, University Medical Center, Johannes Gutenberg-University, Mainz, Germany

Tumor- and Melanoma-Associated Antigens

The basic principle behind cancer immunotherapy is based on the fact that during transformation from healthy to malignant tissues, cancer cells accumulate genetic alterations that translate into changes in their gene-expression profile that can be selectively recognized by immune cells. As discussed in detail in the first chapter of this book (chapter by H.G. Rammensee), tumor-specific B- and T-cell responses play a key role in the immunologic control of tumor cell growth. The first human T-cell epitope representing a tumor antigen was reported by the Boon group more than 20 years ago (van der Bruggen et al. 1991). This seminal study and many others to follow investigated sample specimens derived from patients with malignant melanoma rapidly led to the vision to exploit tumor-associated antigens as targets for antigen-specific immunotherapy

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_14,

for the benefit of patients (Boon 1993). Even more than 10 years later, the biggest fraction of known tumor-associated antigens shown to elicit antigen-specific T-cell responses were melanoma antigens (Boon et al. 2006). It is probably true to state that the contribution of scientists studying tumor-associated antigens and their corresponding immunological responses in malignant melanoma laid the fundament of the field of cancer immunotherapy as we know it today. Numerous immunotherapeutic approaches have been explored which include both local and systemic therapies using either nonspecific immunoactivators and/or treatments that aim to stimulate a specific immune response directed against melanoma antigens. A summary of these approaches is given in Table 1 and will be further discussed in this chapter.

Table 1 Melanoma immunotherapy approaches that have been evaluated in clinical trials and/or are being used in clinical practice

Locoregional tumor immunotherapy strategies
BCG
IL-2
L19-IL-2
IL-12 (plasmid electroporation)
Imiquimod or other topical TLR ligands
DPCP or other contact sensitizers
Nonspecific immunoactivating therapy strategies
Cytokines
IL-2
IFNα
IL-12
"Immune checkpoint inhibitors"
Anti-CTLA-4 – ipilimumab, tremelimumab
Anti-PD-1 – nivolumab, lambrolizumab
Anti-PD-L1 – BMS-936559
Costimulatory molecules
CD40L
CD137
Immune adjuvants
CpG7909
Montanide ISA-51
Poly-ICLC (Hiltonol®)
"Immune conditioning"
Hypofractionated radiotherapy
"Immunogenic" chemotherapy
Oncolytic viruses, e.g., talimogene laherparepvec
Tumor-specific immunotherapies
Melanoma vaccines
Tumor lysates
Short/long peptides or proteins with or without adjuvant with or without cytokines such as IL-2, GM-CSF, IL-12
RNA-based vaccines
DNA- or plasmid-based vaccines
Individualized mutanome vaccines
DC-based vaccines, e.g., DC loaded with tumor lysate, peptides, or RNA
Adoptive T-cell transfer
Tumor-infiltrating lymphocytes
Immune receptor-engineered lymphocytes
Antibodies to melanoma-specific cell surface antigens

IL-2 and IFNα: First Steps Toward Immunologic Control of Malignant Melanoma

The first evidence that melanoma growth might be controlled by the immune system came from the histological demonstration that many primary melanomas are infiltrated with immune cells and that the degree of immune infiltration appeared to correlate with prognosis (Griewank et al. 2013). Moreover, numerous anecdotal case reports described "spontaneous" regression of metastatic lesions in melanoma, a phenomenon that is less frequently seen in other tumors (Bramhall et al. 2013). Thus, melanoma has long been used as a model tumor for immunotherapeutic approaches in preclinical and animal studies, and countless publications using many different kinds of immunotherapies demonstrated their often profound effectiveness for the treatment of murine melanoma. However, clinically relevant immunotherapeutic applications in man remained limited for many years. The first immunotherapeutic agents that entered the clinic were the cytokines IL-2 and IFNα. A series of phase 2 clinical trials performed in the 1990s consistently demonstrated that approx. 10–15 % of melanoma patients exhibited profound and long-lasting tumor regressions after high-dose IL-2 treatment, resulting in the FDA approval of this therapeutic regimen for metastatic melanoma in the USA. Due to potentially severe side effects that may require hospitalization in intensive care units, this treatment is reserved for patients with a healthy cardiovascular system and

a good ECOG performance status. Although high-dose IL-2 therapy was never approved in Europe, many thousand patients have now been treated in the USA, with consistent response rates of 10–15 % and often long-lasting complete responses in some patients (Gogas et al. 2013; Dillman et al. 2012).

The second cytokine that was introduced into clinical practice in melanoma was IFNα. Although no significant efficacy could be demonstrated in stage IV (systemically metastatic) melanoma, a series of ECOG trials led by J. Kirkwood demonstrated that high-dose IFNα treatment led to a significant prolongation of progression-free survival in patients with regional metastases treated in an adjuvant setting (Davar et al. 2013). However, a consistent benefit on overall survival could not be demonstrated. Subsequent trials mostly performed in Europe demonstrated that low-dose IFNα also prolonged progression-free survival but not overall survival in patients with stage II (high-risk primary melanoma, but free of metastases) and stage III (regional metastases) melanoma (Ascierto et al. 2013). Several subsequent meta-analyses calculated a protective effect of IFNα on local recurrence with a hazard ratio of 0.83 and an overall survival benefit with a hazard ratio of 0.91 (Mocellin et al. 2013). As for IL-2, the side effects of systemic immune activation also limit the quality of life of IFNα-treated patients, who often suffer from flu-like symptoms and chronic fatigue as a result of therapy.

In summary, both agents are available for clinical application, but their widespread application is limited by only marginal effectiveness and significant side effects as well as high costs. Thus, recommendations as to the use of these agents in clinical guidelines differ between countries (Pflugfelder et al. 2013; Garbe et al. 2012; Fong and Tanabe 2013), and experts in the field differ in their opinion whether to recommend the use of these agents to patients in general practice.

Local (Intratumoral) Immunotherapy

Localized tumor immunotherapy strategies that are based upon the injection of an immunotherapeutic agent into melanoma metastases or the topical application of an immunoactivator to the skin in the area of cutaneous metastases have been investigated for more than 50 years. Whereas clinical responses to BCG have remained anecdotal (Krone et al. 2005), other locally administered immunotherapeutic agents have been shown to induce tumor regressions in a high percentage of treated metastases, although mostly in small and/or nonrandomized clinical trials. Imiquimod has been reported to be effective for the treatment of lentigo maligna and intracutaneous melanoma metastases (Moon and Spencer 2013; Steinmann et al. 2000). Likewise, topical contact sensitizers such as diphenylcyclopropenone (DPCP) have been reported to induce regression of cutaneous melanoma lesions. When used in combination with the systemic chemotherapeutic agent, DTIC, systemic tumor regressions have also been reported (Damian et al. 2009; Trefzer and Sterry 2005). In addition, intralesional injection of rose bengal has been shown to induce a systemic tumor-specific immune response in murine models of melanoma and in melanoma patients, resulting in tumor cell necrosis and a profound immune infiltration of treated lesions (Toomey et al. 2013; Thompson et al. 2008).

As a more defined therapeutic approach, Weide et al. demonstrated a profound clinical effectivity of locally administered IL-2, with regression of the majority of injected metastatic lesions but no effect on systemic tumor immunity (Weide et al. 2011). Moreover, early clinical trials using L19-IL-2, a tumor-targeting immunocytokine composed of recombinant human IL-2 and the human antibody fragment L19 (specific to a splice domain of fibronectin that is specifically expressed in tumor neo-vasculature), also show promising results (Eigentler et al. 2011; Johannsen et al. 2010). In addition, an IL-12 encoding plasmid also showed clinical efficacy and is being pursued in clinical trials (Cha and Daud 2012; Heinzerling et al. 2005).

Thus, intralesional immunotherapies in general exhibit a surprisingly good clinical efficacy, but often fail to induce systemic tumor immunity. More recently, however, a large phase 3 clinical trial with talimogene laherparepvec, an oncolytic viral vector encoding GM-CSF, has produced

statistically significant clinical efficacy not only for local tumor control of injected lesions but also for systemic lesion control, albeit to a lesser extent (see below), and may thus be a very promising novel therapeutic modality that may soon enter clinical practice.

Checkpoint Inhibitors

The discovery of surface molecules on T cells that either are required for full T-cell activation ("costimulatory molecules") or can downregulate their activation status ("immune checkpoint inhibitors," "coinhibitory molecules") opened the option to control T-cell activation in general by using therapeutic inhibitors or stimulators of these molecules. It was shown that anti-CTLA-4 strongly enhances the primary activation of naïve T cells (Chambers et al. 2001). However, it became evident that besides potentiation of primary T-cell activation, a major mechanism of action of anti-CTLA-4 is that it reactivates T cells that were either "exhausted" or whose activity was suppressed by regulatory T cells (Simpson et al. 2013; Wolchok et al. 2013a). Studies in mice showed that antibodies to CTLA-4 greatly enhanced their capacity to control the growth of melanoma (van Elsas et al. 1999). These results led to the clinical development of therapeutic antibodies against CTLA-4 (ipilimumab by Medarex and subsequently BMS, and of tremelimumab by Pfizer). In 2002 it was first reported that melanoma patients may respond to anti-CTLA-4 therapy (Phan et al. 2003), but it lasted to 2010 until two large phase 3 clinical trials demonstrated a statistically significant survival benefit in approx. 20–25 % of patients treated with ipilimumab (Hodi et al. 2010; Robert et al. 2011).

These trials were the first in history to show a clinical efficacy in terms of a statistically significant overall survival benefit for large patient cohorts with metastatic melanoma. Thus, after decades of negative clinical trial outcomes and an emerging general skepticism about the principal applicability of immunotherapy for human cancers, the demonstration of the clinical effectiveness of ipilimumab and the subsequent approval of this drug for clinical use in the USA and in Europe constitutes the birthdate of immuno-oncology as a treatment option for routine clinical care.

Indeed, two important aspects of tumor biology were elucidated by these studies, since the effectiveness of CTLA-4 blockade demonstrated that (1) natural T-cell-mediated immune responses against melanoma occur spontaneously in many patients and (2) these spontaneous tumor immune responses are often actively shut down in advanced tumor disease. However, during the introduction of ipilimumab into the standard clinical care, it also became evident that a general, unrestricted activation of "exhausted" or actively suppressed T cells also bears the risk of unwanted immune activation, resulting in autoimmune side effects. These side effects occur in approx. 30 % of treated patients and consist of rashes, colitis, autoimmune liver toxicity, and inflammations in various endocrine glands (e.g., thyroiditis, hypophysitis) (Voskens et al. 2013). Especially colitis and hepatitis can be severe, have resulted in fatalities, and may lead to termination of treatment. The full spectrum of side effects is only now becoming clear after approval and more widespread use (Voskens et al. 2013). Thus, the overall risk-benefit ratio of ipilimumab is limited by these side effects, and despite the general enthusiasm about this novel therapeutic option, it needs to be mentioned that due to moderate response rates (only one in five patients will respond) and potentially severe side effects (one in three patients will experience autoimmune events of some degree) the drug is far from being the ideal treatment for metastatic melanoma.

However, more options are within close reach. Besides other antibodies against CTLA-4, antibodies against PD-1, another inhibitory molecule expressed on activated T cells, and against its ligand, PD-1 L, are being developed by several companies, and some of these have reached phase 3 of clinical development (Topalian et al. 2012; Hamid et al. 2013). Especially the antibodies against PD-1, nivolumab and lambrolizumab, have generated very promising early clinical results (Topalian et al. 2012; Hamid et al. 2013),

with superior efficacy (response rates of 30 % and more) and less severe side effects as ipilimumab. This may be due to an additional mechanism of action of PD-1 blocking agents, since its ligand PD-1 L is often expressed by tumor cells and may thus constitute an intrinsic mechanism of T-cell inactivation within solid tumors.

The development of additional checkpoint inhibitors and other immunotherapeutic agents now for the first time also offers the possibility to combine different kinds of immunotherapeutic regimens. In this regard, Wolchok et al. reported recently that a combination of ipilimumab and nivolumab resulted in an objective response rate of 40 % (53 % at the highest dose) with tumor reduction of 80 % or more in metastatic melanoma patients (Wolchok et al. 2013b) – response rates that were never seen before in melanoma patients or in any tumor treated with immunotherapy albeit this was at the expense of serious treatment-related adverse events in 53 % of the patients. Many exciting clinical trials that further explore the clinical efficacy of combined immunotherapy approaches are currently underway or are being planned for the near future.

Interestingly, another highly effective, but primarily nonimmunotherapeutic, strategy is being pursued in melanoma in parallel to the development of immunotherapy. Since it has been discovered that small molecule inhibitors of mutated tyrosine kinases (BRAF, MEK) are potently able to block tumor growth in patients that harbor this particular mutation, several of these substances are being developed for treatment of advanced melanoma. Among these, the inhibitors of V600-mutated BRAF, vemurafenib and dabrafenib, as well as MEK inhibitors, such as trametinib, are already approved for clinical use. Although these substances are highly effective with response rates of up to 80 % and more, their use is limited by the necessity of the respective mutation in the individual patients tumor and by the fact that multiple resistance mechanisms exist that result in tumor escape often within months after treatment onset (Luke and Hodi 2013) which is a common feature of most oncogene-targeted therapies for cancer. Despite the frequent escape, improved overall survival can be observed in patients with advanced melanoma (Chapman et al. 2011), but durable tumor responses have so far only been observed upon tumor immunotherapy strategies such as ipilimumab (Hodi et al. 2010). The chance to combine both treatment approaches could merge the benefits of high response rates obtainable with targeted therapies and durable response rates with immunotherapies and forms the scientific basis for the combination of vemurafenib or dabrafenib with immunomodulatory compounds. In addition preclinical and clinical data suggest that use of BRAF inhibitors in vitro or in vivo (1) results in no detectable negative impact on existing systemic immunity or the de novo generation of tumor-specific T cells in patients (Hong et al. 2012), (2) is associated with enhanced melanoma antigen expression and a more favorable tumor microenvironment in patients with metastatic melanoma (Frederick et al. 2013), (3) may lead to a dramatic increase in the number of tumor-infiltrating lymphocytes in biopsy samples taken from patients before and early during treatment (Wilmott et al. 2012), (4) may restore compromised DC function (Ott et al. 2013), and (5) increases tumor infiltration by T cells and enhances the antitumor activity of adoptive immunotherapy in mice (Liu et al. 2013). As an interesting anecdotal case from our own institution, we noted that a patient that received vemurafenib for only 7 days and terminated treatment due to side effects experienced a slow but continuous tumor regression over the subsequent months without any further treatment, resulting in a complete tumor regression. Taken together, these results suggest that the combination of immunotherapy with BRAF inhibitors will address two complementary modes of action and bears a huge high synergistic potential. In 2011 a first clinical trial combining vemurafenib with ipilimumab has been initiated in patients with malignant melanoma (NCT01400451). Furthermore, two additional phase 1 clinical trials have been recently initiated combining dabrafenib with ipilimumab (NCT01767454 and NCT01940809). It needs to be mentioned that one trial combining ipilimumab with vemurafenib had to be stopped due to high hepatotoxicity (Ribas et al. 2013).

Along these lines, it has been reported that certain modes of radiotherapy may have inflammatory and immunopotentiating effects that can lead to tumor regression also at tumor sites that were not irradiated ("abscopal effect") (Teulings et al. 2013; Postow et al. 2012). Several current clinical trials explore this option further (see Table 2). In addition, Kroemer and Zitvogel have reported that some, but not all, chemotherapeutic agents cause an "inflammatory tumor destruction," resulting in the activation of various signaling pathways that lead to inflammasome activation and the release of inflammatory cytokines and endogenous danger signals activating dendritic cells (Ma et al. 2013; Zitvogel et al. 2013) which is also discussed in greater detail in the chapter J.M. Pitt et al. in this book. Thus, due to the plethora of novel substances, the rapid evolution of new treatment standards, and a multitude of potentially synergistic treatment combinations, the design of clinical trials in melanoma will become both exciting and difficult.

Tumor Vaccines

Although immunotherapies that unleash preexisting spontaneous T-cell immunity and thus are not specific for the tumor or a tumor antigen are currently the only substances with proven clinical efficacy and are thus approved for therapy, it appears logical that a truly tumor-selective form of immunotherapy would require some form of vaccination and the use of a specific tumor antigen. Due to the first discovery of tumor-specific antigens in melanoma by van der Bruggen in 1991 (van der Bruggen et al. 1991), numerous therapeutic approaches aimed at vaccinating patients with peptides, whole proteins, DNA, or RNA encoding these tumor antigens (see other chapters in this book). More recent developments aim at using multipeptide vaccines instead of single tumor antigen peptides (Slingluff et al. 2007) or aim for the identification of mutated tumor antigens in each individual patient and the generation of patient-individualized vaccines (see below). However, the majority of clinical trials in this field so far revealed disappointing therapeutic results and only few trials reached statistical significance in their clinical endpoints. As one of the few trials in this area, a recent phase 2 study that combined a gp100 peptide vaccine with systemic IL-2 treatment generated statistically significant responses (Schwartzentruber et al. 2011). In contrast, a large phase 3 adjuvant study with Mage-A3 peptide vaccination in stage III melanoma yielded no significant protective effect although it remains to be seen whether a prolongation of DFS occurs in the gene signature positive subpopulation defined as the second co-primary endpoint (Gajewski et al. 2010). Nevertheless, multiple current pathways are being explored to exploit single or multiple immunogenic epitopes from known tumor antigens in combination with immunostimulatory adjuvants or other immunotherapeutic agents such as ipilimumab for their clinical efficacy in various stages of melanoma.

Other modes of antigen-specific immunotherapy include the use of tumor antigen-exposed dendritic cells (DC). Since the first discovery that adoptively transferred autologous DC are able to effectively present tumor antigen in murine models of melanoma (Grabbe et al. 1991; Nestle et al. 1998), the understanding of DC physiology has improved significantly, and it became increasingly clear not only that several subpopulations of these cells with often entirely different biological capacities exist but that these cells also can acquire various functional states (Steinman 2012; Grabbe et al. 2000). A phase 3 clinical trial using cytokine-matured DC pulsed with a cocktail of melanoma peptides yielded disappointing clinical results (Schadendorf et al. 2006) possibly also due to a suboptimal quality and number of injected DC. It became clear that multiple variables need to be tested to optimize DC-based immunotherapy in clinical trials. Due to enhanced regulatory requirements and high costs, it is becoming increasingly difficult to further pursue this therapeutic option. Nevertheless, promising results of several laboratories (Schuler 2010; Aarntzen et al. 2012; Wilgenhof et al. 2013; Dannull et al. 2013) and ongoing efforts by many

Table 2 Ongoing clinical trials for immunotherapy of melanoma in 2013

NCT number	Study	Sponsor	Phase
NCT01703754	DNA-based vaccine encoding IL-12 (Ad-RTS-IL-12), combined with the oral activator, veledimex	ZIOPHARM	Phase 2
NCT01753089	Subcutaneous implantation of a DC-activating scaffold that contains GM-CSF and a melanoma antigen (WDVAX)	Dana-Farber Cancer Center	Phase 1
NCT01973322	Autologous DC loaded with autologous tumor lysate or homogenate, combined with hypofractionated radiotherapy and/or IFNα and/or leukapheresis	Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori	Phase 2
NCT00116597	Recombinant vaccinia virus (replication inactivated) expressing 3 melanoma-associated antigens and 2 costimulatory molecules	University Hospital, Basel	Phase 1/2
NCT00243529	Peptide-pulsed vs. RNA-transfected plasmacytoid DC vaccine	Radboud University	Phase 1/2
NCT00429312	Recombinant vaccinia virus (JX-594 = Pexa-Vec)	Jennerex Biotherapeutics	Phase 1/2
NCT00477906	Hapten-modified autologous melanoma cells (M-Vax)	AVAX Technologies	Phase 3
NCT00610311	Canarypox vector (ALVAC)-encoded gp100 combined with IL-2	National Cancer Institute	Phase 2
NCT00672542	DC transfected with tumor antigen RNA (encoding MART-1, tyrosinase, gp100, and MAGE-3) and siRNA (targeting the inducible immunoproteasome beta subunits LMP2, LMP7, and MECL1)	Duke University	Phase 1
NCT00722098	Tumor antigen pulsed DC vaccine plus systemic cyclophosphamide	Baylor Research Institute	Phase 2
NCT00769704	Intratumoral injection of the oncolytic virus talimogene laherparepvec compared to GM-CSF (T-VEC)	Amgen	Phase 3
NCT00948961	An anti-DEC205 linked to the peptide melanoma antigen NY-ESO-1, applied together with resiquimod or poly-ICLC (CDX-1401)	Celldex	Phase 1/2
NCT00961844	DC transfected with hTERT-, survivin-, and tumor cell-derived mRNA, combined with *ex vivo* T-cell expansion and reinfusion	Oslo University Hospital	Phase 1/2
NCT01008527	Poly-ICLC and NY-ESO-1/gp100 (Oncovir)	H. Lee Moffitt Cancer Center and Research Institute	Phase 1
NCT01029873	Interleukin-2/T-cell receptor fusion protein targeting p53 (aa264-272)/HLA-A*0201 (ALT-801)	Altor BioScience Corporation	Phase 1/2
NCT01137006	Anti-TRP-1 monoclonal antibody IMC-20D7S	ImClone	Phase 1
NCT01138410	DNA-based vaccine that encodes a melanoma antigen (TRP2) and a modified monoclonal antibody with T-cell mimotopes expressed within the CDR regions of the antibodies (SCIB1)	Scancell	Phase 1/2
NCT01176461	Multiple class I peptides applied together with adjuvant (montanide ISA 51) plus the anti-PD-1 antibody (BMS-936558)	H. Lee Moffitt Cancer Center and Research Institute	Phase 1

(continued)

Table 2 (continued)

NCT number	Study	Sponsor	Phase
NCT01213472	Multipeptide melanoma vaccine plus adjuvant as an antigen-specific cancer immunotherapeutic (2241658A)	GlaxoSmithKline	Phase 1
NCT01295827	Anti-PD-L1 monoclonal antibody (MK-3475)	Merck Sharp & Dohme	Phase 1
NCT01397708	Adenoviral-transduced hIL-12-expressing autologous DC (INXN-3001) and subsequent oral administration of an activator ligand (INXN-1001)	ZIOPHARM	Phase 1/2
NCT01400451	Ipilimumab-vemurafenib combo	Bristol-Myers Squibb	Phase 1
NCT01435369	Monoclonal antibody against PD-1 (CT-011)	CureTech	Phase 2
NCT01435499	Melanoma vaccine with or without cyclophosphamide (GVAX)	Sidney Kimmel Comprehensive Cancer Center	Phase 1
NCT01437605	recMAGE-A3 + AS15 ASCI with or without poly-ICLC	H. Lee Moffitt Cancer Center and Research Institute	Phase 2
NCT01497808	RADVAX: stereotactic body radiotherapy followed by ipilimumab	Abramson Cancer Center of the University of Pennsylvania	Phase 1/2
NCT01585350	Multipeptide vaccine, applied together with adjuvant (montanide ISA-51 or LPS or poly-ICLC), with topical imiquimod, or with systemic IFN (MELITAC 12.1)	University of Virginia	Phase 1
NCT01654692	Ipilimumab combined with fotemustine	Italian Network for Tumor Biotherapy	Phase 2
NCT01684241	Intranodal administration of an RNA-based cancer vaccine with RBL001/RBL002 targeting two tumor-associated antigens	Ribological	Phase 1
NCT01701674	Ipilimumab with lymphodepletion plus adoptive cell transfer and high-dose IL-2	H. Lee Moffitt Cancer Center and Research Institute	Phase 1
NCT01721746	BMS-936558 to the physician's choice of either dacarbazine or carboplatin and paclitaxel in advanced melanoma patients that have progressed following anti-CTLA-4 therapy	Bristol-Myers Squibb	Phase 3
NCT01723813	MAGE-A3 peptide vaccinations combined with the galectin 3 inhibitor, GM-CT-01	Cliniques universitaires Saint-Luc- Université Catholique de Louvain	Phase 1/2
NCT01729663	Allogeneic, irradiated tumor cell vaccine with BCG and GM-CSF (CSF-470)	Laboratorio Pablo Cassará S.R.L.	Phase 2/3
NCT01740297	Ipilimumab combined with talimogene laherparepvec (T-VEC)	Amgen	Phase 1/2
NCT01740401	Ipilimumab plus low-dose cyclophosphamide	New York University Langone Clinical Cancer Center	Phase 2
NCT01744171	Vaccine therapy (hsp110-gp100 chaperon) in treating patients with advanced stage III–IV melanoma	Roswell Park Cancer Institute	Phase 1
NCT01767454	Dabrafenib ± trametinib in combination with ipilimumab	GlaxoSmithKline	Phase 1
NCT01810016	Ipilimumab combined with a NY-ESO-1 peptide vaccine	Ludwig Institute for Cancer Research	Phase 1
NCT01940809	Ipilimumab with or without dabrafenib, and/or trametinib	National Cancer Institute (NCI)	Phase 1

NCT01946789	IL-15 superagonist (ALT-803)	Altor BioScience Corporation	Phase 1/2
NCT01961115	Orally available IDO inhibitor that targets IDO1 (INCB024360) with the MELITAC 12.1 peptide vaccine (a mixture of 12 class I MHC-restricted melanoma peptides and a class II MHC-restricted tetanus toxoid helper peptide)	Fred Hutchinson Cancer Research Center	Phase 2
NCT01983748	Dendritic cells plus autologous tumor RNA	University Hospital Erlangen	Phase 3
NCT01984242	Anti-PD-L1 as monotherapy (MPDL3280A) or in combination with Avastin (bevacizumab) vs. sunitinib	Hoffmann-La Roche	Phase 2
NCT02035956	Personalized vaccination with IVAC mutanome vaccine with or without initial treatment with RBL001/RBL002	Ribological	Phase 1

Non-comprehensive list retrieved from clinicaltrials.gov

laboratories worldwide indicate that this approach is still actively pursued and may eventually arrive at the point of reliable clinical effectiveness. In this respect, the use of several antigen-encoding RNAs or whole-tumor RNA as a source of tumor antigen(s) seems especially intriguing (Schaft et al. 2005; Gilboa and Vieweg 2004), and optimized modes of DC activation ("tri-mix-DC" (Wilgenhof et al. 2013)) or the use of specific subtypes of DC, such as plasmacytoid DC (Aarntzen et al. 2012), also provides promising approaches.

In addition to employing DC generated *ex vivo* (mostly from precursors in the peripheral blood), several laboratories worldwide aim at the selective targeting of DC in vivo. This requires the use of multifunctional agents that consist of a DC-targeting molecule (e.g., an antibody against a DC-specific surface molecule such as DEC205 or CLEC9a), a tumor antigen, and a DC-activating agent (e.g., a TLR ligand) (Birkholz et al. 2010; Lahoud et al. 2011; Sancho et al. 2008). An anti-DEC205 antibody that is linked to a melanoma antigen is currently in clinical trials (Tsuji et al. 2011); other substances, such as nanoparticle-based multifunctional vaccines that harbor DC-targeting molecules, tumor antigens, and TLR ligands, are in preclinical stages of development (Paulis et al. 2013).

Adoptive T-Cell Transfer

Besides active immunotherapy (vaccines), passive transfer of immune effector cells or tumor-targeting antibodies are other strategies for effective melanoma immunotherapy that are being explored. Results from the Rosenberg group employing *ex vivo* generated tumor-infiltrating lymphocytes (TILs) that are expanded in vitro and administered together with high-dose IL-2 showed very promising clinical effectivity. Especially when combined with a lymphodepleting chemo- and radiotherapy, more than 70 % of treated melanoma patients develop partial or complete tumor regression, with 50 % of them being durable (Rosenberg et al. 2011). Due to the need of very costly and complex laboratory infrastructure, this mode of therapy is currently restricted to a handful of laboratories worldwide, and it is frequently criticized that only highly preselected patients enter these clinical trials. Nevertheless, the response rates of this therapeutic regimen are impressive by any standard, and current improvements in the culture of TILs such as the use of "young TILs" may lead to a more general applicability of this treatment modality in the future (Dudley et al. 2013). A more detailed review of this matter, including the use of TCR-transfected T cells is provided in the chapter by D. Schendel et al. in this book.

Oncolytic Viruses

New treatment options for cancer in general and malignant melanoma in particular may arise from the use of oncolytic viruses (OVs) as also reviewed in the chapter by M.D. Mühlebach et al. in this book. The basic concept of OV is based on the preferred replication and spread of viruses in tumor cells to achieve selective killing of infected tumors. In addition experiments performed in animal models leave no doubt that the activation of antitumor immune responses is yet another important component of the therapeutic benefit of OVs (Wongthida et al. 2011). The application of talimogene laherparepvec (T-VEC) which is an oncolytic herpes simplex virus type 1 encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) for direct injection into melanoma lesions resulted in a 28 % objective response rate in a phase 2 clinical trial. Notably, responding patients demonstrated regression of both injected and noninjected lesions which is in line with the postulated dual mechanism of action that includes both a direct oncolytic effect and a secondary immune-mediated antitumor effect. Based on these preliminary results, a prospective, randomized phase 3 clinical trial in patients with unresectable stage IIIb or c and stage IV melanoma has been initiated (Kaufman and Bines 2010) in 2009. As reported at ASCO 2013, the trial has completed the enrollment of 439 patients, and a planned interim analysis showed a trend toward improved OS. Not far from now, we will

know whether OV therapy will become available as yet another novel treatment option for malignant melanoma. Novel concepts to rationally enhance the ability of OVs to trigger localized inflammatory reactions and to initiate tumor-specific immunity are in development which will surely lead to further clinical trials for more engineered OVs that are also named oncovaccines (Auer and Bell 2012).

Personalized Mutanome Vaccines

All tumors harbor dozens to hundreds of mutations (Vogelstein et al. 2013). The new era of whole-exome and whole-genome sequencing has also paved the way for an in-depth understanding of the genomic landscape of malignant melanomas that, compared to other tumor types, harbor a larger number of genomic changes (Walia et al. 2012). The high number of mutations found in melanomas may indeed translate into an increased number of immunogenic peptide epitopes that contain mutated sequences expressed on the surface of melanoma cells that may not only constitute true neo-epitopes but also give rise to effective antitumoral T-cell responses. The first evidence for the role of mutation-specific T cells in melanoma was obtained in a lab in Mainz about 20 years ago when a p16INK4a-insensitive CDK4 mutant was shown to be targeted by cytolytic T lymphocytes in human melanoma (Wolfel et al. 1995). More systematic analysis performed in T. Wölfel's lab indicated that the response of autologous T cells to a human melanoma may even be dominated by mutated neoantigens (Lennerz et al. 2005). More evidence for the huge potential of mutation-specific T cells in the context of the adoptive transfer of *ex vivo* cultivated tumor-infiltrating lymphocytes that exert sustainable antitumor responses in a high fraction of melanoma patients was recently generated by the Rosenberg group. The immunologic fine dissection of tumor-infiltrating lymphocytes that were prepared for adoptive transfer into melanoma patients revealed the presence of T cells specifically recognizing patient-specific tumor mutations (Robbins et al. 2013). In addition, the Ton Schumacher's group recently reported that the peripheral blood of a melanoma patient responding to ipilimumab showed a strong induction of mutation-specific T cells (van Rooij et al. 2013). As discussed in the chapter by M. van Buuren in this book, these promising results have meanwhile been confirmed in additional patients that responded to treatment with ipilimumab. Given the high rate of mutations in malignant melanoma, it seems straightforward to exploit vaccine approaches targeting immunogenic mutations in malignant melanoma. Recently, the preclinical proof of concept that mutanome vaccines can elicit effective antitumoral immunity has been reached in mice (Castle et al. 2012). As reviewed in the book chapter authored by the CIMT Regulatory Research Group, a regulatory pathway for personalized vaccines targeting multiple unique patient-specific tumor mutations in cancer patients has now been established (Britten et al. 2013). Based on these findings the first two melanoma patients that will soon be treated with personalized mutanome vaccines have been recruited in Mainz.

Concluding Remarks

Melanoma has always been the prototype of a tumor that is under the control of the immune system, and after decades of disappointing immunotherapy trials, due to the advent of immune checkpoint inhibitors, immunotherapy is now clearly established in every day practice of metastatic melanoma, yielding impressive durable complete responses in an increasing percentage of patients. A summary of the current status of clinical development of various immunotherapies in melanoma is given in Fig. 1. Thus, proof of principle that immunotherapy can be an effective mode of tumor treatment has been reached. Although melanoma is currently the only tumor in which this therapeutic regimen has been successful, many others will probably follow shortly. One of the most significant lessons learned in tumor immunotherapy is that the inactivation of spontaneously occurring T-cell-mediated tumor immunity is a frequent and dominant effect in the

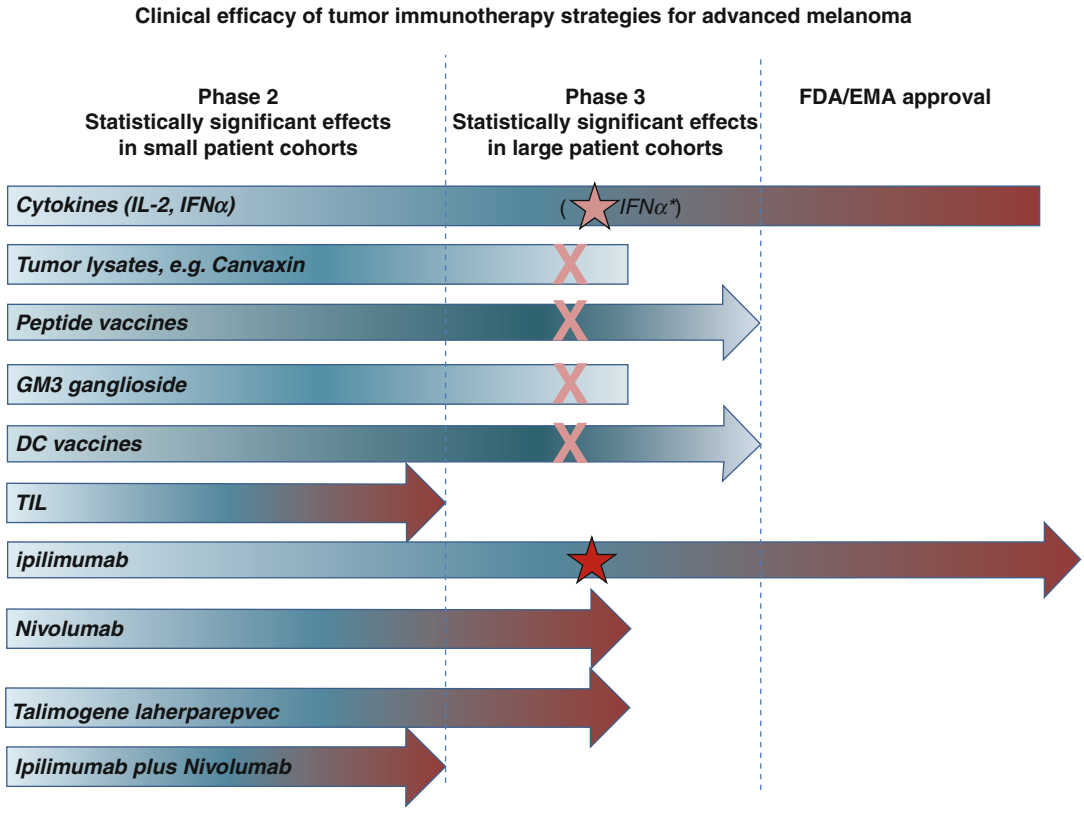

Fig. 1 Summary of the current status of clinical development of various immunotherapies in melanoma

course of tumor progression. Due to the availability of clinically active inhibitors of immune inactivation, specific vaccination approaches may gain clinical efficacy by combined treatment with checkpoint inhibitors, thus opening the perspective for even more effective, yet less toxic, immunotherapies in the future.

Acknowledgment The authors are indebted to Christoph Huber for his constant medical and scientific advice, support, mentorship, and friendship.

References

Aarntzen EH, Schreibelt G, Bol K, Lesterhuis WJ, Croockewit AJ, de Wilt JH, van Rossum MM, Blokx WA, Jacobs JF, Duiveman-de BT, Schuurhuis DH, Mus R, Thielemans K, de Vries IJ, Figdor CG, Punt CJ, Adema GJ (2012) Vaccination with mRNA-electroporated dendritic cells induces robust tumor antigen-specific CD4+ and CD8+ T cells responses in stage III and IV melanoma patients. Clin Cancer Res 18:5460–5470

Ascierto PA, Gogas HJ, Grob JJ, Algarra SM, Mohr P, Hansson J, Hauschild A (2013) Adjuvant interferon alfa in malignant melanoma: an interdisciplinary and multinational expert review. Crit Rev Oncol Hematol 85:149–161

Auer R, Bell JC (2012) Oncolytic viruses: smart therapeutics for smart cancers. Future Oncol 8:1–4

Birkholz K, Schwenkert M, Kellner C, Gross S, Fey G, Schuler-Thurner B, Schuler G, Schaft N, Dorrie J (2010) Targeting of DEC-205 on human dendritic cells results in efficient MHC class II-restricted antigen presentation. Blood 116:2277–2285

Boon T (1993) Tumor antigens recognized by cytolytic T lymphocytes: present perspectives for specific immunotherapy. Int J Cancer 54:177–180

Boon T, Coulie PG, Van den Eynde BJ, van der Bruggen P (2006) Human T cell responses against melanoma. Annu Rev Immunol 24:175–208

Bramhall RJ, Mahady K, Peach AH (2013) Spontaneous regression of metastatic melanoma - Clinical evidence of the abscopal effect. Eur J Surg Oncol 40(1):34–41

Britten CM, Singh-Jasuja H, Flamion B, Hoos A, Huber C, Kallen KJ, Khleif SN, Kreiter S, Nielsen M, Rammensee HG, Sahin U, Hinz T, Kalinke U (2013) The regulatory landscape for actively personalized cancer immunotherapies. Nat Biotechnol 31:880–882

Castle JC, Kreiter S, Diekmann J, Lower M, van de Roemer N, De Graff J, Selmi A, Diken M, Boegel S, Paret C, Koslowski M, Kuhn AN, Britten CM, Huber C, Tureci O, Sahin U (2012) Exploiting the mutanome for tumor vaccination. Cancer Res 72:1081–1091

Cha E, Daud A (2012) Plasmid IL-12 electroporation in melanoma. Hum Vaccin Immunother 8:1734–1738

Chambers CA, Kuhns MS, Egen JG, Allison JP (2001) CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. Annu Rev Immunol 19:565–594

Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, Dummer R, Garbe C, Testori A, Maio M, Hogg D, Lorigan P, Lebbe C, Jouary T, Schadendorf D, Ribas A, O'Day SJ, Sosman JA, Kirkwood JM, Eggermont AM, Dreno B, Nolop K, Li J, Nelson B, Hou J, Lee RJ, Flaherty KT, McArthur GA (2011) Improved survival with vemurafenib in melanoma with BRAF V600E mutation. N Engl J Med 364:2507–2516

Couzin-Frankel J. 2013. Breakthrough of the year (2013) Cancer immunotherapy. Science 342:1432–1433

Damian DL, Shannon KF, Saw RP, Thompson JF (2009) Topical diphencyprone immunotherapy for cutaneous metastatic melanoma. Australas J Dermatol 50: 266–271

Dannull J, Haley NR, Archer G, Nair S, Boczkowski D, Harper M, De RN, Pickett N, Mosca PJ, Burchette J, Selim MA, Mitchell DA, Sampson J, Tyler DS, Pruitt SK (2013) Melanoma immunotherapy using mature DCs expressing the constitutive proteasome. J Clin Invest 123:3135–3145

Davar D, Tarhini AA, Kirkwood JM (2013) Adjuvant immunotherapy of melanoma and development of new approaches using the neoadjuvant approach. Clin Dermatol 31:237–250

Dillman RO, Barth NM, VanderMolen LA, Mahdavi K, McClure SE (2012) Should high-dose interleukin-2 still be the preferred treatment for patients with metastatic melanoma? Cancer Biother Radiopharm 27:337–343

Dudley ME, Gross CA, Somerville RP, Hong Y, Schaub NP, Rosati SF, White DE, Nathan D, Restifo NP, Steinberg SM, Wunderlich JR, Kammula US, Sherry RM, Yang JC, Phan GQ, Hughes MS, Laurencot CM, Rosenberg SA (2013) Randomized selection design trial evaluating CD8+−enriched versus unselected tumor-infiltrating lymphocytes for adoptive cell therapy for patients with melanoma. J Clin Oncol 31:2152–2159

Eigentler TK, Weide B, de Braud F, Spitaleri G, Romanini A, Pflugfelder A, Gonzalez-Iglesias R, Tasciotti A, Giovannoni L, Schwager K, Lovato V, Kaspar M, Trachsel E, Menssen HD, Neri D, Garbe C (2011) A dose-escalation and signal-generating study of the immunocytokine L19-IL2 in combination with dacarbazine for the therapy of patients with metastatic melanoma. Clin Cancer Res 17:7732–7742

Fong ZV, Tanabe KK (2013) Comparison of Melanoma Guidelines in the United States, Canada, Europe, Australia and New Zealand A critical appraisal and comprehensive review. Br J Dermatol 10

Frederick DT, Piris A, Cogdill AP, Cooper ZA, Lezcano C, Ferrone CR, Mitra D, Boni A, Newton LP, Liu C, Peng W, Sullivan RJ, Lawrence DP, Hodi FS, Overwijk WW, Lizee G, Murphy GF, Hwu P, Flaherty KT, Fisher DE, Wargo JA (2013) BRAF inhibition is associated with enhanced melanoma antigen expression and a more favorable tumor microenvironment in patients with metastatic melanoma. Clin Cancer Res 19:1225–1231

Gajewski TF, Louahed J, Brichard VG (2010) Gene signature in melanoma associated with clinical activity: a potential clue to unlock cancer immunotherapy. Cancer J 16:399–403

Garbe C, Peris K, Hauschild A, Saiag P, Middleton M, Spatz A, Grob JJ, Malvehy J, Newton-Bishop J, Stratigos A, Pehamberger H, Eggermont AM (2012) Diagnosis and treatment of melanoma. European consensus-based interdisciplinary guideline – update 2012. Eur J Cancer 48:2375–2390

Gilboa E, Vieweg J (2004) Cancer immunotherapy with mRNA-transfected dendritic cells. Immunol Rev 199:251–263

Gogas H, Polyzos A, Kirkwood J (2013) Immunotherapy for advanced melanoma: fulfilling the promise. Cancer Treat Rev 39:879–885

Grabbe S, Bruvers S, Gallo RL, Knisely TL, Nazareno R, Granstein RD (1991) Tumor antigen presentation by murine epidermal cells. J Immunol 146: 3656–3661

Grabbe S, Kampgen E, Schuler G (2000) Dendritic cells: multi-lineal and multi-functional. Immunol Today 21:431–433

Griewank KG, Ugurel S, Schadendorf D, Paschen A (2013) New developments in biomarkers for melanoma. Curr Opin Oncol 25:145–151

Hamid O, Robert C, Daud A, Hodi FS, Hwu WJ, Kefford R, Wolchok JD, Hersey P, Joseph RW, Weber JS, Dronca R, Gangadhar TC, Patnaik A, Zarour H, Joshua AM, Gergich K, Elassaiss-Schaap J, Algazi A, Mateus C, Boasberg P, Tumeh PC, Chmielowski B, Ebbinghaus SW, Li XN, Kang SP, Ribas A (2013) Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. N Engl J Med 369:134–144

Heinzerling L, Burg G, Dummer R, Maier T, Oberholzer PA, Schultz J, Elzaouk L, Pavlovic J, Moelling K (2005) Intratumoral injection of DNA encoding human interleukin 12 into patients with metastatic melanoma: clinical efficacy. Hum Gene Ther 16:35–48

Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbe C, Peschel C, Quirt I, Clark

JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ (2010) Improved Survival with Ipilimumab in Patients with Metastatic Melanoma. N Engl J Med 363:711–723

Hong DS, Vence L, Falchook G, Radvanyi LG, Liu C, Goodman V, Legos JJ, Blackman S, Scarmadio A, Kurzrock R, Lizee G, Hwu P (2012) BRAF(V600) inhibitor GSK2118436 targeted inhibition of mutant BRAF in cancer patients does not impair overall immune competency. Clin Cancer Res 18: 2326–2335

Johannsen M, Spitaleri G, Curigliano G, Roigas J, Weikert S, Kempkensteffen C, Roemer A, Kloeters C, Rogalla P, Pecher G, Miller K, Berndt A, Kosmehl H, Trachsel E, Kaspar M, Lovato V, Gonzalez-Iglesias R, Giovannoni L, Menssen HD, Neri D, de Braud F (2010) The tumour-targeting human L19-IL2 immunocytokine: preclinical safety studies, phase I clinical trial in patients with solid tumours and expansion into patients with advanced renal cell carcinoma. Eur J Cancer 46:2926–2935

Kaufman HL, Bines SD (2010) OPTIM trial: a Phase III trial of an oncolytic herpes virus encoding GM-CSF for unresectable stage III or IV melanoma. Future Oncol 6:941–949

Krone B, Kolmel KF, Henz BM, Grange JM (2005) Protection against melanoma by vaccination with Bacille Calmette-Guerin (BCG) and/or vaccinia: an epidemiology-based hypothesis on the nature of a melanoma risk factor and its immunological control. Eur J Cancer 41:104–117

Lahoud MH, Ahmet F, Kitsoulis S, Wan SS, Vremec D, Lee CN, Phipson B, Shi W, Smyth GK, Lew AM, Kato Y, Mueller SN, Davey GM, Heath WR, Shortman K, Caminschi I (2011) Targeting antigen to mouse dendritic cells via Clec9A induces potent CD4 T cell responses biased toward a follicular helper phenotype. J Immunol 187:842–850

Lennerz V, Fatho M, Gentilini C, Frye RA, Lifke A, Ferel D, Wolfel C, Huber C, Wolfel T (2005) The response of autologous T cells to a human melanoma is dominated by mutated neoantigens. Proc Natl Acad Sci U S A 102:16013–16018

Liu C, Peng W, Xu C, Lou Y, Zhang M, Wargo JA, Chen JQ, Li HS, Watowich SS, Yang Y, Tompers FD, Cooper ZA, Mbofung RM, Whittington M, Flaherty KT, Woodman SE, Davies MA, Radvanyi LG, Overwijk WW, Lizee G, Hwu P (2013) BRAF inhibition increases tumor infiltration by T cells and enhances the antitumor activity of adoptive immunotherapy in mice. Clin Cancer Res 19:393–403

Luke JJ, Hodi FS (2013) Ipilimumab, vemurafenib, dabrafenib, and trametinib: synergistic competitors in the clinical management of BRAF mutant malignant melanoma. Oncologist 18:717–725

Ma Y, Galluzzi L, Zitvogel L, Kroemer G (2013) Autophagy and cellular immune responses. Immunity 39:211–227

Mocellin S, Lens MB, Pasquali S, Pilati P, Chiarion SV (2013) Interferon alpha for the adjuvant treatment of cutaneous melanoma. Cochrane Database Syst Rev 6:CD008955. doi: 10.1002/14651858.CD008955.pub2. CD008955

Moon SD, Spencer JM (2013) Clearance of invasive melanoma with topical imiquimod. J Drugs Dermatol 12:107–108

Nestle FO, Alijagic S, Gilliet M, Sun Y, Grabbe S, Dummer R, Burg G, Schadendorf D (1998) Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med 4:328–332

Ott PA, Henry T, Baranda SJ, Frleta D, Manches O, Bogunovic D, Bhardwaj N (2013) Inhibition of both BRAF and MEK in BRAF(V600E) mutant melanoma restores compromised dendritic cell (DC) function while having differential direct effects on DC properties. Cancer Immunol Immunother 62:811–822

Paulis LE, Mandal S, Kreutz M, Figdor CG (2013) Dendritic cell-based nanovaccines for cancer immunotherapy. Curr Opin Immunol 25:389–395

Pflugfelder A, Kochs C, Blum A, Capellaro M, Czeschik C, Dettenborn T, Dill D, Dippel E, Eigentler T, Feyer P, Follmann M, Frerich B, Ganten MK, Gartner J, Gutzmer R, Hassel J, Hauschild A, Hohenberger P, Hubner J, Kaatz M, Kleeberg UR, Kolbl O, Kortmann RD, Krause-Bergmann A, Kurschat P, Leiter U, Link H, Loquai C, Loser C, Mackensen A, Meier F, Mohr P, Mohrle M, Nashan D, Reske S, Rose C, Sander C, Satzger I, Schiller M, Schlemmer HP, Strittmatter G, Sunderkotter C, Swoboda L, Trefzer U, Voltz R, Vordermark D, Weichenthal M, Werner A, Wesselmann S, Weyergraf AJ, Wick W, Garbe C, Schadendorf D (2013) Malignant melanoma S3-guideline "diagnosis, therapy and follow-up of melanoma". J Dtsch Dermatol Ges 11(Suppl 6):1–116–1–126

Phan GQ, Yang JC, Sherry RM, Hwu P, Topalian SL, Schwartzentruber DJ, Restifo NP, Haworth LR, Seipp CA, Freezer LJ, Morton KE, Mavroukakis SA, Duray PH, Steinberg SM, Allison JP, Davis TA, Rosenberg SA (2003) Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. Proc Natl Acad Sci U S A 100:8372–8377

Postow MA, Callahan MK, Barker CA, Yamada Y, Yuan J, Kitano S, Mu Z, Rasalan T, Adamow M, Ritter E, Sedrak C, Jungbluth AA, Chua R, Yang AS, Roman RA, Rosner S, Benson B, Allison JP, Lesokhin AM, Gnjatic S, Wolchok JD (2012) Immunologic correlates of the abscopal effect in a patient with melanoma. N Engl J Med 366:925–931

Ribas A, Hodi FS, Callahan M, Konto C, Wolchok J (2013) Hepatotoxicity with combination of vemurafenib and ipilimumab. N Engl J Med 368:1365–1366

Robbins PF, Lu YC, El-Gamil M, Li YF, Gross C, Gartner J, Lin JC, Teer JK, Cliften P, Tycksen E, Samuels Y, Rosenberg SA (2013) Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. Nat Med 19: 747–752

Robert C, Thomas L, Bondarenko I, O'Day S, Weber J, Garbe C, Lebbe C, Baurain JF, Testori A, Grob JJ, Davidson N, Richards J, Maio M, Hauschild A, Miller WH Jr, Gascon P, Lotem M, Harmankaya K, Ibrahim

R, Francis S, Chen TT, Humphrey R, Hoos A, Wolchok JD (2011) Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. N Engl J Med 364:2517–2526

Rosenberg SA, Yang JC, Sherry RM, Kammula US, Hughes MS, Phan GQ, Citrin DE, Restifo NP, Robbins PF, Wunderlich JR, Morton KE, Laurencot CM, Steinberg SM, White DE, Dudley ME (2011) Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. Clin Cancer Res 17:4550–4557

Sancho D, Mourao-Sa D, Joffre OP, Schulz O, Rogers NC, Pennington DJ, Carlyle JR, Reis e Sousa C (2008) Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin. J Clin Invest 118: 2098–2110

Schadendorf D, Ugurel S, Schuler-Thurner B, Nestle FO, Enk A, Brocker EB, Grabbe S, Rittgen W, Edler L, Sucker A, Zimpfer-Rechner C, Berger T, Kamarashev J, Burg G, Jonuleit H, Tuttenberg A, Becker JC, Keikavoussi P, Kampgen E, Schuler G (2006) Dacarbazine (DTIC) versus vaccination with autologous peptide-pulsed dendritic cells (DC) in first-line treatment of patients with metastatic melanoma: a randomized phase III trial of the DC study group of the DeCOG. Ann Oncol 17:563–570

Schaft N, Dorrie J, Thumann P, Beck VE, Muller I, Schultz ES, Kampgen E, Dieckmann D, Schuler G (2005) Generation of an optimized polyvalent monocyte-derived dendritic cell vaccine by transfecting defined RNAs after rather than before maturation. J Immunol 174:3087–3097

Schuler G (2010) Dendritic cells in cancer immunotherapy. Eur J Immunol 40:2123–2130

Schwartzentruber DJ, Lawson DH, Richards JM, Conry RM, Miller DM, Treisman J, Gailani F, Riley L, Conlon K, Pockaj B, Kendra KL, White RL, Gonzalez R, Kuzel TM, Curti B, Leming PD, Whitman ED, Balkissoon J, Reintgen DS, Kaufman H, Marincola FM, Merino MJ, Rosenberg SA, Choyke P, Vena D, Hwu P (2011) gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. N Engl J Med 364:2119–2127

Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, Roddie C, Henry JY, Yagita H, Wolchok JD, Peggs KS, Ravetch JV, Allison JP, Quezada SA (2013) Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. J Exp Med 210:1695–1710

Slingluff CL Jr, Petroni GR, Chianese-Bullock KA, Smolkin ME, Hibbitts S, Murphy C, Johansen N, Grosh WW, Yamshchikov GV, Neese PY, Patterson JW, Fink R, Rehm PK (2007) Immunologic and clinical outcomes of a randomized phase II trial of two multipeptide vaccines for melanoma in the adjuvant setting. Clin Cancer Res 13:6386–6395

Steinman RM (2012) Decisions about dendritic cells: past, present, and future. Annu Rev Immunol 30:1–22

Steinmann A, Funk JO, Schuler G, von den Driesch P (2000) Topical imiquimod treatment of a cutaneous melanoma metastasis. J Am Acad Dermatol 43: 555–556

Teulings HE, Tjin EP, Willemsen KJ, Krebbers G, van Noesel CJ, Kemp EH, Nieuweboer-Krobotova L, van der Veen JP, Luiten RM (2013) Radiation-induced melanoma-associated leucoderma, systemic anti-melanoma immunity and disease-free survival in a patient with advanced-stage melanoma: a case report and immunological analysis. Br J Dermatol 168: 733–738

Thompson JF, Hersey P, Wachter E (2008) Chemoablation of metastatic melanoma using intralesional Rose Bengal. Melanoma Res 18:405–411

Toomey P, Kodumudi K, Weber A, Kuhn L, Moore E, Sarnaik AA, Pilon-Thomas S (2013) Intralesional injection of rose bengal induces a systemic tumor-specific immune response in murine models of melanoma and breast cancer. PLoS One 8:e68561

Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Horn L, Drake CG, Pardoll DM, Chen L, Sharfman WH, Anders RA, Taube JM, McMiller TL, Xu H, Korman AJ, Jure-Kunkel M, Agrawal S, McDonald D, Kollia GD, Gupta A, Wigginton JM, Sznol M (2012) Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 366:2443–2454

Trefzer U, Sterry W (2005) Topical immunotherapy with diphenylcyclopropenone in combination with DTIC and radiation for cutaneous metastases of melanoma. Dermatology 211:370–371

Tsuji T, Matsuzaki J, Kelly MP, Ramakrishna V, Vitale L, He LZ, Keler T, Odunsi K, Old LJ, Ritter G, Gnjatic S (2011) Antibody-targeted NY-ESO-1 to mannose receptor or DEC-205 in vitro elicits dual human CD8+ and CD4+ T cell responses with broad antigen specificity. J Immunol 186:1218–1227

van der Bruggen P, Traversari C, Chomez P, Lurquin C, De PE, Van den Eynde B, Knuth A, Boon T (1991) A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 254: 1643–1647

van Elsas A, Hurwitz AA, Allison JP (1999) Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. J Exp Med 190:355–366

van Rooij N, van Buuren MM, Philips D, Velds A, Toebes M, Heemskerk B, van Dijk LJ, Behjati S, Hilkmann H, El AD, Nieuwland M, Stratton MR, Kerkhoven RM, Kesmir C, Haanen JB, Kvistborg P, Schumacher TN (2013) Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. J Clin Oncol 31:e439–e442

Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA Jr, Kinzler KW (2013) Cancer genome landscapes. Science 339:1546–1558

Voskens CJ, Goldinger SM, Loquai C, Robert C, Kaehler KC, Berking C, Bergmann T, Bockmeyer CL, Eigentler T, Fluck M, Garbe C, Gutzmer R, Grabbe S, Hauschild A, Hein R, Hundorfean G, Justich A, Keller U, Klein C, Mateus C, Mohr P, Paetzold S, Satzger I, Schadendorf D, Schlaeppi M, Schuler G, Schuler-Thurner B, Trefzer U, Ulrich J, Vaubel J, von Moos R, Weder P, Wilhelm T, Goppner D, Dummer R, Heinzerling LM (2013) The price of tumor control: an analysis of rare side effects of anti-CTLA-4 therapy in metastatic melanoma from the ipilimumab network. PLoS One 8:e53745

Walia V, Mu EW, Lin JC, Samuels Y (2012) Delving into somatic variation in sporadic melanoma. Pigment Cell Melanoma Res 25:155–170

Weide B, Eigentler TK, Pflugfelder A, Leiter U, Meier F, Bauer J, Schmidt D, Radny P, Pfohler C, Garbe C (2011) Survival after intratumoral interleukin-2 treatment of 72 melanoma patients and response upon the first chemotherapy during follow-up. Cancer Immunol Immunother 60:487–493

Wilgenhof S, Van Nuffel AM, Benteyn D, Corthals J, Aerts C, Heirman C, Van Riet I, Bonehill A, Thielemans K, Neyns B (2013) A phase IB study on intravenous synthetic mRNA electroporated dendritic cell immunotherapy in pretreated advanced melanoma patients. Ann Oncol 24:2686–2693

Wilmott JS, Long GV, Howle JR, Haydu LE, Sharma RN, Thompson JF, Kefford RF, Hersey P, Scolyer RA (2012) Selective BRAF inhibitors induce marked T-cell infiltration into human metastatic melanoma. Clin Cancer Res 18:1386–1394

Wolchok JD, Hodi FS, Weber JS, Allison JP, Urba WJ, Robert C, O'Day SJ, Hoos A, Humphrey R, Berman DM, Lonberg N, Korman AJ (2013a) Development of ipilimumab: a novel immunotherapeutic approach for the treatment of advanced melanoma. Ann N Y Acad Sci 1291:1–13

Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, Segal NH, Ariyan CE, Gordon RA, Reed K, Burke MM, Caldwell A, Kronenberg SA, Agunwamba BU, Zhang X, Lowy I, Inzunza HD, Feely W, Horak CE, Hong Q, Korman AJ, Wigginton JM, Gupta A, Sznol M (2013b) Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med 369:122–133

Wolfel T, Hauer M, Schneider J, Serrano M, Wolfel C, Klehmann-Hieb E, De Plaen E, Hankeln T, Meyer zum Buschenfelde KH, Beach D (1995) A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science 269:1281–1284

Wongthida P, Diaz RM, Galivo F, Kottke T, Thompson J, Melcher A, Vile R (2011) VSV oncolytic virotherapy in the B16 model depends upon intact MyD88 signaling. Mol Ther 19:150–158

Zitvogel L, Galluzzi L, Smyth MJ, Kroemer G (2013) Mechanism of action of conventional and targeted anticancer therapies: reinstating immunosurveillance. Immunity 39:74–88

Prostate Cancer Vaccines Generated with the Disruptive RNActive® Technology Follow the Path Paved by Sipuleucel-T and Prostvac-VF

Karl-Josef Kallen

Introduction

Prostate carcinoma is the most common cancer in men and the second most common cause of cancer death in the United States, while in Europe, it is the number three of diagnosed cancers after breast and colorectal carcinoma (Ferlay et al. 2013; Jemal et al. 2011). Early diagnosis offers the chance for curative interventions by surgical treatment with prostatectomy or curative radiotherapy. Yet around one third of patients experience a rise of prostate-specific antigen (PSA) following the initial curative treatment (Scher et al. 2008). This indicates recurrence of the tumor and may happen after variable lengths of time, even after many years. Patients with this so-called biochemical relapse are then treated with androgen deprivation therapy which effectively lowers serum concentrations of PSA in most patients, induces tumor regression, and relieves symptoms. However, the treatment is not durable and a rising PSA level under antihormonal treatment indicates reactivated androgen-receptor signaling and defines the inexorably fatal castrate-resistant state (Ryan et al. 2013). Tumor recurrence may be locoregional or reflect overt metastasis, but in many cases the tumor is undetectable. Patients with castrate-resistant prostate carcinoma (CRPC) often have a good performance status with either no or only mild symptoms from the cancer. However, this period of watchful waiting is of limited duration, and the onset of symptomatic diseases signals the necessity of therapeutic intervention. Ten years ago, docetaxel given every 3 weeks was established as the standard treatment for patients with symptomatic metastatic disease which prolonged median overall survival by 2.4 months (Tannock et al. 2004). In the past 3–4 years, a number of new treatment options have opened up for patients that are progressive after docetaxel treatment. These include the novel taxane derivative cabazitaxel (de Bono et al. 2010) and the second-generation antiandrogens abiraterone (de Bono et al. 2011) and enzalutamide (Scher et al. 2012). The alpha emitter radium-223 dichloride (radium-223) which selectively targets bone metastases with alpha particles was recently approved for the treatment of bone metastases in advanced prostate cancer. Importantly, though, also patients with asymptomatic or mildly symptomatic CRPC could be offered a new therapeutic option with sipuleucel-T, the first active immunotherapy approved in humans. This article will reflect on the success story of sipuleucel-T, novel cancer vaccines in development for patients with CRPC, and discuss some options to augment the efficacy of such immunotherapies by integration with other therapeutic modalities.

K.-J. Kallen, MD, PhD
CureVac GmbH,
Paul Ehrlich Str. 15, Tübingen 72076, Germany
e-mail: kl@curevac.com

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_15,

Sipuleucel-T

Sipuleucel-T is an active cellular immunotherapy (Small et al. 2006). Autologous peripheral-blood mononuclear cells (PBMCs), which include antigen-presenting cells (APCs), are cultured and activated ex vivo with PA2024, a recombinant fusion protein. PA2024 contains the prostate antigen prostatic acid phosphatase (PAP) fused to the immunostimulating granulocyte–macrophage colony-stimulating factor. PBMCs thus stimulated are then infused into patients at week 0, 2, and 4. Three phase III studies in patients with CRPC yielded essentially very similar results (Table 1). The median overall survival of patients with symptomatic or mildly asymptomatic CRPC with Gleason scores ≤7 was increased by 4.3 to 23.2 months in the cumulative analysis of the first two trials (Higano et al. 2009) and by 4.1 to 25.8 months in the pivotal IMPACT trial, leading to the approval of sipuleucel-T in 2010 (Kantoff et al. 2010a). Antibody titers against prostatic acid phosphatase exceeding threshold levels were observed in more patients treated with sipuleucel-T than placebo (28.5 % (43/151 patients) vs. 1.4 % (1/70 patients)). T-cell proliferation exceeding an index value of 5 after stimulation with PAP was also higher in sipuleucel-T than in placebo-treated patients (27.5 % (15/55 patients) vs. 8.0 % (2/25 patients)). The improved survival in patients receiving sipuleucel-T was associated with a very favorable adverse event profile that consisted mainly of grades I–II chills, fever, headache, and influenza-like illness, consistent with observations in the preceding studies (Higano et al. 2009; Small et al. 2006).

Conspicuously, the confirmed survival advantage was achieved without a difference in median progression-free survival. This raised doubts as to the validity of these results. It was argued that survival was worse in older than in younger patients in the placebo groups and that PBMCs were only reinfused in the sipuleucel-T, but not the placebo arm which might have confounded the survival analyses (Huber et al. 2012). However, in a detailed response to this critique, the study authors and an independent group of experts replied that these issues had been rigorously considered in the review process and were found either to be untenable or to result from chance (age concern) or to be unbacked by historical data and if anything to have rather favored the control arm (PBMC reinfusion) (Gulley et al. 2012; Kantoff et al. 2012). It was also pointed out that 63.7 % of placebo patients had received apc8015f (cryopreserved PBMCs stimulated with PA2024, i.e., cryopreserved sipuleucel-T) at some time after conclusion of study treatment and that there was a positive survival trend also in these patients compared to non-receivers (23.8 vs. 11.6 months), supporting the general conclusion of a sipuleucel-T-dependent survival effect (Kantoff et al. 2010a).

Immunological data obtained from consenting patients of the three sipuleucel-T phase III trials published were also suggestive of a positive immune effect on survival (Sheikh et al. 2013). Sipuleucel-T appeared to induce activation of APCs (6.5-fold increase of $CD54^+$ cells over controls) already after the first administration, which became somewhat stronger by the booster administrations (10.5-fold). Sipuleucel-T treatment appeared to elicit more PA2024- and/or PAP-specific cellular responses than placebo (sipuleucel-T 60 % (61/102) T-cell proliferation and 48 % (49/102) for IFNγ ELISPOT vs. placebo 6 % (3/51) T-cell proliferation and 13 % [7/52] for IFNγ ELISPOT). PA2024- and/or PAP-specific humoral responses were more frequent and higher in sipuleucel-T-treated patients than in placebo controls (68 % (102/151) vs. 3 % (2/70)). In a subset of immunologically evaluated patients from the IMPACT trial ($n = 156$), at least one post-baseline immune reaction to PA2024 and/or PAP correlated with improved survival (HRs 0.46–0.53) which even reached statistical significance in all cases.

Prostvac-VF

The dissociation of progression-free and overall survival was also observed with the cancer vaccine Prostvac-VF. Prostvac-VF is a viral vector cancer vaccine that encodes PSA as a target tumor antigen and a triad of three different costimulatory molecules (B7.1 (CD80), ICAM-1(CD54), LFA-3 (CD58); together designated

Table 1 Summary of pivotal trials demonstrating survival advantages in the past 10 years and recent immunotherapy trials

Drugs tested	Patients	Patient number Randomization scheme	Median radiographic PFS	Median OS	Ref.
Docetaxel q3w vs. docetaxel q1w vs. mitoxantrone	Progressive metastatic CRPC	$n=1{,}006$ 1:1:1 randomization	Not determined	18.9 vs. 16.5 months (docetaxel q3w vs. mitoxantrone) +2.4 months HR 0.76 (0.62–0.94), $p=0.009$	Tannock et al. (2004)
Docetaxel and estramustine vs. mitoxantrone	Progressive metastatic CRPC (stage D1 or D2)	$n=674$ 1:1 randomization	6.3 vs. 3.2 months +3.1 months $p<0.001$	17.5 months vs. 15.6 months +1.9 months HR 0.80 (0.67–0.97), $p=0.02$	Petrylak et al. (2004)
Cabazitaxel vs. mitoxantrone	Metastatic CRPC progressive on docetaxel	$n=755$ 1:1 randomization	2.8 vs. 1.4 months +1.4 months HR 0.74 (0.64–0.86), $p<0.0001$	15.1 months vs. 12.7 months +2.4 months HR 0.70 (0.59–0.83), $p<0.0001$	de Bono et al. (2010)
Abiraterone vs. placebo	Metastatic CRPC progressive on docetaxel	$n=1{,}195$ 2:1 randomization	Progression-free survival 5.6 months vs. 3.6 months +2.0 months $p<0.001$	14.8 vs. 10.9 months +3.9 months HR 0.65 (0.54–0.77), $p<0.001$)	de Bono et al. (2011)
Abiraterone vs. placebo	Metastatic CRPC without prior chemotherapy	$n=1{,}088$ 1:1 randomization study unblinded after planned interim analysis performed after 43 % of expected events (deaths)	16.5 vs. 8.3 +8.2 months HR[a] 0.53 (0.45–0.62), $p<0.001$	Abiraterone plus prednisone – median not reached – vs. 27.2 months for placebo plus prednisone alone HR 0.75 (0.61–0.93), $p=0.01$, predefined efficacy boundary not crossed	Ryan et al. (2013)
Enzalutamide vs. placebo	Metastatic CRPC after previous therapy with docetaxel	$n=1{,}199$ 2:1 randomization	8.3 vs. 2.9 months +5.4 months HR 0.40 (0.35–0.47), $p<0.001$	18.4 vs. 13.6 months +4.8 months HR 0.63 (0.53–0.75), $p<0.001$	Scher et al. (2012)
Radium-223 vs. placebo	Pts. refractory or ineligible to docetaxel or refusing this therapy	$n=921$ 2:1 randomization	15.6 vs. 9.8 months[b] +3.6 months HR 0.70 (0.58–0.83), $p<0.001$	14.9 vs. 11.3 months +3.6 months HR 0.70 (0.58–0.83), $p<0.001$	Parker et al. (2013)
Sipuleucel-T vs. placebo					

(continued)

Table 1 (continued)

Drugs tested	Patients	Patient number Randomization scheme	Median radiographic PFS	Median OS	Ref.
Integrated analysis of studies D9901 and D9902A	Asymptomatic or minimally symptomatic metastatic CRPC without cancer-related bone pain or visceral metastases	$n=225$ 2:1 randomization	11.1 vs. 9.7 months +1.4 months Hazard ratio 1.26 (0.95–1.68), $p=0.111$ n.s.	23.2 vs. 18.9 months +4.3 months Hazard ratio 1.50 (1.10–2.05), $p=0.011$	Higano et al. (2009), Small et al. (2006)
IMPACT trial	Asymptomatic or minimally symptomatic metastatic CRPC, initial Gleason score ≤ 7	$n=512$ 2:1 randomization	No difference	25.8 vs. 21.7 months +4.1 months HR 0.78 (0.61–0.98), $p=0.03$	Kantoff et al. (2010a)
Prostvac-VF phase IIb	Symptomatic or mildly symptomatic CRPC, Gleason score ≤ 7 without visceral disease	$n=125$ 2:1 randomization	No difference Hazard ratio=0.88 (95 % CI, 0.57–1.38), $p=0.60$	25.1. vs. 16.6 +8.1 months HR 0.56 (0.37–0.85), $p=0.0061$	Kantoff et al. (2010b)

[a]HR, hazard ratio; 95 % confidence interval in brackets
[b]Time to first symptomatic skeletal event

PSA-TRICOM) to enhance immunogenicity (Hodge et al. 1999). The use of two recombinant viral vector systems allows a heterologous prime–boost strategy: a vaccinia vector is used for the prime, whereas recombinant fowlpox vector is used for a total of five boosts to avoid neutralizing antibody formation (Arlen et al. 2007). Prostvac-VF is administered with GM-CSF (granulocyte–macrophage colony-stimulating factor) as an adjuvant. In a controlled phase IIb trial in patients with metastatic CRPC, Gleason scores ≤ 7 and no visceral metastases, Prostvac-VF improved median overall survival by a remarkable +8.5 to 25.1 months (HR 0.56 (95 % CI, 0.37–0.85)), a clinically relevant and statistically highly significant effect ($p=0.0061$) (Kantoff et al. 2010b). Adverse events were mild and similar to those observed in the IMPACT study, the only grade III event was an injection site cellulitis. Crossover occurred in approximately 47.5 % (19/40) of placebo patients.

Despite this impressive gain in overall survival, yet again there was no difference in progression-free survival between Prostvac-VF and placebo. Moreover, PSA responses were infrequent (only one patient had a PSA reduction of >80 %) and thus mirrored a similar observation in the IMPACT trial, where PSA reductions >50 % were only observed in 8 of 311 (2.6 %) patients in the sipuleucel-T arm compared to 2 of 153 placebo patients (1.3 %) (Kantoff et al. 2010a). The paradox dissociation between improved overall survival on the one hand and the absence of prolonged progression-free survival, clear PSA responses or objective responses on the other hand has recently attracted much attention.

A possible explanation is that an intermittent immunotherapy may increase sensitivity to subsequent therapies. Docetaxel administered after PSA-TRICOM enhanced the induction of T-cells and patients appeared to profit longer from sequential PSA-Tricom/docetaxol therapy than from docetaxol alone, an effect that may have played a role in the observed dissociation of PFS and OS described above (Arlen et al. 2006; Garnett et al. 2008). A study performed with the antiandrogen nilutamide reported that patients appeared to survive longer when nilutamide was

administered after PSA-TRICOM than before (Arlen et al. 2005; Madan et al. 2008).

Alternatively or additionally, immunotherapies may alter the growth rates of tumors. Tumor growth rates were estimated from serial PSA measurements in five different phase II trials including one experimental PSA vaccine (PSA-TRICOM) trial (Stein et al. 2011). A reduction in growth rates indicated more effective chemotherapy. The PSA-TRICOM vaccine appeared to exert clear clinical benefit not during vaccination, but by the development of a beneficial, sustained immune response that appeared to slow tumor growth. Graphical modeling of the clinical impact of reduced growth rates readily showed that chemotherapy may reduce tumor burden, but without delayed tumor growth rate death would rapidly occur after chemotherapy failure. By contrast, a decreased tumor growth rate in the model was able to prolong survival beyond that achieved with chemotherapy even in the absence of clear tumor reductions (Gulley and Drake 2011; Madan et al. 2012a). It would be expected that this effect is stronger in smaller tumors that would allow also more time for the immune response to build up (Gulley et al. 2011), a fact with far reaching corollaries and broad applicability for the clinical development not just of prostate cancer vaccines and immunotherapies (Gulley et al. 2011; Gulley and Drake 2011).

Indeed, clinical evidence exists to support the validity of this view. In a pharmacodynamic study with Prostvac-VF (Gulley et al. 2010), patients with a predicted Halabi survival longer than 18 months benefitted by long-term survival from vaccination, while there was no such benefit in patients with predicted Halabi survival shorter than 18 months. Presumably this reflects lower tumor burden or less aggressive tumors (Gulley et al. 2011). PSA was the strongest baseline prognostic factor for survival in the IMPACT study (Schellhammer et al. 2013). The OS hazard ratio for patients in the lowest baseline PSA quartile ($\leq$ 22.1 ng/mL) was 0.51 (95 % confidence interval, 0.31–0.85) compared with no statistically different effect (HR 0.84 (95 % confidence interval, 0.55–1.29)) for patients in the highest PSA quartile (>134 ng/mL). The estimated median survival improvement was 13.0 months (HR 0.51 (95 % confidence interval, 0.31–0.85)) in the lowest baseline PSA quartile compared to 2.8 months in the highest quartile. Observations of clinical benefit of immunotherapies have also been made at earlier stages of prostate carcinoma. A pilot trial of the MUC1-targeting cancer vaccine L-BLP25 in men with biochemical failure after radical prostatectomy showed an increase of the PSA doubling time by more than 50 % in 6 of 16 patients. 1 of 10 men completing the full 1-year maintenance treatment period maintained a stable PSA level throughout (North et al. 2006). A DNA vaccine targeting PAP caused T-cell proliferation in 41 % and CD8+ T-cells in 14 % patients with D0 disease (biochemical failure) after definitive first-line prostatectomy and/ or radiotherapy (McNeel et al. 2009). The PSA doubling time increased from a median 6.5 months pretreatment to 8.5 months on-treatment ($p=0.033$) and 9.3 months in the 1-year post-treatment period ($p=0.054$). Two patients had decreasing PSA values on treatment and one post treatment. A controlled trial with sipuleucel-T in 176 in somewhat unclearly defined patients (randomized 2:1) with PSA recurrence after radical prostatectomy and on 3–4 months of androgen-suppressive therapy resulted in a 48 % increase of the PSA doubling time ($p=0.038$), but no effect on time to biochemical failure defined as serum PSA level $\geq$3.0 ng/mL (Beer et al. 2011). While these trials point to possible activity of cancer vaccines at the very early stage of prostate carcinoma, they are hypothesis generating at best. Yet the time scales of progression at early stages of prostate carcinoma pose very severe, almost prohibitive challenges to clinical development in this early clinical setting. Clearly, predictive surrogate markers defining long-term progression and survival are needed to overcome these obstacles.

Some support for the "small is more accessible" view can be obtained from a phase IIb study with L-BLP25 in patients with inoperable NSCLC stage IIIB/IV. The median overall survival after treatment with L-BLP25 was not reached in patients with locoregional NSCLC stage IIIB, while the best supportive care arm had a median overall survival of just 13.3 months (adjusted

HR 0.524 (95 %) CI: 0.261–1.052; $p=0.069$) (Butts et al. 2005). There was no difference in survival between the more advanced patients with NSCLC stage IIIB with malignant pleural effusion or stage IV and the control arm. In 806 patients with inoperable NSCLC stage IIIA/IIIB, L-BLP25 improved median overall survival to 30.8 months (L-BLP25) compared to 20.6 months in control patients (HR 0.78 (95 % CI 0.64–0.95, $p=0.016$)) following concurrent chemoradiotherapy, while median OS in patients vaccinated after equential chemoradiotherapy was not statistically different from non-vaccinated patients (Butts et al. 2013). Hence, not only the tumor burden but also the treatment preceding the immunotherapy under investigation will have to be considered for clinical studies.

New Perspectives for Nucleotide-Based Vaccination: The RNActive® Technology

Approaches to use nucleotide-encoded antigens instead of peptide-, protein-, or vector-encoded vaccines have been pursued mostly with DNA vaccines. Though long avoided for its perceived instability, messenger RNA (mRNA) appears to be a promising alternative to DNA for engineering nucleotide-based vaccines (Hoerr et al. 2000; Kallen and Thess 2014; Kallen et al. 2013). The so-called RNActive® technology developed by CureVac rests on two components: one is a sequence-engineered mRNA with highly enhanced and prolonged expression that encodes a protein antigen, and the second component is the same mRNA complexed to protamine that results in immune activation via TLR7 (Fotin-Mleczek et al. 2011). Essentially, the RNActive® vaccines seek to combine the flexibility and nimbleness of a nucleotide-based antigen-coding approach together with good and long-lasting immunogenicity.

The minimal mRNA structure is a protein-encoding open reading frame (ORF) flanked by two essential elements at the 5′- and 3′-end, the 7-methyl-guanosine cap structure at the 5′-end of the RNA and the poly(A) tail at the 3′-end (Banerjee 1980; Wickens 1990). Untranslated regions (UTR) at the 5′-end (between cap and ORF) and the 3′-end (between ORF and poly(A)-tail) of the ORF also influence protein expression (Schlake et al. 2012). Enhanced and prolonged protein expression was achieved by engineering the nucleotide sequence of the mRNA elements with experimental and computational methods without affecting the primary amino acid sequence of the ORF using only the naturally occurring nucleotides A, G, C, and U (T) (Schlake et al. 2012). Following the ultrahigh purification of this sequence, modified mRNA leads to an expression of the reporter gene luciferase ($t_{1/2}\sim 2$ h) augmented by 4–5 orders of magnitude in various test systems comparing the most recent mRNA version to earllier ones (Fig. 1). The temporal expression of luciferase peaked after 24–48 h with the expression after 72 h matching peak expression of previous generations. The protein expression kinetics of such enhanced RNA molecules resemble that of proteins after an influenza virus infection (Julkunen et al. 2001).

Complexation of the expression-enhanced mRNA leads to the formation of nanoparticles of around 250–350 nm in size which activate the immune system via the endosomal TLR7 (toll-like receptor 7) (Fotin-Mleczek et al. 2011; Kallen et al. 2013; Scheel et al. 2005). In vitro experiments demonstrated uptake of the uncomplexed mRNA (particle size around 50 nm) by an ATP-dependent process into cytoplasm and lysosomes (Lorenz et al. 2011). The final formulation of an RNActive vaccine is obtained by mixing the immunostimulating mRNA/protamine complexes with the antigen expressing nonencapsulated, “naked” sequence-engineered mRNA. An optimal mixture of the two components was identified that ensures both good antigen expression and good immunostimulation after intradermal administration (Fotin-Mleczek et al. 2011). Such vaccines can be comparatively easily produced by the transcription of target RNA by RNA polymerases from a linearized plasmid DNA template, followed by enzymatic destruction of the DNA template by DNAses and purification of the resulting mRNA by precipitation and chromatographic methods according to size (detailed

description in Kallen and Thess 2014; Ketterer et al. 2008; Pascolo 2004, 2006). This process results in very pure RNA products, and while it has been mainly used to produce standard mRNA sizes of a few kilobases, mRNAs of sizes up to 15 kilobases are also feasible.

The two-component, self-adjuvanted RNActive vaccines consistently induce strong and balanced immune responses which comprise Th1 and Th2, humoral and cellular, as well as effector and memory responses. In several different tumor models, powerful CD4+ and CD8+ T-cell responses resulted in good antitumor activity (Fotin-Mleczek et al. 2011, 2012). The approach, however, is not limited to tumor applications which allow frequent vaccine administration. Influenza vaccines were generated with the RNActive® technology that elicited protective, long-lived humoral immune responses against various influenza strains after intradermal prime–boost administration in several species including mice, ferrets, and large pigs (Petsch et al. 2012). Importantly, intradermal administration was as immunogenic as intranodal administration (Kallen et al. 2013)

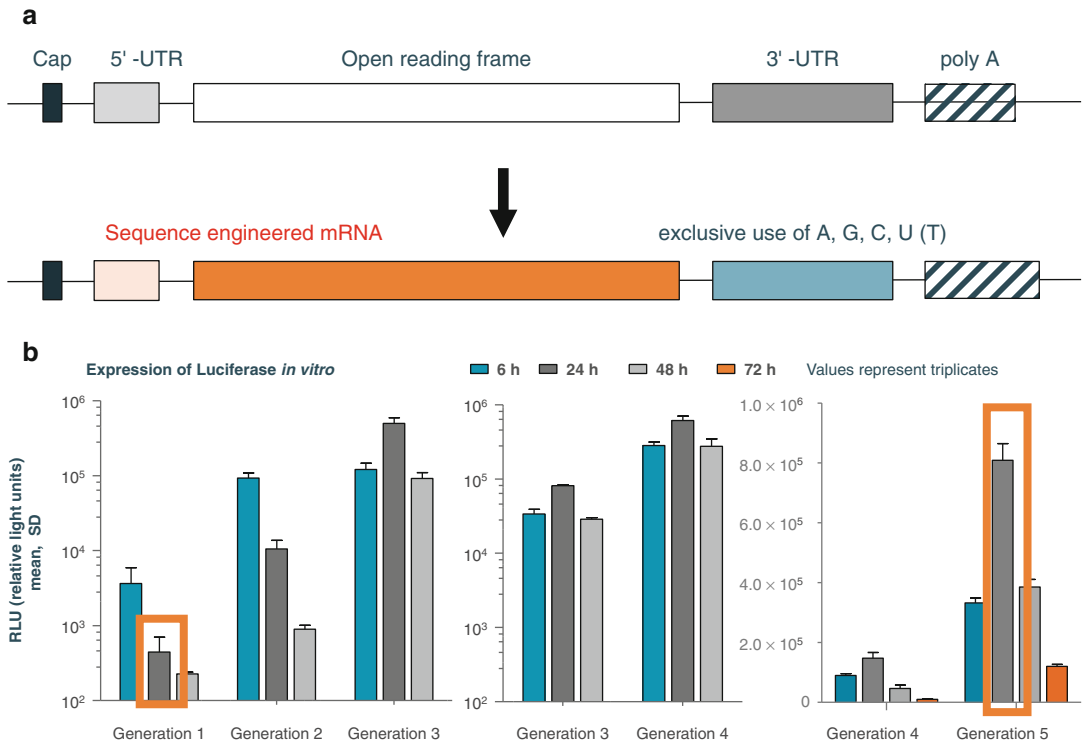

Fig. 1 Strongly enhanced *p*rotein expression by sequence-engineered mRNA used in RNActive® vaccines. (**a**) Using only the natural nucleotides A, G, C, and U, all elements of the classical mRNA structure (cap, (optional) 5′-UTR, open reading frame, (optional) 3′-UTR, poly(A)-tail) were sequence optimized without affecting the primary amino acid sequence encoded by the open reading. (**b**) Effect on in vitro luciferase expression (PpLuc) encoded by different generations of sequence-engineered mRNAs produced in the past years. Generational differences result from optimization of the nucleotide content of the ORF or incorporation of different 3′- or 5′-UTRs or combinations thereof. The mRNA generations 1–4 were electroporated into HeLa cells (generations 1–4). Generations 4 and 5 were compared in human dermal fibroblasts after lipofection. The luciferase level was determined at 6, 24, and 48 h or 72 h post transfection. The dynamic range of the assay prevents a comparison of all mRNA generations in one experiment. (**c**) Luciferase-encoding mRNA (generation 5), optimized for translation and stability, was injected intradermally (i.d.) into a BALB/c mouse (four injection sites). The luciferase expression was visualized in the skin by optical imaging at various time points after mRNA injection. (**d**) Quantitative expression of luciferase over time until 9 days after mRNA injection. *Left panel* results are on a linear scale; *right panel* results are on a semilogarithmic scale (The figure is an adaptation from Schlake et al. (2012))

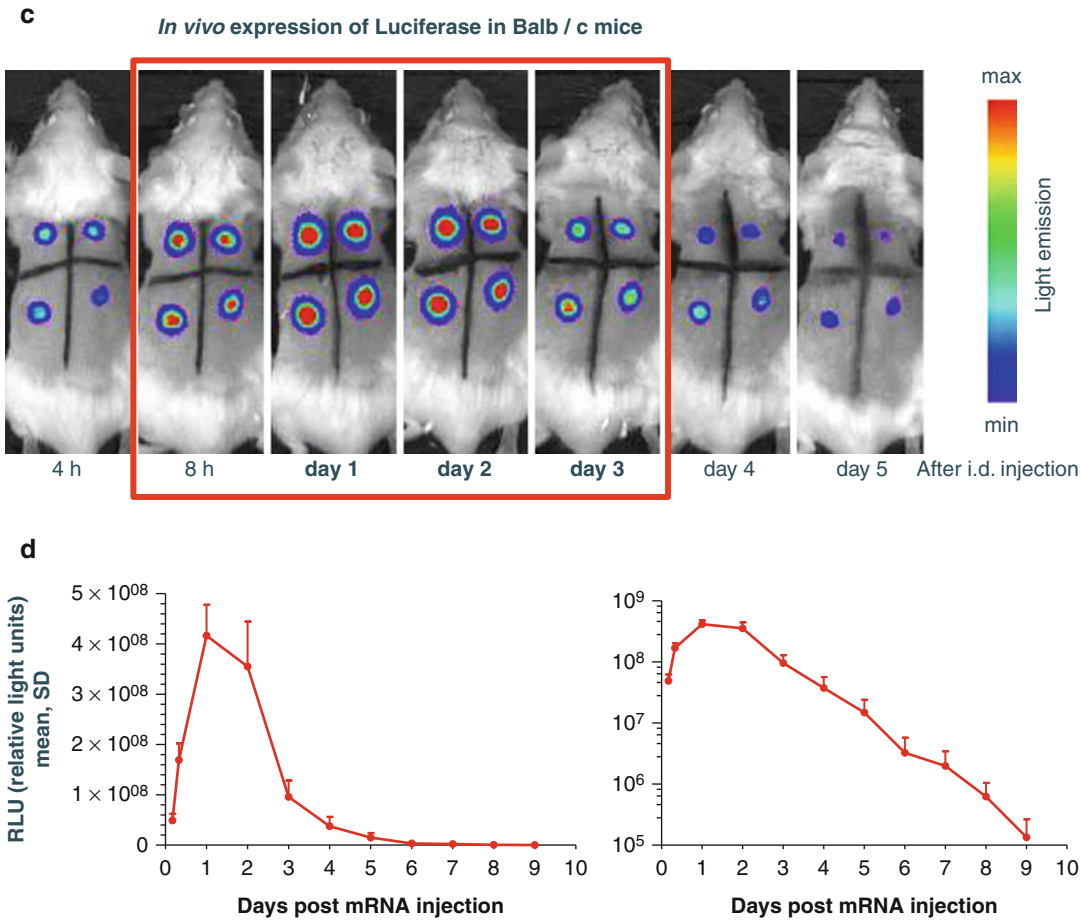

Fig. 1 (continued)

obliviating the need for the more complicated intranodal administration (Kreiter et al. 2010, 2011; Van Lint et al. 2012).

The reasons for the good activity of RNActive vaccines after intradermal administration may reflect the enhanced protein expression capacity of RNActive vaccines and activation of the immune system via TLR7. TLR7/8 agonists were critically important for the development of a novel dendritic cell (DC) stimulation protocol which induced strong Th1 responses (Spranger et al. 2010, 2012). Similarly, small molecule TLR7/8 agonists were shown to localize to an MCH class II containing compartment of human plasmacytoid dendritic cells (Iavarone et al. 2011; Russo et al. 2011). The production of the type I interferons (comprising 12 IFNα subtypes, IFNβ1, IFNε, IFNκ, and IFNω) by these cells appears to be essential for strong Th1 and memory responses, which are required to reject tumors (Desmet and Ishii 2012; Diamond et al. 2011). Hence, activation of TLR7/8 pathway might be a very favorable successful vaccination strategy against cancer, but also against chronic infections (Bernstein et al. 2012; Mbow et al. 2010; Walsh et al. 2012). Due to the varying expression of different TLRs between different DCs (Desmet and Ishii 2012) which have differing preferred locations that can be affected by inflammation in a patient (Hartmann et al. 2003, 2006; Naik et al. 2006; Wollenberg et al. 2002), vaccination efficiency is likely to depend also on the route of administration. Hence, translation of preclinical results to the clinic also requires

consideration of the effect of different routes of administration.

mRNA-Based Vaccines in Clinical Studies

Three clinical trials with mRNA-based vaccines were initiated with precursors of RNActive vaccines almost a decade ago. In the first trial, 15 patients with melanoma stage III or IV were vaccinated by direct intradermal injection of an autologous mRNA library prepared from metastatic lesions together with granulocyte–macrophage colony-stimulating factor (GM-CSF) as an adjuvant (Weide et al. 2008). Two patients had a mixed response, and a favorable clinical course was observed in 5 patients, but an objective response could not be observed. Humoral responses were found in four patients and a possible transient increases in CD4+ and CD8+ T-cell frequencies.

The next trial employed protamine-stabilized mRNAs (coding for Melan-A, tyrosinase, gp100, MAGE-A1, MAGE-A3, and survivin) for intradermal vaccination of 21 patients with metastatic melanoma, again with GM-CSF as adjuvant (Weide et al. 2009). Ten patients received keyhole limpet hemocyanin (KLH) in addition to the vaccine. No grade III adverse event was observed, and an antigen-specific T-cell response was detected in two of four immunologically evaluable patients. KLH had caused a decrease of Foxp3+/CD4+ T-regulatory cells in patients receiving it in addition to the vaccine and GM-CSF, whereas myeloid-suppressor cells (CD11b+HLA-DRlo monocytes) were reduced in the patients not receiving it. Seven patients had measurable disease; one of them developed a partial response of lung metastases after 12 vaccinations. In another patient, a bone metastasis was detected and surgically removed 16 months after the start of vaccination. This patient remained relapse free.

In addition to melanoma patients, 30 patients with stage IV renal cell cancer were immunized with a naked mRNA vaccine encoding mucin 1(MUC1), carcinoembryonic antigen (CEA), human epidermal growth factor receptor 2 (Her-2/neu), telomerase, survivin, and melanoma-associated antigen 1 (MAGE-A1) with GM-CSF as adjuvant (Rittig et al. 2011). The second cohort of 16 patients received a more intensive induction schedule than the first 14 patients, and both cohorts received monthly vaccinations afterward. The vaccination was well tolerated. 12 of 17 immunologically evaluable patients showed an immune response. One patient in the first cohort had a confirmed partial response. Another patient that required paracentesis every second day experienced decline of the paracentesis frequency in line with a decline of the tumor marker CA-125 and regression of abdominal tumor sites. Ultimately, he remained free of paracentesis for >3 months.

First Clinical Steps with Self-Adjuvanted RNActive Vaccines

Self-adjuvanted RNActive® vaccines were first investigated in two phase I/IIa trials in prostate carcinoma and NSCLC (non-small cell lung cancer). The four tumor-associated antigens, PSA (prostate-specific antigen), PSCA (prostate stem cell antigen), PSMA (prostate-specific membrane antigen), and STEAP1 (six transmembrane epithelial antigen of the prostate 1), were selected for the first-in-man phase I/IIa study in patients with advanced prostate cancer designated CV9103 (Kübler et al. 2011). In the second trial in patients with NSCLC stage IIIB/IV disease that had at least stable disease after first-line platinum-based chemotherapy or chemoradiation, an antigen cocktail (CV9201) of the five tumor-associated antigens, MAGE-C1, MAGE-C2, NY-ESO-1, survivin, and 5T4, was used (Sebastian et al. 2011; Sebastian et al. 2012).

In the prostate carcinoma study, five intradermal vaccinations with CV9103 were administered over a 6-month period. CV9103 was well tolerated despite the advanced nature of the disease. An unexpectedly high number of vaccinated, imunologically evaluable patients, around 80 %, developed an increased number of

antigen-specific T-cells independent of their HLA background (Kübler et al. 2011). This is in line with the suggestion that mRNA-based vaccination is a method to overcome HLA restriction of tumor patients (Van Nuffel et al. 2012). Antigen-specific T-cell immune responses were detected against all antigens administered independent of their cellular localization. Antigen-unspecific B-cells appeared to be increased, and natural killer (NK)-cells tended to show an activated phenotype. Most patients with an antigen-specific immune response reacted against more than one antigen. Multi-antigen responses in response to a multi-peptide vaccine appeared to be associated with increased survival in a recent study in patients with renal cell carcinoma (Walter et al. 2012). While the prostate carcinoma study was not designed to assess clinical efficacy, individual patients showed interesting clinical courses suggestive of clinical benefit. The overall survival of vaccines and its correlation with immune responses are presently analyzed; intermediate analyses however indicate a very encouraging survival trend and an association of improved survival with the immune response (Kübler, manuscript in preparation).

The results described above were considered strong enough by CureVac to launch a controlled phase IIb study (ClinicalTrials.gov number NCT01817738) in patients with castrate-resistant prostate carcinoma with asymptomatic or minimally symptomatic metastasis to systematically investigate the clinical efficacy of CV9104, a further developed version of CV9103 using two more antigens, MUC1 and PAP (prostate acid phosphatase). The study will employ more frequent vaccination in the induction phase followed by maintenance vaccinations at increasingly prolonged intervals. The primary endpoint of this study of around 180 patients recruited in eight European countries is overall survival. A number of secondary endpoints will analyze biomarker and the mechanism of action and ascertain the link between immune response and hopefully much improved survival. Particular importance will be given to the impact of the intermittent RNActive® immunotherapy on the response to subsequent therapies with any of the newly approved drugs and the duration of that response in the vaccination arm compared to the placebo arm (see Table 1).

In the phase I/IIa NSCLC, patients were vaccinated with up to five intradermal administrations of CV9201. The more life-threatening disease of these patients necessitated a change from the originally planned 15-week period to a more intensive vaccination schedule of 7 weeks in the phase IIa part of the study. Similar to CV9103, the NSCLC cocktail CV9201 was well tolerated also in patients receiving the intensified vaccination schedule. An antigen-specific humoral and cellular immune response was determined in roughly two thirds of the treated patients, and a similar proportion showed a multi-antigen response. Pre-germinal center B-cells (pGCB) were significantly increased by a factor of at least two and up to more than tenfold in more than half of the patients and associated with an increase of total CD4+ effector T-cells during treatment. Together, more than 80 % of the treated NSCLC patients had a detectable antigen-specific immune response and/or an increase in germinal center B-cells despite their heavy pre-treatment with platinum-based chemotherapy (Sebastian, manuscript in preparation). Based on these results, own preclinical observations, and an increasing body of evidence (Burnette et al. 2011; Lee et al. 2009; Liang et al. 2013; Reits et al. 2006) that points to a strongly positive activity interplay between combinations of radiation and immunotherapy, a phase Ib study has been launched in metastatic NSCLC patients to ascertain a preclinically observed synergistic effect between vaccination with RNActive® vaccines and radiotherapy in the clinic (ClinicalTrials.gov number NCT01915524).

Potential Ways to Enhance the Efficacy of Cancer Immunotherapies by Rationale Combinations

The clinical data recently published and emerging now suggest that immunotherapies could provide substantial clinical benefit to patients with prostate cancer. These could be made more efficient

by combinations. The checkpoint inhibitor ipilimumab (anti-CTLA4) was recently approved for the treatment of advanced melanoma, since it provided long-term survival benefits albeit at substantial toxicity in about 50 % of the patients (Hodi et al. 2010; Robert et al. 2011). Inhibitors of the PD1/PD-L1 pathway have also demonstrated clinical efficacy (Brahmer et al. 2012; Hamid et al. 2013; Topalian et al. 2012). However, the combination of simultaneous anti-CTLA4 and anti-PD1 blockade achieved a stunning 53 % response rate in patients with advanced melanoma (Wolchok et al. 2013). Most of these responses were deep (tumor reduction >80 %) and lasted for more than 24 weeks. A grade 3/4 toxicity rate of greater than 50 %, however, is probably not acceptable at earlier disease stages such as CRPC. The combination of active immunotherapies such as RNActive® proved to be largely synergistic with an anti-CTLA4 antibody in mice bearing already large tumors and resulted in long-term survival of half of the animals treated with the combination, whereas all of the mice receiving single-agent treatment died (Fotin-Mleczek et al. 2012). Combinations with other checkpoint inhibitors also proved high antitumor activity in mice (unpublished observations), and favorable antitumor responses were also achieved by rationale combinations of RNActive vaccines with chemotherapies (Fotin-Mleczek et al. 2012). A phase I study of Prostvac and ipilimumab in metastatic CRPC did not observed exacerbation of ipilimumab-associated immune-related adverse events, but observed a PSA decline from baseline in 58 % (14/24) patients and a >50 % decline in 6 patients (Madan et al. 2012b). These encouraging results need further testing in controlled trials and widened to the inclusion of other checkpoint inhibitors. If the preclinical synergistic effects between active immunotherapies such as RNActive vaccines and checkpoint inhibitors can be translated to the clinic, strong antitumor effects might be achievable at reduced doses of the checkpoint inhibitors with fewer side effects. Conversely, large tumors that do not respond to single agent therapy might be controlled by the combination without the risk of increased side effects. Given that the antitumor efficacy of radiotherapy appears to be largely dependent on the immune system (Burnette et al. 2011; Lee et al. 2009; Liang et al. 2013; Reits et al. 2006), it will also be interesting to see whether the recently approved alpha emitter radium-223 (Parker et al. 2013) might act synergistically with active immune therapies such as sipuleucel-T, Prostvac-VF, or RNActive vaccines.

Summary

After several decades of failed efforts, sipuleucel-T was the first active immunotherapy approved for the treatment of CRPC. This success has been followed by promising results with Prostvac-FV, a viral vector-based vaccine targeting PSA. Noteworthy, nucleotide-based vaccines appear to have come of age not in the form of DNA vaccines, but vaccines using mRNA. A prostate cancer vaccine, CV9103, engineered with the RNActive® technology that uses an expression-enhanced and self-adjuvanted mRNA has successfully passed a phase I/IIa study and demonstrated good immunogenicity of the encoded prostate cancer antigens as well as a very favorable safety profile. These results and the availability of other immunotherapeutic moieties such as the checkpoint inhibitor and classical and novel radiotherapies will allow to build even more effective cancer therapies by exploiting the combinatorial potential of immunological principles. It appears that that cancer immunotherapy has now reached a degree of maturity where success in clinical medicine will no longer be just anecdotal, but also be convincing in randomized trials.

Literature

Arlen PM, Gulley JL, Todd N, Lieberman R, Steinberg SM, Morin S, Bastian A, Marte J, Tsang KY, Beetham P et al (2005) Antiandrogen, vaccine and combination therapy in patients with nonmetastatic hormone refractory prostate cancer. J Urol 174:539–546. doi:10.1158/1078-0432.CCR-07-0588

Arlen PM, Gulley JL, Parker C, Skarupa L, Pazdur M, Panicali D, Beetham P, Tsang KY, Grosenbach DW, Feldman J et al (2006) A randomized phase II study of concurrent docetaxel plus vaccine versus vaccine

alone in metastatic androgen-independent prostate cancer. Clin Cancer Res 12:1260–1269
Arlen PM, Skarupa L, Pazdur M, Seetharam M, Tsang KY, Grosenbach DW, Feldman J, Poole DJ, Litzinger M, Steinberg SM et al (2007) Clinical safety of a viral vector based prostate cancer vaccine strategy. J Urol 178:1515–1520
Banerjee AK (1980) 5′-terminal cap structure in eucaryotic messenger ribonucleic acids. Microbiol Rev 44:175–205
Beer TM, Bernstein GT, Corman JM, Glode LM, Hall SJ, Poll WL, Schellhammer PF, Jones LA, Xu Y, Kylstra JW et al (2011) Randomized trial of autologous cellular immunotherapy with sipuleucel-T in androgen-dependent prostate cancer. Clin Cancer Res 17: 4558–4567
Bernstein DI, Cardin RD, Bravo FJ, Earwood J, Clark JR, Li Y, Mishra P, Li C, Nayak BP, Miller AT, et al (2012) Topical SMIP-7.7, a toll-like receptor 7 agonist, protects against genital herpes simplex virus-2 disease in the guinea pig model of genital herpes. Antivir Chem Chemother. doi:10.3851/IMP2499
Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsi K et al (2012) Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med 366:2455–2465
Burnette BC, Liang H, Lee Y, Chlewicki L, Khodarev NN, Weichselbaum RR, Fu YX, Auh SL (2011) The efficacy of radiotherapy relies upon induction of type i interferon-dependent innate and adaptive immunity. Cancer Res 71:2488–2496
Butts C, Murray N, Maksymiuk A, Goss G, Marshall E, Soulieres D, Cormier Y, Ellis P, Price A, Sawhney R et al (2005) Randomized phase IIB trial of BLP25 liposome vaccine in stage IIIB and IV non-small-cell lung cancer. J Clin Oncol 23:6674–6681
Butts CA, Socinski M, Mitchell P, Thatcher N, Havel L, Krzakowski MJ, Nawrocki S, Ciuleanu TE, Bosquée L, Perez JMT, et al (2013) START: a phase III study of L-BLP25 cancer immunotherapy for unresectable stage III non-small cell lung cancer. J Clin Oncol 31(suppl):abstr 7500
de Bono JS, Oudard S, Ozguroglu M, Hansen S, Machiels JP, Kocak I, Gravis G, Bodrogi I, Mackenzie MJ, Shen L et al (2010) Prednisone plus cabazitaxel or mitoxantrone for metastatic castration-resistant prostate cancer progressing after docetaxel treatment: a randomised open-label trial. Lancet 376:1147–1154
de Bono JS, Logothetis CJ, Molina A, Fizazi K, North S, Chu L, Chi KN, Jones RJ, Goodman OB Jr, Saad F et al (2011) Abiraterone and increased survival in metastatic prostate cancer. N Engl J Med 364: 1995–2005
Desmet CJ, Ishii KJ (2012) Nucleic acid sensing at the interface between innate and adaptive immunity in vaccination. Nat Rev Immunol 12:479–491
Diamond MS, Kinder M, Matsushita H, Mashayekhi M, Dunn GP, Archambault JM, Lee H, Arthur CD, White JM, Kalinke U et al (2011) Type I interferon is selectively required by dendritic cells for immune rejection of tumors. J Exp Med 208:1989–2003
Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JW, Comber H, Forman D, Bray F (2013) Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. Eur J Cancer 49:1374–1403
Fotin-Mleczek M, Duchardt KM, Lorenz C, Pfeiffer R, Ojkic-Zrna S, Probst J, Kallen KJ (2011) Messenger RNA-based vaccines with dual activity induce balanced TLR-7 dependent adaptive immune responses and provide antitumor activity. J Immunother 34:1–15
Fotin-Mleczek M, Zanzinger K, Heidenreich R, Lorenz C, Thess A, Duchardt KM, Kallen KJ (2012) Highly potent mRNA based cancer vaccines represent an attractive platform for combination therapies supporting an improved therapeutic effect. J Gene Med 14:428–439
Garnett CT, Schlom J, Hodge JW (2008) Combination of docetaxel and recombinant vaccine enhances T-cell responses and antitumor activity: effects of docetaxel on immune enhancement. Clin Cancer Res 14:3536–3544
Gulley JL, Drake CG (2011) Immunotherapy for prostate cancer: recent advances, lessons learned, and areas for further research. Clin Cancer Res 17:3884–3891
Gulley JL, Arlen PM, Madan RA, Tsang KY, Pazdur MP, Skarupa L, Jones JL, Poole DJ, Higgins JP, Hodge JW et al (2010) Immunologic and prognostic factors associated with overall survival employing a poxviral-based PSA vaccine in metastatic castrate-resistant prostate cancer. Cancer Immunol Immunother 59:663–674
Gulley JL, Madan RA, Schlom J (2011) Impact of tumour volume on the potential efficacy of therapeutic vaccines. Curr Oncol 18:e150–e157
Gulley JL, Leitman SF, Dahut W, Schlom J (2012) Re: interdisciplinary critique of sipuleucel-T as immunotherapy in castration-resistant prostate cancer. J Natl Cancer Inst 104:1106; author reply 1109–1112
Hamid O, Robert C, Daud A, Hodi FS, Hwu WJ, Kefford R, Wolchok JD, Hersey P, Joseph RW, Weber JS et al (2013) Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. N Engl J Med 369:134–144
Hartmann E, Wollenberg B, Rothenfusser S, Wagner M, Wellisch D, Mack B, Giese T, Gires O, Endres S, Hartmann G (2003) Identification and functional analysis of tumor-infiltrating plasmacytoid dendritic cells in head and neck cancer. Cancer Res 63:6478–6487
Hartmann E, Graefe H, Hopert A, Pries R, Rothenfusser S, Poeck H, Mack B, Endres S, Hartmann G, Wollenberg B (2006) Analysis of plasmacytoid and myeloid dendritic cells in nasal epithelium. Clin Vaccine Immunol 13:1278–1286
Higano CS, Schellhammer PF, Small EJ, Burch PA, Nemunaitis J, Yuh L, Provost N, Frohlich MW (2009) Integrated data from 2 randomized, double-blind, placebo-controlled, phase 3 trials of active cellular

immunotherapy with sipuleucel-T in advanced prostate cancer. Cancer 115:3670–3679
Hodge JW, Sabzevari H, Yafal AG, Gritz L, Lorenz MG, Schlom J (1999) A triad of costimulatory molecules synergize to amplify T-cell activation. Cancer Res 59:5800–5807
Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC et al (2010) Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 363:711–723
Hoerr I, Obst R, Rammensee HG, Jung G (2000) In vivo application of RNA leads to induction of specific cytotoxic T lymphocytes and antibodies. Eur J Immunol 30:1–7
Huber ML, Haynes L, Parker C, Iversen P (2012) Interdisciplinary critique of sipuleucel-T as immunotherapy in castration-resistant prostate cancer. J Natl Cancer Inst 104:273–279
Iavarone C, Ramsauer K, Kubarenko AV, Debasitis JC, Leykin I, Weber AN, Siggs OM, Beutler B, Zhang P, Otten G et al (2011) A point mutation in the amino terminus of TLR7 abolishes signaling without affecting ligand binding. J Immunol 186:4213–4222
Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. CA Cancer J Clin 61:69–90
Julkunen I, Sareneva T, Pirhonen J, Ronni T, Melen K, Matikainen S (2001) Molecular pathogenesis of influenza A virus infection and virus-induced regulation of cytokine gene expression. Cytokine Growth Factor Rev 12:171–180
Kallen K-J, Thess A (2014) A development that may evolve into a revolution in medicine: mRNA as the basis for novel, nucleotide-based vaccines and drugs. Ther Adv Vaccines 2:10–31. doi:10.1177/2051013613508729
Kallen KJ, Heidenreich R, Schnee M, Petsch B, Schlake T, Thess A, Baumhof P, Scheel B, Koch SD, Fotin-Mleczek M (2013) A novel, disruptive vaccination technology: Self-adjuvanted RNActive ((R)) vaccines. Hum Vaccin Immunother 9:2263–2276
Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, Redfern CH, Ferrari AC, Dreicer R, Sims RB et al (2010a) Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med 363:411–422
Kantoff PW, Schuetz TJ, Blumenstein BA, Glode LM, Bilhartz DL, Wyand M, Manson K, Panicali DL, Laus R, Schlom J et al (2010b) Overall survival analysis of a phase II randomized controlled trial of a Poxviral-based PSA-targeted immunotherapy in metastatic castration-resistant prostate cancer. J Clin Oncol 28:1099–1105
Kantoff PW, Higano CS, Small EJ, Whitmore JB, Frohlich MW, Schellhammer PF (2012) Re: interdisciplinary critique of sipuleucel-T as immunotherapy in castration-resistant prostate cancer. J Natl Cancer Inst 104:1107–1109; author reply 1109–1112
Ketterer T, von der Mülbe F, Reidel L, Mutzke T (2008) Method for purifying rna on a preparative scale by means of hplc. Patent WO2008077592
Kreiter S, Selmi A, Diken M, Koslowski M, Britten CM, Huber C, Tureci O, Sahin U (2010) Intranodal vaccination with naked antigen-encoding RNA elicits potent prophylactic and therapeutic antitumoral immunity. Cancer Res 70:9031–9040
Kreiter S, Diken M, Selmi A, Tureci O, Sahin U (2011) Tumor vaccination using messenger RNA: prospects of a future therapy. Curr Opin Immunol 23: 399–406
Kübler H, Maurer T, Stenzl A, Feyerabend S, Steiner U, Schostak M, Schultze-Seemann W, vom Dorp F, Pilla L, Viatali G et al (2011) Final analysis of a phase I/IIa study with CV9103, an intradermally administered prostate cancer immunotherapy based on self-adjuvanted mRNA. J Clin Oncol 29(suppl):abstr 4535
Lee Y, Auh SL, Wang Y, Burnette B, Wang Y, Meng Y, Beckett M, Sharma R, Chin R, Tu T et al (2009) Therapeutic effects of ablative radiation on local tumor require CD8+ T cells: changing strategies for cancer treatment. Blood 114:589–595
Liang H, Deng L, Chmura S, Burnette B, Liadis N, Darga T, Beckett MA, Lingen MW, Witt M, Weichselbaum RR et al (2013) Radiation-induced equilibrium is a balance between tumor cell proliferation and T cell-mediated killing. J Immunol 190:5874–5881
Lorenz C, Fotin-Mleczek M, Roth G, Becker C, Dam TC, Verdurmen WP, Brock R, Probst J, Schlake T (2011) Protein expression from exogenous mRNA: uptake by receptor-mediated endocytosis and trafficking via the lysosomal pathway. RNA Biol 8:627–636
Madan RA, Gulley JL, Schlom J, Steinberg SM, Liewehr DJ, Dahut WL, Arlen PM (2008) Analysis of overall survival in patients with nonmetastatic castration-resistant prostate cancer treated with vaccine, nilutamide, and combination therapy. Clin Cancer Res 14:4526–4531
Madan RA, Bilusic M, Heery C, Schlom J, Gulley JL (2012a) Clinical evaluation of TRICOM vector therapeutic cancer vaccines. Semin Oncol 39:296–304
Madan RA, Mohebtash M, Arlen PM, Vergati M, Rauckhorst M, Steinberg SM, Tsang KY, Poole DJ, Parnes HL, Wright JJ et al (2012b) Ipilimumab and a poxviral vaccine targeting prostate-specific antigen in metastatic castration-resistant prostate cancer: a phase 1 dose-escalation trial. Lancet Oncol 13:501–508
Mbow ML, De Gregorio E, Valiante NM, Rappuoli R (2010) New adjuvants for human vaccines. Curr Opin Immunol 22:411–416
McNeel DG, Dunphy EJ, Davies JG, Frye TP, Johnson LE, Staab MJ, Horvath DL, Straus J, Alberti D, Marnocha R et al (2009) Safety and immunological efficacy of a DNA vaccine encoding prostatic acid phosphatase in patients with stage D0 prostate cancer. J Clin Oncol 27:4047–4054
Naik SH, Metcalf D, van Nieuwenhuijze A, Wicks I, Wu L, O'Keeffe M, Shortman K (2006) Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. Nat Immunol 7:663–671
North SA, Graham K, Bodnar D, Venner P (2006) A pilot study of the liposomal MUC1 vaccine BLP25 in

prostate specific antigen failures after radical prostatectomy. J Urol 176:91–95
Parker C, Nilsson S, Heinrich D, Helle SI, O'Sullivan JM, Fossa SD, Chodacki A, Wiechno P, Logue J, Seke M et al (2013) Alpha emitter radium-223 and survival in metastatic prostate cancer. N Engl J Med 369: 213–223
Pascolo S (2004) Messenger RNA-based vaccines. Expert Opin Biol Ther 4:1285–1294
Pascolo S (2006) Vaccination with messenger RNA. Methods Mol Med 127:23–40
Petrylak DP, Tangen CM, Hussain MH, Lara PN Jr, Jones JA, Taplin ME, Burch PA, Berry D, Moinpour C, Kohli M et al (2004) Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. N Engl J Med 351: 1513–1520
Petsch B, Schnee M, Vogel AB, Lange E, Hoffmann B, Voss D, Schlake T, Thess A, Kallen KJ, Stitz L et al (2012) Protective efficacy of in vitro synthesized, specific mRNA vaccines against influenza A virus infection. Nat Biotechnol 30:1210–1216
Reits EA, Hodge JW, Herberts CA, Groothuis TA, Chakraborty M, Wansley EK, Camphausen K, Luiten RM, de Ru AH, Neijssen J et al (2006) Radiation modulates the peptide repertoire, enhances MHC class I expression, and induces successful antitumor immunotherapy. J Exp Med 203:1259–1271
Rittig SM, Haentschel M, Weimer KJ, Heine A, Muller MR, Brugger W, Horger MS, Maksimovic O, Stenzl A, Hoerr I et al (2011) Intradermal vaccinations with RNA coding for TAA generate CD8+ and CD4+ immune responses and induce clinical benefit in vaccinated patients. Mol Ther 19:990–999
Robert C, Thomas L, Bondarenko I, O'Day S, Weber J, Garbe C, Lebbe C, Baurain JF, Testori A, Grob JJ et al (2011) Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. N Engl J Med 364:2517–2526
Russo C, Cornella-Taracido I, Galli-Stampino L, Jain R, Harrington E, Isome Y, Tavarini S, Sammicheli C, Nuti S, Mbow ML et al (2011) Small molecule Toll-like receptor 7 agonists localize to the MHC class II loading compartment of human plasmacytoid dendritic cells. Blood 117:5683–5691
Ryan CJ, Smith MR, de Bono JS, Molina A, Logothetis CJ, de Souza P, Fizazi K, Mainwaring P, Piulats JM, Ng S et al (2013) Abiraterone in metastatic prostate cancer without previous chemotherapy. N Engl J Med 368:138–148
Scheel B, Teufel R, Probst J, Carralot JP, Geginat J, Radsak M, Jarrossay D, Wagner H, Jung G, Rammensee HG et al (2005) Toll-like receptor-dependent activation of several human blood cell types by protamine-condensed mRNA. Eur J Immunol 35:1557–1566
Schellhammer PF, Chodak G, Whitmore JB, Sims R, Frohlich MW, Kantoff PW (2013) Lower baseline prostate-specific antigen is associated with a greater overall survival benefit from sipuleucel-T in the Immunotherapy for Prostate Adenocarcinoma Treatment (IMPACT) trial. Urology 81:1297–1302
Scher HI, Halabi S, Tannock I, Morris M, Sternberg CN, Carducci MA, Eisenberger MA, Higano C, Bubley GJ, Dreicer R et al (2008) Design and end points of clinical trials for patients with progressive prostate cancer and castrate levels of testosterone: recommendations of the Prostate Cancer Clinical Trials Working Group. J Clin Oncol 26:1148–1159
Scher HI, Fizazi K, Saad F, Taplin ME, Sternberg CN, Miller K, de Wit R, Mulders P, Chi KN, Shore ND et al (2012) Increased survival with enzalutamide in prostate cancer after chemotherapy. N Engl J Med 367:1187–1197
Schlake T, Thess A, Fotin-Mleczek M, Kallen KJ (2012) Developing mRNA-vaccine technologies. RNA Biol 9:1319–1330
Sebastian M, von Boehmer L, Zippelius A, Mayer F, Reck M, Atanackovic D, Thomas M, Schneller F, Stoehlmacher J, Goekkurt E et al (2011) Messenger RNA vaccination in NSCLC: Findings from a phase I/IIa clinical trial. J Clin Oncol 29(suppl):abstr 2584
Sebastian M, von Boehmer L, Zippelius A, Mayer F, Reck M, Atanackovic D, Thomas M, Schneller F, Stoehlmacher-William J, Goekkurt E et al (2012) Messenger RNA vaccination and B-cell responses in NSCLC patients. J Clin Oncol 30(suppl):abstr 2573
Sheikh NA, Petrylak D, Kantoff PW, Dela Rosa C, Stewart FP, Kuan LY, Whitmore JB, Trager JB, Poehlein CH, Frohlich MW et al (2013) Sipuleucel-T immune parameters correlate with survival: an analysis of the randomized phase 3 clinical trials in men with castration-resistant prostate cancer. Cancer Immunol Immunother 62:137–147
Small EJ, Schellhammer PF, Higano CS, Redfern CH, Nemunaitis JJ, Valone FH, Verjee SS, Jones LA, Hershberg RM (2006) Placebo-controlled phase III trial of immunologic therapy with sipuleucel-T (APC8015) in patients with metastatic, asymptomatic hormone refractory prostate cancer. J Clin Oncol 24:3089–3094
Spranger S, Javorovic M, Burdek M, Wilde S, Mosetter B, Tippmer S, Bigalke I, Geiger C, Schendel DJ, Frankenberger B (2010) Generation of Th1-polarizing dendritic cells using the TLR7/8 agonist CL075. J Immunol 185:738–747
Spranger S, Frankenberger B, Schendel DJ (2012) NOD/scid IL-2Rg(null) mice: a preclinical model system to evaluate human dendritic cell-based vaccine strategies in vivo. J Transl Med 10:30
Stein WD, Gulley JL, Schlom J, Madan RA, Dahut W, Figg WD, Ning YM, Arlen PM, Price D, Bates SE et al (2011) Tumor regression and growth rates determined in five intramural NCI prostate cancer trials: the growth rate constant as an indicator of therapeutic efficacy. Clin Cancer Res 17:907–917
Tannock IF, de Wit R, Berry WR, Horti J, Pluzanska A, Chi KN, Oudard S, Theodore C, James ND, Turesson

I et al (2004) Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. N Engl J Med 351:1502–1512

Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB et al (2012) Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 366:2443–2454

Van Lint S, Goyvaerts C, Maenhout S, Goethals L, Disy A, Benteyn D, Pen J, Bonehill A, Heirman C, Breckpot K et al (2012) Preclinical evaluation of TriMix and antigen mRNA-based antitumor therapy. Cancer Res 72:1661–1671

Van Nuffel AM, Wilgenhof S, Thielemans K, Bonehill A (2012) Overcoming HLA restriction in clinical trials: Immune monitoring of mRNA-loaded DC therapy. Oncoimmunology 1:1392–1394

Walsh KB, Teijaro JR, Zuniga EI, Welch MJ, Fremgen DM, Blackburn SD, von Tiehl KF, Wherry EJ, Flavell RA, Oldstone MB (2012) Toll-like receptor 7 is required for effective adaptive immune responses that prevent persistent virus infection. Cell Host Microbe 11:643–653

Walter S, Weinschenk T, Stenzl A, Zdrojowy R, Pluzanska A, Szczylik C, Staehler M, Brugger W, Dietrich PY, Mendrzyk R et al (2012) Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. Nat Med 18:1254–1261

Weide B, Carralot JP, Reese A, Scheel B, Eigentler TK, Hoerr I, Rammensee HG, Garbe C, Pascolo S (2008) Results of the first phase I/II clinical vaccination trial with direct injection of mRNA. J Immunother 31:180–188

Weide B, Pascolo S, Scheel B, Derhovanessian E, Pflugfelder A, Eigentler TK, Pawelec G, Hoerr I, Rammensee HG, Garbe C (2009) Direct injection of protamine-protected mRNA: results of a phase 1/2 vaccination trial in metastatic melanoma patients. J Immunother 32:498–507

Wickens M (1990) How the messenger got its tail: addition of poly(A) in the nucleus. Trends Biochem Sci 15:277–281

Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, Segal NH, Ariyan CE, Gordon RA, Reed K et al (2013) Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med 369:122–133

Wollenberg A, Wagner M, Gunther S, Towarowski A, Tuma E, Moderer M, Rothenfusser S, Wetzel S, Endres S, Hartmann G (2002) Plasmacytoid dendritic cells: a new cutaneous dendritic cell subset with distinct role in inflammatory skin diseases. J Invest Dermatol 119:1096–1102

Recent Developments in the Active Immunotherapy of Renal Cell Cancer

Harpreet Singh-Jasuja

Introduction

Renal cell carcinoma (RCC) is the adenocarcinoma of the kidney and the most common form of kidney cancer. More than 300,000 people are newly affected by RCC every year globally (Ferlay et al. 2012). The most common subtype of RCC is clear-cell RCC which comprises about 75 % of RCCs. For two decades, cytokine-based immunotherapy was the standard of care for the management of RCC. In the last 10 years, a better understanding of the tumor biology of RCC has led to the approval of a number of novel agents targeting angiogenesis and has led to an improvement of patient outcomes. However, almost all patients eventually develop a resistance to antiangiogenic agents, and the extension of overall survival (by each individual agent on its own) has remained relatively modest. These observations as well as recent remarkable developments in the field of cancer immunotherapy have strongly resurged the interest in immunotherapeutic approaches for treatment of RCC. This chapter summarizes the recent developments in the treatment of RCC through immunotherapies approved or in clinical development as well as the immunological role of targeted therapies.

H. Singh-Jasuja, PhD
Immatics Biotechnologies GmbH,
Paul-Ehrlich-Str. 15, Tuebingen 72076, Germany
e-mail: singh@immatics.com

Cytokine-Based Immunotherapy

Before approval of the first tyrosine kinase inhibitors (TKIs) in the mid of the last decade, cytokine-based immunotherapy involving interleukin-2 and/or interferon-α was the major therapeutic option in the management of advanced RCC. A small proportion of patients (10–15 %) demonstrate complete radiological response after treatment with these cytokines, and approx. 60–70 % of these complete responses are sustainable after long-term follow-up (Fyfe et al. 1996). Although the role of cytokines has been significantly decreased since the advent of targeted therapies, high-dose cytokine therapy remains the only approved therapeutic regimen that can result in durable objective responses and possibly even to cure. Particularly with high-dose IL-2, due to toxicity concerns, the therapy is often limited only to younger patients who are fit enough to tolerate the therapy. Efforts to expand the proportion of responders as well as to better prospectively select for patients responding to IL-2 are ongoing.

Antiangiogenic Targeted Therapy

In the last decade, angiogenic targets, particularly vascular endothelial growth factor (VEGF) or its receptor (VEGFR), key mediators of neoangiogenesis, and the mammalian target of rapamycin (mTOR), a key mediator of tissue growth, proliferation, and angiogenesis, were

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_16,

moved into the focus of RCC treatment (Mihaly et al. 2012). Since 2005, seven drugs addressing these targets have been approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA). Four agents, sorafenib (first approved 2005), sunitinib (2006), pazopanib (2009), and axitinib (2012), directly inhibit VEGFR; two agents inhibit mTOR, temsirolimus (2007) and everolimus (2009); and one agent is an antibody binding directly to VEGF, bevacizumab plus interferon-α (approved in 2009). While these agents were the first after two decades to demonstrate significant clinical benefit (i.e., partial responses and stable diseases in 60–70 % of patients) in the management of RCC, durable responses are very rare, and eventually all patients develop resistances to these targeted agents. Efforts to increase the efficacy have led to combination of targeted agents which were strongly limited by the addition of individual toxicities of these drugs as well as newly unexpected toxicities. Thus, development of most of such combinations has been discontinued.

Immunological Effects of Targeted Therapies

It is known that VEGF is an immunosuppressive molecule, and anti-VEGF strategies have demonstrated enhancement of immune activity including improving endogenous dendritic cell (DC) function (Gabrilovich et al. 1999), decrease of numbers of regulatory T cells (Hipp et al. 2008; Finke et al. 2008), and enhancement of T-cell infiltration into tumor tissue (Shrimali et al. 2010). For this reason, a rationale for combination of direct VEGF inhibition by bevacizumab – an antibody preventing VEGF to bind to its receptor – and active immunotherapy can be envisioned.

With regard to TKIs, we explored in detail sorafenib and sunitinib and identified differences in their effects on immune cells (Hipp et al. 2008). Sorafenib, but not sunitinib, inhibited the function of human DCs in vitro in response to TLR ligands as well as by their impaired ability to migrate and stimulate T-cell responses. These inhibitory effects were mediated by inhibition of PI3 and MAP kinases and NF-kappa B signaling. To analyze the effects of both TKIs on cytotoxic T-cell induction in vivo, mice were pretreated with sorafenib or sunitinib and immunized with OVA peptide. Sorafenib, but not sunitinib, application significantly reduced the induction of antigen-specific T cells. On the other hand, the number of regulatory T cells was reduced in peripheral blood mononuclear cells from mice treated with sunitinib. In the clinical setting, we found that second-line metastatic RCC patients who had been treated first-line with sunitinib – in contrast to patients pretreated with cytokines or sorafenib – showed significantly reduced numbers of Foxp3+ regulatory T cells in peripheral blood (Walter et al. 2012). Additionally, sunitinib has been described to dramatically decrease the number of myeloid-derived suppressor cells (MDSC) in the clinical setting (Finke et al. 2013). These results indicate that sunitinib may be suitable for (and may even be synergistic in) combination with immunotherapeutic approaches, while the parallel application of sorafenib is expected to have immunosuppressive effects.

The immunological effects of other targeted therapies used in the treatment of RCC are less described. Axitinib, a TKI of VEGF receptors, showed superior antitumor efficacy in terms of overall survival in a murine melanoma model, when combined with vaccination with OVA peptide-loaded DCs engineered to produce IL12p70. Axitinib was also associated with a reduction of MDSC populations and activation and recruitment of type-1, vaccine-induced CD8+ T cells into the tumor (Bose et al. 2012). We have investigated various TKIs in vitro in an allogeneic response setting (unpublished data). Mice were treated for 7 days with physiologically relevant doses of axitinib, tivozanib, pazopanib, sorafenib, or sunitinib. Proliferation of CD8+ and CD4+ T cells in vitro in response to allogeneic spleen cells was compared to those of untreated mice. For both types of responses, axitinib did not change the response significantly, tivozanib and pazopanib had modestly inhibitive effects, and sorafenib had clearly inhibitive effects confirming the observations above. Sunitinib was the only TKI that dramatically increased both CD4+ and CD8+ allogeneic responses.

Autologous Cancer Vaccines

In contrast to targeted therapies, therapeutic cancer vaccines do not directly engage against the tumor cells but attempt to stimulate an antitumor immune response. Such a response is thought to rely on the availability of tumor-associated antigens (TAAs). As initially only very few RCC-associated antigens were available, first attempts of therapeutic vaccination in the clinical setting were based on autologous approaches, also described as passively personalized immunotherapy. Randomized clinical trials were conducted with two autologous vaccines, both conducted in the adjuvant setting of RCC: one using autologous tumor cell lysate (Reniale) and one using autologous Gp96/Grp94 heat shock protein preparations (vitespen), both generated or isolated from the patient's tumor. In the randomized phase III trial for Reniale, 5-year progression-free survival rates showed a trend in favor of the vaccinated over untreated patients (77 % vs. 68 %) (Jocham et al. 2004). However, the European Medicines Agency (EMA) did not grant marketing authorization for Reniale, presumably on the basis of methodological flaws and required further clinical studies. For vitespen, the final analysis of the primary endpoint, recurrence-free survival, in the randomized phase III trial did not show any difference in the vaccine vs. the observation group (Wood et al. 2008). Another autologous vaccine in late-stage development is AGS-003 comprising DCs loaded with autologous RNA isolated from the patient's tumor. In a nonrandomized phase II trial combining AGS-003 in combination with sunitinib, the median OS was reported to be 30.2 months (Figlin et al. 2012). A randomized phase III trial is ongoing (NCT01582672).

Novel Renal Cell Cancer Antigens

The principal disadvantage of autologous vaccination approaches is the lack of knowledge on the nature of the administered TAAs. The administration of molecularly defined TAAs offers several advantages: TAAs can be delivered synthetically in a drug-like fashion (e.g., as peptides or RNA), off-the-shelf without being diluted by non-tumor-associated antigens, without restrictions with regard to the number of vaccinations applied, and without the major challenges in manufacturing and logistics associated with autologous approaches. Moreover, as the nature of the applied TAA is known, the TAA itself can be used to monitor antigen-specific immune responses in peripheral blood and the tumor tissue. Such biological endpoints can be tested for association with clinical endpoints. While the latter does not constitute proof of efficacy, it allows confirming the hypothesized mechanism of action and can also trigger better and informed decisions for the choice of immunomodulation and further combination therapy.

For a long time, the number of known RCC-associated antigens was restricted and thus their application as vaccines was typically limited to single antigens. Discovery of novel RCC-associated antigens is reliably possible through several avenues: (1) to study the expression of TAAs in RCC as well as specific T-cell responses against such RCC-directed antigens (Neumann et al. 1998; Dorrschuck et al. 2004) or (2) through direct elution of HLA-restricted peptides from tumor cell lines or preferably primary tumor tissue (Rammensee et al. 1993). We have performed the latter and identified hundreds of novel RCC-associated antigens directly from RCC tissue using the XPRESIDENT discovery platform, also known as the Tuebingen approach (Weinschenk et al. 2002; Singh-Jasuja et al. 2004; Kruger et al. 2005): HLA-peptide complexes are isolated from tumor cells by affinity chromatography, and high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) is used to isolate and sequence high numbers of HLA-restricted peptides (typically hundreds to thousands from one tumor specimen). These peptides (if their sequence can be unambiguously confirmed) are de facto *naturally presented* tumor-associated peptides (TUMAPs). The analysis of a number of different RCC tissues quickly leads to a saturation of newly discovered peptides which marks the point where the technically accessible immunopeptidome for a given tumor and HLA type has been acquired. From the RCC immunopeptidome, those TUMAPs can be selected that are

derived from highly overexpressed TAAs. Such selected TUMAPs can be used to also perform in vitro priming in order to determine their immunogenicity and further select those TUMAPs with highest biological efficacy (Walter et al. 2012).

Antigen-Based Cancer Vaccines

Following antigen-based cancer vaccines have been or are being developed in advanced-stage clinical trials:

MVA-5T4

MVA-5T4 is a Modified Ankara Viral vector encoding the full-length 5T4 antigen which is highly overexpressed in RCC. A randomized phase III study assessed overall survival in metastatic RCC patients treated with MVA-5T4 or placebo in combination with the standard of care (sunitinib, IL-2, or IFN-a). However, no significant differences in overall survival were observed (Amato et al. 2010), and the failure was partly attributed to the heterogeneous nature of the clinical trial design not restricted to one combination partner.

IMA901

We have employed the XPRESIDENT platform as described above to identify suitable TUMAPs for RCC therapy. Such selected TUMAPs were combined to a multi-peptide vaccine termed IMA901 composed of 9 HLA class I-restricted and 1 HLA class II-restricted peptides (Walter et al. 2012). A total of 96 HLA-A*02-positive subjects with advanced RCC were treated with IMA901 in two consecutive studies. In the phase I study, the T-cell responses of patients to multiple TUMAPs were associated with better disease control and lower numbers of prevaccine forkhead box P3 (FOXP3)-positive T-regulatory cells (Treg). Based on the latter result, in the subsequent trial, all patients received the vaccine and were randomized to receive one dose (300 mg/m^2) of cyclophosphamide ahead of the first vaccination or not. This phase II trial showed that low-dose cyclophosphamide reduced the number of Treg and confirmed that immune responses to multiple TUMAPs were associated with longer overall survival. Furthermore, among the six predefined populations of MDSC, two were shown to be prognostic for overall survival which constitutes the first indication of a clinical significance of MDSC in RCC patients. Based on this observation and for reasons described above, a combination of IMA901 vaccine and sunitinib was rationalized. A randomized phase III study to determine the clinical benefit of treatment with IMA901 in combination with sunitinib is ongoing (Rini et al. 2011).

Immune Resistance in RCC patients

A number of pathways of resistance of tumor cells to immune cells as well as dysfunctionality of immune cells have been described in various cancers including RCC.

The role of downmodulation of HLA class I expression and single components of the antigen-processing machinery in tumor cells is discussed controversially. While some reports demonstrate such defects in RCC lesions (Seliger et al. 2003), others have rather observed upregulation of HLA class I expression in RCC tissue compared to healthy kidney tissue (Stickel et al. 2011) and even, unexpectedly, HLA class II expression (Dengjel et al. 2006) which is normally absent on kidney tissue.

As described above, we have observed abundance of Treg and MDSC in peripheral blood of RCC patients. Two key mechanisms by which MDSCs cause T-cell dysfunction have been reported previously: depletion of arginine, which induces T-cell receptor ζ chain downregulation, and the generation of reactive oxygen species, which induces T-cell tyrosine nitration. Indeed, we found that TCR-ζ expression was significantly lower and nitrotyrosine expression by T cells significantly higher in patients compared with controls. Additionally, TCR-ζ expression

was significantly inversely correlated with numbers of five of the six measured MDSC species (Walter et al. 2012). Low-dose cyclophosphamide and sunitinib may be appropriate measures to affect Treg and MDSC numbers and function, but other agents are also being explored.

Programmed death-1 (PD-1), a member of the B7-CD28 family, is a co-inhibitory receptor expressed by T cells. PD-1 ligates with PD-L1 (B7-H1, CD274) which is frequently expressed by cancer cells including RCC and strongly inversely correlated with poor prognosis (Thompson et al. 2006). Likewise, the analysis of TILs isolated from RCC patients revealed that PD-1 expression in TILs is highly associated with PD-L1 expression on cancer cells and negatively associated with prognosis of such patients (Thompson et al. 2007).

Immune Checkpoint Blockade

Two well-studied immune checkpoint molecules expressed by T cells are CTLA-4 (CD152) and PD1. Both receptors exhibit an inhibitory activity on T cells upon ligation and play a critical role in the negative regulation of T-cell responses. Thus, antibodies blocking such co-inhibitory receptors can "release the brakes" on T cells and are a highly promising therapeutic strategy employed in a number of cancers.

Anti-CTLA-4 Antibody

CTLA-4 expressed by T cells inhibits T-cell proliferation and function upon ligation by CD80 or CD86 expressed by professional antigen-presenting cells. The anti-CTLA-4 antibody ipilimumab has been recently approved for the treatment of advanced melanoma based on two randomized phase III clinical trials. However, treatment is often associated with serious immune-related adverse events (irAE). A phase II study of ipilimumab was conducted in 61 patients with metastatic RCC (Yang et al. 2007). Five of 40 patients at the higher of two administered doses experienced partial responses. While a third of all patients experienced grade 3 or 4 immune-mediated toxicity, there was a highly significant association between irAE and tumor regression. Though these results indicate efficacy of ipilimumab in RCC, the response rate in this initial trial seems relatively modest, particularly in light of the toxicity profile.

Anti-PD1/PD-L1 Antibody

A large phase I study of the single-agent anti-PD1 antibody nivolumab (BMS-936558) developed by Bristol-Myers Squibb conducted in refractory solid tumors showed early evidence of efficacy in RCC patients, while the administration of the antibody was tolerated relatively well. Out of a total of 33 RCC patients treated with anti-PD1 antibody, nine observed objective responses with duration of responses of almost 2 years in some of the patients at the time of the report. Moreover, in a subgroup of patients where immunohistochemical analysis could be performed on pretreatment tumor specimens, intratumoral PD-L1 expression was clearly associated with response to anti-PD1 intervention (Topalian et al. 2012). A randomized phase III study targeted to recruit more than 800 pretreated advanced RCC patients treated with either nivolumab or the mTOR inhibitor everolimus is currently ongoing (NCT01668784).

Another anti-PD1 antibody is lambrolizumab (MK-3475) developed by Merck Inc. Very little information on the single-agent effect of lambrolizumab in RCC patients is available so far, but a large phase I/II trial in combination with the TKI pazopanib (developed by GlaxoSmithKline) is currently in preparation and planned to recruit more than 200 advanced RCC patients (NCT02014636).

MPDL3280A (RG7446) is an antibody developed by Roche targeting PD-L1 on tumor cells and has been explored in a phase I study in 53 advanced RCC patients. Objective responses occurred in 6 out of 47 evaluable patients with most of these responses being durable, and disease control was observed in 34 of 47 patients. Interestingly, the response rate was higher in PD-L1-expressing tumors (Cho et al. 2013).

A randomized phase II study targeting accrual of 150 patients of MPDL3280A as monotherapy or in combination with bevacizumab compared to sunitinib is in preparation (NCT01984242).

Taken together, anti-PD1/PD-L1 approaches constitute a promising novel approach for treatment of RCC while offering a relatively benign safety profile.

Conclusion and Outlook

Active immunotherapy is a highly promising therapeutic strategy for the treatment of RCC. This has been particularly highlighted by the efficacy of anti-PD1 antibodies as initially shown in early-stage clinical trials and to be confirmed by the currently ongoing phase III trials. It seems likely that these antibodies will become part of the standard of care in RCC alongside targeted therapies. Additionally, despite a number of failures in the past, therapeutic vaccines remain promising for several reasons:

1. Though immune resistance mechanisms remain abundant in RCC, our knowledge on these mechanisms is strongly increasing, allowing combination of therapeutic vaccination with agents that provide counteractive measures. Besides certain targeted therapies, blocking immune-inhibitory receptors such as PD-1 or their ligands expressed on tumor tissue such as PD-L1 can be envisioned as attractive partners for cancer vaccines. Importantly, it appears that anti-PD1/PD-L1 requires the existence of T cells infiltrating the tumor tissue. This is only the case in a minority of RCC patients. Thus, starting with therapeutic vaccination to drive RCC-specific T cells into the tumor followed by anti-PD1/PD-L1 intervention to "release the brakes" on these vaccine-induced T cells may be the "dream team" of cancer immunotherapy.
2. Most previously used immunomodulators in conjunction with vaccines exhibit limited properties. A number of novel immunomodulators and antigen delivery systems are now available that will most likely lead to T-cell responses of higher magnitude and quality. Moreover, an increasing number of functional markers to differentiate successful T cells from non-successful ones are becoming available, allowing to explore the most effective way of immunomodulation. Immunomodulators may not be restricted to classical immune adjuvants, but also include standard therapies with immunological effects such as targeted but even classical chemotherapies.
3. The knowledge on relevant cancer antigens has strongly increased over the recent past. This allows moving beyond single antigens towards multiple antigens applied simultaneously for vaccination. Indeed, association of T-cell responses to multiple TAAs is not restricted to RCC only (Walter et al. 2012) but has also been observed in colorectal cancer (unpublished data) and melanoma (Fay et al. 2006). Moreover, latest advances in mass spectrometry as well as in next-generation sequencing (NGS) allow for the first time to fully personalize the antigen composition for every patient based on a comprehensive analysis of the cancer immunopeptidome (Rammensee et al. 2002) and the mutanome (Castle et al. 2012). Indeed, the first clinical trials utilizing such actively personalized vaccines (APVACs) as described by the Regulatory Research Group of the Association of Cancer Immunotherapy (CIMT) (Britten et al. 2013) are currently in preparation in glioblastoma (GAPVAC 2014), melanoma (MERIT Consortium 2014), and liver cancer (HEPAVAC Consortium 2014).

Treatment of advanced RCC is clearly advancing towards higher degrees of personalization. Guided by biomarkers measured with surgical resection and accompanied by a comprehensive genomic and immune cell analysis, the RCC patient of the future will be eligible for a whole regimen, for instance, comprising vaccination with highly personalized (including mutated) antigens tailored to individual tumor antigen profiling combined with immunomodulators fitted to the status of immune resistance mechanisms as well as immune checkpoint blockade and targeted therapy. Based on today's knowledge, this seems to be the most likely path toward moving from a survival advantage of currently only few additional months to years of more and better life for an RCC patient.

Disclosure The author is a shareholder and an employee of immatics biotechnologies which has a commercial interest in the development of IMA901, a therapeutic vaccine for the treatment of renal cell carcinoma.

Acknowledgment The author would like to thank Christoph Huber and Hans-Georg Rammensee for their guiding voices in the always exciting but challengingly complex and somewhat confusing universe of cancer immunology.

References

Amato RJ, Hawkins RE, Kaufman HL et al (2010) Vaccination of metastatic renal cancer patients with MVA-5T4: a randomized, double-blind, placebo-controlled phase III study. Clin Cancer Res 16:5539–5547

Bose A, Lowe DB, Rao A et al (2012) Combined vaccine+axitinib therapy yields superior antitumor efficacy in a murine melanoma model. Melanoma Res 22:236–243

Britten CM, Singh-Jasuja H, Flamion B et al (2013) The regulatory landscape for actively personalized cancer immunotherapies. Nat Biotechnol 31:880–882

Castle JC, Kreiter S, Diekmann J et al (2012) Exploiting the mutanome for tumor vaccination. Cancer Res 72:1081–1091

Cho D, Sosman JA, Sznol M et al (2013) Clinical activity, safety, and biomarkers of MPDL3280A, an engineered PD-L1 antibody in patients with metastatic renal cell carcinoma (mRCC). J Clin Oncol 31(15_Suppl):Abstract 4505

Dengjel J, Nastke MD, Gouttefangeas C et al (2006) Unexpected abundance of HLA class II presented peptides in primary renal cell carcinomas. Clin Cancer Res 12:4163–4170

Dorrschuck A, Schmidt A, Schnurer E et al (2004) CD8+ cytotoxic T lymphocytes isolated from allogeneic healthy donors recognize HLA class Ia/Ib-associated renal carcinoma antigens with ubiquitous or restricted tissue expression. Blood 104:2591–2599

Fay JW, Palucka AK, Paczesny S et al (2006) Long-term outcomes in patients with metastatic melanoma vaccinated with melanoma peptide-pulsed CD34(+) progenitor-derived dendritic cells. Cancer Immunol Immunother 55:1209–1218

Ferlay J, Soerjomataram I, Ervik M et al (2012) GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11. http://globocan.iarc.fr/Default.aspx. Accessed 10 Jan 2014

Figlin RA, Amin A, Dudek A et al (2012) Phase II study combining personalized dendritic cell (DC)-based therapy, AGS-003, with sunitinib in metastatic renal cell carcinoma (mRCC). J Clin Oncol 30(5_Suppl):Abstract 348

Finke JH, Rini B, Ireland J et al (2008) Sunitinib reverses type-1 immune suppression and decreases T-regulatory cells in renal cell carcinoma patients. Clin Cancer Res 14:6674–6682

Finke JH, Rayman PA, Ko JS et al (2013) Modification of the tumor microenvironment as a novel target of renal cell carcinoma therapeutics. Cancer J 19:353–364

Fyfe GA, Fisher RI, Rosenberg SA et al (1996) Long-term response data for 255 patients with metastatic renal cell carcinoma treated with high-dose recombinant interleukin-2 therapy. J Clin Oncol 14:2410–2411

Gabrilovich DI, Ishida T, Nadaf S et al (1999) Antibodies to vascular endothelial growth factor enhance the efficacy of cancer immunotherapy by improving endogenous dendritic cell function. Clin Cancer Res 5:2963–2970

GAPVAC (2014) The Glioma Actively Personalized Vaccine Consortium. http://www.gapvac.eu. Accessed 10 Jan 2014

HEPAVAC Consortium (2014) Cancer vaccine development for hepatocellular carcinoma. http://www.hepavac.eu. Accessed 10 Jan 2014

Hipp MM, Hilf N, Walter S et al (2008) Sorafenib, but not sunitinib, affects function of dendritic cells and induction of primary immune responses. Blood 111:5610–5620

Jocham D, Richter A, Hoffmann L et al (2004) Adjuvant autologous renal tumour cell vaccine and risk of tumour progression in patients with renal-cell carcinoma after radical nephrectomy: phase III, randomised controlled trial. Lancet 363:594–599

Kruger T, Schoor O, Lemmel C et al (2005) Lessons to be learned from primary renal cell carcinomas: novel tumor antigens and HLA ligands for immunotherapy. Cancer Immunol Immunother 54:826–836

MERIT Consortium (2014) Mutanome Engineered RNA Immuno-Therapy (MERIT) Project. http://www.merit-consortium.eu. Accessed 10 Jan 2014

Mihaly Z, Sztupinszki Z, Surowiak P et al (2012) A comprehensive overview of targeted therapy in metastatic renal cell carcinoma. Curr Cancer Drug Targets 12:857–872

Neumann E, Engelsberg A, Decker J et al (1998) Heterogeneous expression of the tumor-associated antigens RAGE-1, PRAME, and glycoprotein 75 in human renal cell carcinoma: candidates for T-cell-based immunotherapies? Cancer Res 58:4090–4095

Rammensee HG, Falk K, Rotzschke O (1993) Peptides naturally presented by MHC class I molecules. Annu Rev Immunol 11:213–244

Rammensee HG, Weinschenk T, Gouttefangeas C et al (2002) Towards patient-specific tumor antigen selection for vaccination. Immunol Rev 188:164–176

Rini BI, Eisen T, Stenzl A et al (2011) IMA901 Multipeptide Vaccine Randomized International Phase III Trial (IMPRINT): a randomized, controlled study investigating IMA901 multipeptide cancer vaccine in patients receiving sunitinib as first-line therapy for advanced/metastatic RCC. J Clin Oncol 29(15_Suppl):Abstract TPS183

Seliger B, Atkins D, Bock M et al (2003) Characterization of human lymphocyte antigen class I antigen-processing machinery defects in renal cell carcinoma

lesions with special emphasis on transporter-associated with antigen-processing down-regulation. Clin Cancer Res 9:1721–1727

Shrimali RK, Yu Z, Theoret MR et al (2010) Antiangiogenic agents can increase lymphocyte infiltration into tumor and enhance the effectiveness of adoptive immunotherapy of cancer. Cancer Res 70:6171–6180

Singh-Jasuja H, Emmerich NP, Rammensee HG (2004) The Tubingen approach: identification, selection, and validation of tumor-associated HLA peptides for cancer therapy. Cancer Immunol Immunother 53:187–195

Stickel JS, Stickel N, Hennenlotter J et al (2011) Quantification of HLA class I molecules on renal cell carcinoma using Edman degradation. BMC Urol 11:1

Thompson RH, Kuntz SM, Leibovich BC et al (2006) Tumor B7-H1 is associated with poor prognosis in renal cell carcinoma patients with long-term follow-up. Cancer Res 66:3381–3385

Thompson RH, Dong H, Lohse CM et al (2007) PD-1 is expressed by tumor-infiltrating immune cells and is associated with poor outcome for patients with renal cell carcinoma. Clin Cancer Res 13:1757–1761

Topalian SL, Hodi FS, Brahmer JR et al (2012) Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 366:2443–2454

Walter S, Weinschenk T, Stenzl A et al (2012) Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. Nat Med 18:1254–1261

Weinschenk T, Gouttefangeas C, Schirle M et al (2002) Integrated functional genomics approach for the design of patient-individual antitumor vaccines. Cancer Res 62:5818–5827

Wood C, Srivastava P, Bukowski R et al (2008) An adjuvant autologous therapeutic vaccine (HSPPC-96; vitespen) versus observation alone for patients at high risk of recurrence after nephrectomy for renal cell carcinoma: a multicentre, open-label, randomised phase III trial. Lancet 372:145–154

Yang JC, Hughes M, Kammula U et al (2007) Ipilimumab (anti-CTLA4 antibody) causes regression of metastatic renal cell cancer associated with enteritis and hypophysitis. J Immunother 30: 825–830

Current Status of Immunotherapy in Gastroesophageal Cancer

Stefan Kasper and Martin Schuler

Introduction

Cancers of the stomach (GC), the gastroesophageal junction (GEJ), and the distal esophagus are the second leading cause of cancer deaths worldwide (Ferlay et al. 2010). Incidence rates and tumor localization vary considerably between geographical regions thus implying genetic and environmental factors in disease pathophysiology. Adenocarcinomas of the distal stomach are dominant in Eastern Europe, Asia, and South America. In contrast, cancers located in the proximal stomach or at the GEJ are more prevalent in Western Europe and North America (Kamangar et al. 2006). This is attributed to the high association of *Helicobacter pylori* infection and additional nutritional and socioeconomic risk factors with distal GC in less-developed countries (Parkin 2004). In contrast, risk factors for adenocarcinomas of the GEJ and distal esophagus such as obesity and gastroesophageal reflux disease have become more prevalent in countries adopting a "Western" lifestyle. Accordingly, increased incidence rates have been observed in Western Europe and North America (Cervantes et al. 2007).

S. Kasper
Department of Medical Oncology,
West German Cancer Center, University Hospital Essen, University Duisburg-Essen, Essen, Germany
e-mail: stefan.kasper@uk-essen.de

M. Schuler (✉)
Department of Medical Oncology,
West German Cancer Center, University Hospital Essen, University Duisburg-Essen, Essen, Germany

German Cancer Consortium (DKTK),
Heidelberg, Germany
e-mail: martin.schuler@uk-essen.de

Current Treatment Options for Localized, Locally Advanced, and Metastatic Disease

Surgical resection and, for very early stage cancers, endoscopic mucosal resection remain the standard of care for localized cancers of the upper gastrointestinal (GI) tract. However, the majority of patients are diagnosed with locally advanced tumors, regional lymph node involvement, or metastatic disease. Based on the MAGIC and ACCORD trials, perioperative chemotherapy has been established as the standard of care in Western Europe for patients with locally advanced GEJ/GC scheduled for surgical resection including extensive (D2) lymphadenectomy (Cunningham et al. 2006; Ychou et al. 2011). Systemic treatments in the perioperative setting are largely based on platinum and fluoropyrimidine agents alone or in combination with taxanes or anthracyclines. Preoperative radiochemotherapy is another option for patients with advanced adenocarcinomas of the GEJ and the distal esophagus. In particular, patients achieving a complete pathological response following induction therapy appear to benefit from this approach (van Hagen et al. 2012; Stahl et al. 2009). In contrast, postoperative

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_17,

radiochemotherapy has evolved as a standard in North American centers based on the SWOG/Intergroup 0116 trial, which mainly enrolled patients with less extensive (D0 or D1) lymph node dissection (Smalley et al. 2012). Adjuvant chemotherapies following gastrectomy and D2 lymph node dissection have improved disease specific and overall survival. However, this effect so far was confined to Asian populations (Sasako et al. 2011). Despite these progresses, more than 50 % of patients undergoing potentially curative multimodal therapy for locally advanced GEJ/GC ultimately relapse. Palliative chemotherapy prolongs survival and improves cancer-related symptoms in these patients as well as in patients with primary metastatic disease (Wagner et al. 2006). When taxanes are added to platin- and fluoropyrimidine-based regimens, remission rates and disease control are enhanced. However, the median survival time of patients with metastatic disease stagnates at approximately 12 months (Van Cutsem et al. 2006; Cunningham et al. 2006; Ychou et al. 2011).

Recent insights into the biology of gastrointestinal cancers have nominated potential targets for novel molecular therapies and biomarkers for stratification of patient populations. Here we review the current status and future developments of clinical immunotherapy in GEJ/GC.

Growth Factor Receptors as Therapeutic Targets

Growth factor receptors are receptor tyrosine kinase proteins located in the plasma membrane. Their physiological function is to transduce signals derived from locally and systemically secreted ligands into the cell to promote proliferation and survival. Ligand binding induces homo- or heterodimerization of the receptor molecules, which facilitates autophosphorylation and activation of receptor tyrosine kinase as well as the recruitment of adaptor molecules to form signaling complexes at the inner cell membrane. Growth factor receptor signaling can be deregulated in cancer by various mechanisms including aberrantly increased receptor expression, autocrine or paracrine ligand secretion, and somatic mutations leading to structurally altered receptors with enhanced or constitutive activation. Two pharmacologic strategies have been devised to target growth factor receptors in GI cancers: (1) Monoclonal antibodies (moAb) binding epitopes of the extracellular domain of growth factor receptors can prevent receptor-ligand interaction, receptor dimerization, and thus activation. In addition, moAbs of specific isotypes may trigger immunological mechanisms such as antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), or recruitment of effector cells of the adaptive immune system, which lead to elimination of antibody-marked cells. Antibodies or recombinant receptor-antibody fusion proteins may also be used to neutralize ligands such as the vascular endothelial growth factor (VEGF), which contribute to tumor progression or can be conjugated with cytotoxic drugs and can serve as transporter molecules. (2) Small molecule compounds have been developed that enter the cell and interfere with the enzymatic function of receptor tyrosine kinases or intracellular signaling molecules to inhibit aberrant signal transduction. Most compounds that have been clinically explored inhibit the kinase activity by competing with adenosine triphosphate (ATP) binding in the catalytic domain. While small molecule tyrosine kinase inhibitors so far failed to show substantial clinical activity in phase III trials, several monoclonal antibodies were approved for the treatment of advanced GC/GEJ in the last years or are in advanced clinical evaluation.

Targeting Receptors of the ERBB Family

Monoclonal antibodies have been devised to target receptors of the ERBB family, including EGFR and HER2/ERBB2. The EGFR is highly expressed in up to 50 % of gastroesophageal adenocarcinomas and was associated with dismal outcome in patients with metastatic disease.

In contrast to EGFR, HER2 is an "orphan receptor" lacking a natural ligand. It unfolds its activity by heterodimerization with other ERBB family receptors, such as EGFR and HER4. While HER2 overexpression in breast cancer clearly associates with more aggressive tumor biology and disease course (Slamon et al. 1989), its prognostic value in GC is less clear. Highly recurrent somatic mutations leading to structural alterations of the EGFR have not been described in this disease (Kim et al. 2007; Okines et al. 2011). Overexpression of the HER2/ERBB2 receptor is observed in 20–30 % of GC, predominantly of the intestinal subtype, and GEJ cancers (Tanner et al. 2005; Kim et al. 2008). Due to intratumoral heterogeneity of receptor expression and divergent staining characteristics, specific skills are demanded from the pathologist to detect HER2-positive GC by immunohistochemistry. Ambiguous cases are thus validated by probing *HER2* gene amplification using in situ hybridization techniques (Grabsch et al. 2010).

Antibodies Targeting EGFR

Cetuximab (chimeric IgG1) and panitumumab (human IgG2) are the first anti-EGFR moAbs approved for the treatment of metastatic colorectal cancer based on activity as single agents and in combination with chemotherapy (Bokemeyer et al. 2009; Cunningham et al. 2004; Douillard et al. 2010; Peeters et al. 2010; Van Cutsem et al. 2007; Van Cutsem et al. 2009). Besides its direct cytotoxic activity, cetuximab is capable to mediate antibody-dependent cellular cytotoxicity (ADCC) in vitro and in vivo (Kasper et al. 2013). In contrast, panitumumab is unable to induce classical, natural killer (NK) cell-mediated ADCC due to its IgG_2 isotype. It was found that patients with colorectal cancers harboring somatic mutations of *RAS* oncogenes do not benefit from anti-EGFR antibody therapy (Douillard et al. 2013; De Roock et al. 2010). Cetuximab is also approved for the treatment of recurrent or metastatic head and neck squamous cell carcinoma (HNSCC), either in combination with radiotherapy in locally advanced disease or in combination with platinum-based chemotherapy (Vermorken et al. 2008; Bonner et al. 2006).

Anti-EGFR moAbs have been extensively studied in patients with metastatic adenocarcinomas of the stomach and GEJ. Several phase I and phase II trials combining moAbs with chemotherapy revealed promising activity and acceptable toxicity profiles (Han et al. 2009; Moehler et al. 2011; Lordick et al. 2010; Kim et al. 2011; Pinto et al. 2007; Pinto et al. 2009; Okines et al. 2010; Rao et al. 2010; Trarbach et al. 2013). Based on these results, two large phase III studies were conducted in patients with advanced GEJ/GC. The EXPAND trial evaluated the combination of cetuximab with capecitabine and cisplatin (Lordick et al. 2013). The addition of cetuximab provided no significant benefit in terms of progression-free survival (PFS), overall survival (OS), or overall response rate (ORR, Table 1). Similar results were obtained by the REAL-3 study, which explored the addition of panitumumab to the EOX (epirubicin, oxaliplatin, and capecitabine) chemotherapy regimen (Waddell et al. 2013). Based on toxicities observed in a prior dose-finding study (Okines et al. 2010), the chemotherapy doses in the panitumumab arm of REAL-3 had to be modified (mEOX), whereas the control received fully dosed chemotherapy. Interestingly, panitumumab combined with mEOX resulted in inferior PFS and OS as compared to EOX (Table 1). Based on these two trials, anti-EGFR moAbs in combination with chemotherapy are not recommended for the treatment of patients with metastatic adenocarcinoma of the stomach or GEJ. Defining the role of anti-EGFR moAbs in preoperative radiochemotherapy or perioperative chemotherapy of patients with locally advanced disease is subject to ongoing trials (NeoPECX, NCT01234324; SAKK75/08, NCT01107639).

Antibodies Targeting HER2

The IgG1 moAbs trastuzumab and pertuzumab prevent heterodimerization of the HER2/ERBB2 receptor, trigger receptor internalization, and

Table 1 Antibodies targeting growth factor receptors and angiogenesis

Author	Indication	Study name	Agents	*N*	ORR [%]	PFS [months]	OS [months]
Lordick et al. (2013)	Metastatic GC and GEJ	EXPAND phase III	Capecitabine/ cisplatin ± cetuximab	904	30 vs. 29; $p=0.77$	4.4 vs. 5.9; HR 1.09 [95 % CI 0.92–1.29; $p=0.32$]	9.4 vs. 10.7; HR 1.0 [95 % CI 0.87–1.17; $p=0.95$]
Waddell et al. (2013)	Metastatic GC and GEJ	REAL3 phase III	Epirubicin/oxaliplatin/ capecitabine (EOX) vs. mEOX + panitumumab	553	46 vs. 42; $p=0.42$	6.0 vs. 7.4; HR 1.22 [95 % CI 0.98–1.52; $p=0.068$]	8.8 vs. 11.3; HR1.37 [95 % CI 1.07–1.76; $p=0.013$]
Bang et al. (2010)	Metastatic GC and GEJ; Her2/ neu positive	ToGA phase III	Fluoropyrimidine/ cisplatin ± trastuzumab	594	47 vs. 35; $p=0.002$	6.7 vs. 5.5; HR 0.71 [95 % CI 0.59–0.85; $p=0.0002$]	13.8 vs. 11.1; HR 0.74 [95 % CI 0.60–0.91; $p=0.005$]; [a]16.0 vs. 11.8; HR 0.65 [95 % CI 0.51–0.83; $p=0.036$]
Ohtsu et al. (2011)	Metastatic GC and GEJ	AVAGAST phase III	Capecitabine/ cisplatin ± bevacizumab	774	46 vs. 37.4; $p=0.0315$	6.7 vs. 5.3; HR 0.80 [95 % CI 0.68–0.93; $p=0.0037$]	12.1 vs. 10.1; HR 0.87 [95 % CI 0.73–1.03; $p=0.1002$];
Fuchs et al. (2013)	Second-line GC and GEJ	REGARD phase III	BSC ± ramucirumab (2:1)	355	49 vs. 23 (DCR)	2.1 vs. 1.3; HR 0.483; [95 % CI 0.376–0.620; $p<0.0001$]	5.2 vs. 3.8; HR 0.776 [95 % CI 0.603–0.998; $p=0.0473$]

BSC best supportive care, *FISH* fluorescence in situ hybridization, *IHC* immunohistochemistry, *m* modified
[a]Explorative subgroup analysis Her2/neu IHC3+ and IHC2+/FISH+

mediate ADCC. T-DM1 is a conjugate of trastuzumab and the cytotoxic agent mertansine. After binding and internalization of the construct, mertansine is released and destroys cancer cells by interacting with tubulin. Trastuzumab is approved as single agent and in combination with chemotherapy and antihormonal therapy for the adjuvant and palliative treatment of HER2-positive breast cancer (Slamon et al. 2001; Smith et al. 2007; Goldhirsch et al. 2013). Against this background, a phase III study, ToGA, was conducted to explore the addition of trastuzumab to cisplatin/fluoropyrimidine-based chemotherapy in the treatment of metastatic HER2-positive GEJ/GC (Bang et al. 2010). The addition of trastuzumab significantly increased the ORR and prolonged PFS and OS (Table 1). In an explorative post hoc analysis, the subgroup of patients with high (IHC 3+) or intermediate HER2 expression (IHC2+) and gene amplification (FISH+) benefited the most (Table 1). Accordingly, trastuzumab combined with platin-/fluoropyrimidine-based chemotherapy has become the standard of care for metastatic, HER2-positive GEJ/GC. The role of trastuzumab in the perioperative therapy of locally advanced HER2-positive cancers is being explored in trials such as the Her-FLOT study (NCT01472029). The moAb pertuzumab was developed to target a different epitope of the HER2/ERBB2 receptor than trastuzumab. Recently, pertuzumab was approved in combination with trastuzumab and docetaxel for the first-line treatment of metastatic HER2-positive breast cancer based on the CLEOPATRA study (Baselga et al. 2012). The ongoing JACOB study (NCT01774786) explores the value of adding pertuzumab to trastuzumab and chemotherapy in metastatic HER2-positive GEJ/GC (Table 2). T-DM1 has recently been approved for the treatment of HER2-positive trastuzumab-resistant metastatic breast cancer as monotherapy based on the positive results of the EMILIA trial (Verma et al. 2012). In HER2-positive GEJ/GC recently, T-DM1 is being explored in second-line treatment after failure of platinum-based chemotherapy in the GATSBY study (NCT01641939) (Table 2).

Antibodies Targeting MET (Hepatocyte Growth Factor Receptor)

The MET receptor tyrosine kinase is physiologically expressed by stem cells. It is essential for embryonic development and wound healing. Hepatocyte growth factor (HGF), its natural ligand, is mainly secreted by mesenchymal cells. In experimental systems, HGF/MET signals mediate cell migration, invasion, and angiogenesis. The former is associated with a particular phenotype termed epithelial-to-mesenchymal transition (EMT). Aberrant MET expression and signaling has been observed in several cancers including papillary renal cell carcinoma, non-small cell lung cancer, hepatocellular carcinoma, and glioblastoma. This can be associated with *MET* gene amplification (Peters and Adjei 2012). Approximately 10–15 % of GC exhibit *MET* amplification, and high MET protein expression is found in up to 30 % of cases. Both have been associated with poor prognosis (Bachleitner-Hofmann et al. 2008; Nakajima et al. 1999; Lee et al. 2012). Several moAbs and small molecule inhibitors have been developed to target MET. Recently, two large randomized phase III studies have been initiative that combine anti-MET moAbs, onartuzumab, and rilotumumab, with chemotherapy in treatment of patients with advanced GEJ/GC overexpressing MET. In the MetGastric (NCT01662869) trial, 800 patients will be treated with a modified FOLFOX6 protocol with or without onartuzumab (Table 2). In the RILOMET-1 (NCT01697072) trial, 450 patients will receive ECX with or without combined rilotumumab (Table 2). Definition and prospective validation of robust biomarkers indicative of treatment benefit from MET targeting will be critical for the success of anti-MET moAbs.

Targeting Angiogenesis

Tumor angiogenesis is considered an essential "hallmark" of cancer (Hanahan and Weinberg 2011). During tumor progression, an angiogenic switch is postulated to occur in response to

Table 2 Novel immunotherapeutics in clinical trials

Target	Immunotherapeutic	Mode of action	Combination agents	Indication	Study name	N	PI/Author
Claudin 18.2	Claudiximab (IMAB362)	ADCC, CDC, direct cytotoxicity	Monotherapy	Claudin 18.2 positive metastatic GC and GEJ further line	Phase Ib/II (NCT00909025)	15/34	Schuler et al. (2013)
Claudin 18.2	Claudiximab (IMAB362)	ADCC, CDC, direct cytotoxicity	Epirubicin/oxaliplatin/ fluoropyrimidine	Claudin 18.2 overexpressing metastatic GC and GEJ first line	FAST phase II (NCT01630083)	210	Schuler M, Essen, Germany
HER2/ neu	Pertuzumab	ADCC, CDC, direct cytotoxicity	Cisplatin/ fluoropyrimidine + trastuzumab	Her2/neu-positive metastatic GC and GEJ first line	JACOB phase III (NCT01774786)	780	Hoffmann-La Roche
HER2/ neu	T-DM1	ADCC, CDC, direct cytotoxicity, mertansine conjugated	Monotherapy vs. taxane	Her2/neu-positive metastatic GC and GEJ, second line	GATSBY phase II/ III (NCT01641939)	412	Hoffmann-La Roche
cMET	Onartuzumab	ADCC, CDC, direct cytotoxicity	Oxaliplatin/folinic acid/5-fluorouracil (mFOLFOX6)	cMET positive metastatic GC and GEJ first line	MetGastric (NCT01662869)	800	Hoffmann-La Roche
cMET	Rilotumumab	ADCC, CDC, direct cytotoxicity	Epirubicin/cisplatin/capecitabine	cMET positive metastatic GC and GEJ, first line	RILOMET-1, phase III (NCT01697072)	450	Amgen
CTLA4	Tremelimumab	Immune checkpoint inhibition	Monotherapy	Metastatic GC and GEJ, second line	Phase II	18	Ralph et al. (2010)
CTLA4	Ipilimumab	Immune checkpoint inhibition	Monotherapy vs. standard of care after first-line chemotherapy	Maintenance after first-line therapy	Phase II (NCT01585987)	114	Bristol-Myers Squibb
EpCAM	Catumaxomab	Trifunctional antibody binding EpCAM-positive cancer cells, T cells, and Fcγ-receptor-positive immune cells	Monotherapy vs. paracentesis only	Malignant ascites due to EpCAM-positive cancer	Phase III (NCT00836654)	258	Heiss et al. (2010)
EpCAM	Catumaxomab	Trifunctional antibody binding EpCAM-positive cancer cells, T cells, and Fcγ-receptor-positive immune cells	Monotherapy followed by chemotherapy with 5-fluorouracil, folinic acid, oxaliplatin, docetaxel	Peritoneal carcinomatosis in patients with GC/GEJ, neoadjuvant	AIO-STO-0110, phase II (NCT01784900)	42	Lordick F, Leibzig, Germany
EpCAM	Catumaxomab	Trifunctional antibody binding EpCAM-positive cancer cells, T cells, and Fcγ-receptor-positive immune cells	Monotherapy after complete surgical resection of peritoneal carcinomatosis	Peritoneal carcinomatosis in patients with GC/GEJ after complete resection, adjuvant	Phase II (NCT01784900)	120	Gustave Roussy, Cancer Campus, Grand Paris, France

ADCC antibody-dependent cellular cytotoxicity, *CDC* complement-dependent cytotoxicity

hypoxia and nutrient depletion of the tumor. Formation of new blood vessels or sprouting from existing ones is particularly initiated by vascular endothelial growth factor (VEGF), which is secreted by tumor and stroma cells. High VEGF expression in tumor specimens correlated with poor prognosis in GC patients (Chen et al. 2013). Two alternative pharmacological strategies have been devised to modulate angiogenic signaling in cancer therapy: (1) depletion of angiogenic factors by moAbs such as the anti-VEGF antibody bevacizumab, or recombinant receptor-antibody fusion proteins such as aflibercept and (2) targeting angiogenic receptors with moAbs such as the anti-VEGFR2 antibody ramucirumab or small tyrosine kinase inhibitors. While the role of bevacizumab is not conclusively determined, recent study reports suggest activity of ramucirumab in second-line treatment of metastatic GEJ/GC.

Anti-VEGF Antibody Therapy

Bevacizumab is a humanized moAb targeting VEGF-A. It has been approved in combination with chemotherapy for the treatment of metastatic colorectal, ovarian, and non-small cell lung cancers and in combination with interferon-alpha for the treatment of metastatic renal cell cancer. It is still approved for combination therapy of metastatic breast cancer in Europe. The large randomized phase III study AVAGAST evaluated the addition of bevacizumab to palliative chemotherapy with capecitabine and cisplatin in patients with advanced GEJ/GC. Median PFS and ORR were improved in the bevacizumab arm (Table 1), but the primary endpoint of the study, prolongation of OS, was not met (Ohtsu et al. 2011). Retrospective subgroup analyses suggested that patients with high levels of VEGF-A and low levels of neuropilin-1 in the serum benefitted the most. Also, a positive effect of bevacizumab was observed in patients treated at US centers (Ohtsu et al. 2011; Van Cutsem et al. 2012). An ongoing randomized phase III trial of the UK Medical Research Council in patients with locally advanced GEJ/GC explores the effect of adding bevacizumab to perioperative therapy with epirubicin, capecitabine, and cisplatin (MAGIC-B, NCT00450203). In summary, there is no data to support the use of bevacizumab in palliative treatment of GEJ/GC, whereas its role in perioperative therapy remains to be defined.

Anti-VEGFR Antibody Therapy

The anti-VEGFR2 moAb ramucirumab showed single-agent activity and a favorable toxicity profile in a randomized phase III study (REGARD) in pretreated patients with advanced GEJ/GC. Median PFS and OS with ramucirumab were comparable to results obtained in trials of second-line chemotherapy (Fuchs et al. 2013; Table 1). Another randomized phase III study (RAINBOW, NCT01170663) compared the efficacy of paclitaxel in combination with ramucirumab with paclitaxel plus placebo in second-line treatment of GEJ/GC. Recently, primary analysis demonstrated an improvement of OS for the combination of paclitaxel with ramucirumab of two months (9.63 months versus 7.36 months) as compared to paclitaxel plus placebo (Wilke et al. 2014).

Targeting Cell-Cell Adhesion Molecules

Cell adhesion molecules (CAMs) are expressed by epithelial cells and interact with the extracellular matrix and cell-cell adhesion. In addition, CAMs are involved in functions including signal transduction, cell motility, and inflammation. Loss of intercellular adhesion or desquamation of cells from the extracellular matrix allows cancer cells to escape from their site of origin and invade into the lymphatic or blood system. Circulating cancer cells use adhesion molecules to establish distant metastases in organs different from the site of the primary tumor. Accordingly, CAMs represent an attractive therapeutic target for moAb.

Malignant ascites due to peritoneal carcinomatosis is a common clinical manifestation of advanced GC/GEJ. Complete surgical resection followed by hyperthermic intraperitoneal chemotherapy (HIPEC) is a treatment option that

may achieve long-lasting disease control in selected patients with peritoneal carcinomatosis. However, only a minority of patients with metastatic GC/GEJ is suited for this aggressive procedure with high perioperative morbidity and mortality rates. An alternative palliative approach is the intraperitoneal application of the trifunctional IgG1 antibody catumaxomab. Catumaxomab binds to the epithelial cell antigen EpCAM on cancer cells and the human CD3 T cell antigen. Via its rat Fc region, catumaxomab engages immune effector cells such as natural killer cells (NK), dendritic cells (DC), and macrophages. This interaction is thought to induce a complex immune reaction leading to elimination of tumor cells. In a randomized clinical phase II/III trial (NCT00836654), patients with chemotherapy-refractory ascites due to EpCAM-positive tumors were treated with paracentesis only or with paracentesis followed by intraperitoneal injection of catumaxomab (Heiss et al. 2010). The application of the trifunctional moAb significantly prolonged the paracentesis-free interval and resulted in numerically prolonged overall survival (Table 2). These results lead to the approval of catumaxomab for the therapy of refractory ascites due to EpCAM-positive cancers. Currently, several trials are ongoing to explore the efficacy of catumaxomab in neoadjuvant or adjuvant settings before and after surgical resection of peritoneal carcinomatosis in patients with GC/GEJ (NCT01784900, NCT01784900; Table 2).

An in silico screen for tetraspanin receptors specifically expressed on cancer cells nominated claudin 18.2 as a putative antibody target in GEJ/GC (Sahin et al. 2008). Claudiximab, a chimeric moAb against claudin 18.2 with optimized ADCC, CDC, and direct cytotoxic activities, is in advanced early clinical development in patients with metastatic GEJ/GC. Safety, toxicities, and recommended phase II doses of claudiximab were established in monotherapy trials enrolling heavily pretreated patients with claudin 18.2-positive adenocarcinoma of the stomach or GEJ, which revealed signals of moderate single-agent activity (Schuler et al. 2013). Currently, a randomized phase II study, FAST, explores the addition of claudiximab to first-line chemotherapy with EOX in patients with claudin 18.2-positive GEJ/GC (NCT01630083, Table 2).

Targeting Immunoregulatory Signals

Recently, the so-called immune checkpoint inhibitors, antibodies targeting T cell regulatory molecules such as CTLA4, PD-1, or PD-L1, have emerged as potentially effective agents over a wide range of tumor entities (Wolchok et al. 2013; Hamid et al. 2013; Brahmer et al. 2012; Topalian et al. 2012; Hodi et al. 2010). In the corresponding phase I trials with these new immunotherapeutics, only a few patients with GC or GEJ were included. In a phase II trial, the anti-CTLA4 antibody tremelimumab was explored as second-line treatment in 18 patients with advanced GC/GEJ (Ralph et al. 2010). Despite an overall low response rate in the entire study population, one patient had a long-lasting partial remission, thus providing an interesting clinical signal (Table 2). Recently, a phase II trial evaluating the role of ipilimumab as maintenance therapy after platinum-based chemotherapy in advanced GC/GEJ was initiated (NCT01585987, Table 2). Currently, the role of "immune checkpoint inhibitors" in GEJ/GC treatment is not determined, but these agents clearly hold promise.

Antigen-Specific, Active Immunotherapies

The introduction of antigen-specific immunotherapies in GEJ/GC has been hampered by inefficient vaccination strategies and the lack of clinically feasible, cancer-specific antigens. HER2, which is overexpressed in approximately 20 % of cases in principle, constitutes an appropriate target. However, this is already effectively addressed by monoclonal antibodies and, more recently, by antibody-toxin conjugates. These comparators have a high level of clinical activity, which will be hard to match by current vaccine-based or cellular immunotherapies. Moreover,

the extratumoral expression of HER2 poses a risk for long-term toxicity once an effective T cell response has been evoked. From our perspective, individualized vaccine approaches following resection of high-risk cancer will constitute a clinically feasible window for further exploration of curative active immunotherapy in GEJ/GC.

Conclusion

Multiple clinical trials have been conducted to explore antibodies targeting ERBB family receptors and angiogenic signaling in the treatment of advanced and metastatic GEJ/GC. So far, trastuzumab has shown convincing clinical activity in patients defined by high intratumoral expression of a biomarker, the antibody target HER2. Recent evidence suggests a role for the anti-VEGFR2 antibody ramucirumab in treatment of patients relapsing after chemotherapy. The identification and validation of novel targets as well as of predictive biomarkers to define patient populations responsive to a specific targeted intervention has been highly successful in treatment of breast cancer or metastatic adenocarcinoma of the lung. It is expected that further insights into GEJ/GC biology and immunobiology will enable progress in this devastating disease.

References

Bachleitner-Hofmann T, Sun MY, Chen CT, Tang L, Song L, Zeng Z, Shah M, Christensen JG, Rosen N, Solit DB, Weiser MR (2008) HER kinase activation confers resistance to MET tyrosine kinase inhibition in MET oncogene-addicted gastric cancer cells. Mol Cancer Ther 7:3499–3508. doi:10.1158/1535-7163.MCT-08-0374

Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, Lehle M, Rüschoff J, Kang YK, ToGA Trial Investigators (2010) Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomized controlled trial. Lancet 376:687–697

Baselga J, Cortés J, Kim SB, Im SA, Hegg R, Im YH, Roman L, Pedrini JL, Pienkowski T, Knott A, Clark E, Benyunes MC, Ross G, Swain SM, CLEOPATRA Study Group (2012) Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. N Engl J Med 366:109–119. doi:10.1056/NEJMoa1113216

Bokemeyer C, Bondarenko I, Makhson A, Hartmann JT, Aparicio J, de Braud F, Donea S, Ludwig H, Schuch G, Stroh C, Loos AH, Zubel A, Koralewski P (2009) Fluorouracil, leucovorin, and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer. J Clin Oncol 27:663–671. doi:10.1200/JCO.2008.20.8397

Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, Jones CU, Sur R, Raben D, Jassem J, Ove R, Kies MS, Baselga J, Youssoufian H, Amellal N, Rowinsky EK, Ang KK (2006) Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med 354:567–578

Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsi K, Pitot HC, Hamid O, Bhatia S, Martins R, Eaton K, Chen S, Salay TM, Alaparthy S, Grosso JF, Korman AJ, Parker SM, Agrawal S, Goldberg SM, Pardoll DM, Gupta A, Wigginton JM (2012) Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med 366:2455–2465. doi:10.1056/NEJMoa1200694

Cervantes A, Rodríguez Braun E, Pérez Fidalgo A, Chirivella González I (2007) Molecular biology of gastric cancer. Clin Transl Oncol 9:208–215, Review

Chen J, Zhou SJ, Zhang Y, Zhang GQ, Zha TZ, Feng YZ, Zhang K (2013) Clinicopathological and prognostic significance of galectin-1 and vascular endothelial growth factor expression in gastric cancer. World J Gastroenterol 19:2073–2079. doi:10.3748/wjg.v19.i13.2073

Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, Bets D, Mueser M, Harstrick A, Verslype C, Chau I, Van Cutsem E (2004) Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. N Engl J Med 351:337–345

Cunningham D, Allum WH, Stenning SP, Thompson JN, Van de Velde CJ, Nicolson M, Scarffe JH, Lofts FJ, Falk SJ, Iveson TJ, Smith DB, Langley RE, Verma M, Weeden S, Chua YJ, MAGIC Trial Participants (2006) Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. N Engl J Med 355:11–20

De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, Kalogeras KT, Kotoula V, Papamichael D, Laurent-Puig P, Penault-Llorca F, Rougier P, Vincenzi B, Santini D, Tonini G, Cappuzzo F, Frattini M, Molinari F, Saletti P, De Dosso S, Martini M, Bardelli A, Siena S, Sartore-Bianchi A, Tabernero J, Macarulla T, Di Fiore F, Gangloff AO, Ciardiello F, Pfeiffer P, Qvortrup C, Hansen TP, Van Cutsem E, Piessevaux H, Lambrechts D, Delorenzi M, Tejpar S (2010) Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium

analysis. Lancet Oncol 11:753–762. doi:10.1016/S1470-2045(10)70130-3

Douillard JY, Siena S, Cassidy J, Tabernero J, Burkes R, Barugel M, Humblet Y, Bodoky G, Cunningham D, Jassem J, Rivera F, Kocákova I, Ruff P, Błasińska-Morawiec M, Šmakal M, Canon JL, Rother M, Oliner KS, Wolf M, Gansert J (2010) Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. J Clin Oncol 28:4697–4705. doi:10.1200/JCO.2009.27.4860

Douillard JY, Oliner KS, Siena S, Tabernero J, Burkes R, Barugel M, Humblet Y, Bodoky G, Cunningham D, Jassem J, Rivera F, Kocákova I, Ruff P, Błasińska-Morawiec M, Šmakal M, Canon JL, Rother M, Williams R, Rong A, Wiezorek J, Sidhu R, Patterson SD (2013) Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. N Engl J Med 369:1023–1034. doi:10.1056/NEJMoa1305275

Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 127:2893–2917. doi:10.1002/ijc.25516, PubMed PMID: 21351269

Fuchs CS, Tomasek J, Yong CJ, Dumitru F, Passalacqua R, Goswami C, Safran H, Dos Santos LV, Aprile G, Ferry DR, Melichar B, Tehfe M, Topuzov E, Zalcberg JR, Chau I, Campbell W, Sivanandan C, Pikiel J, Koshiji M, Hsu Y, Liepa AM, Gao L, Schwartz JD, Tabernero J; for the REGARD Trial Investigators (2013) Ramucirumab monotherapy for previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (REGARD): an international, randomised, multicentre, placebo-controlled, phase 3 trial. Lancet. doi:10.1016/S0140-6736(13)61719-5, pii:S0140-6736(13)61719-5.

Goldhirsch A, Gelber RD, Piccart-Gebhart MJ, de Azambuja E, Procter M, Suter TM, Jackisch C, Cameron D, Weber HA, Heinzmann D, Dal Lago L, McFadden E, Dowsett M, Untch M, Gianni L, Bell R, Köhne CH, Vindevoghel A, Andersson M, Brunt AM, Otero-Reyes D, Song S, Smith I, Leyland-Jones B, Baselga J, Herceptin Adjuvant (HERA) Trial Study Team (2013) 2 years versus 1 year of adjuvant trastuzumab for HER2-positive breast cancer (HERA): an open-label, randomised controlled trial. Lancet 382:1021–1028

Grabsch H, Sivakumar S, Gray S, Gabbert HE, Müller W (2010) HER2 expression in gastric cancer: rare, heterogeneous and of no prognostic value – conclusions from 924 cases of two independent series. Cell Oncol 32:57–65. doi:10.3233/CLO-2009-0497

Hamid O, Robert C, Daud A, Hodi FS, Hwu WJ, Kefford R, Wolchok JD, Hersey P, Joseph RW, Weber JS, Dronca R, Gangadhar TC, Patnaik A, Zarour H, Joshua AM, Gergich K, Elassaiss-Schaap J, Algazi A, Mateus C, Boasberg P, Tumeh PC, Chmielowski B, Ebbinghaus SW, Li XN, Kang SP, Ribas A (2013) Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. N Engl J Med 369:134–144. doi:10.1056/NEJMoa1305133

Han SW, Oh DY, Im SA, Park SR, Lee KW, Song HS, Lee NS, Lee KH, Choi IS, Lee MH, Kim MA, Kim WH, Bang YJ, Kim TY (2009) Phase II study and biomarker analysis of cetuximab combined with modified FOLFOX6 in advanced gastric cancer. Br J Cancer 100:298–304. doi:10.1038/sj.bjc.6604861

Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144:646–674. doi:10.1016/j.cell.2011.02.013. Review

Heiss MM, Murawa P, Koralewski P, Kutarska E, Kolesnik OO, Ivanchenko VV, Dudnichenko AS, Aleknaviciene B, Razbadauskas A, Gore M, Ganea-Motan E, Ciuleanu T, Wimberger P, Schmittel A, Schmalfeldt B, Burges A, Bokemeyer C, Lindhofer H, Lahr A, Parsons SL (2010) The trifunctional antibody catumaxomab for the treatment of malignant ascites due to epithelial cancer: results of a prospective randomized phase II/III trial. Int J Cancer 127:2209–2221. doi:10.1002/ijc.25423

Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbé C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ (2010) Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 363:711–723. doi:10.1056/NEJMoa1003466

Kamangar F, Dores GM, Anderson WF (2006) Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. J Clin Oncol 24:2137–2150

Kasper S, Breitenbuecher F, Reis H, Brandau S, Worm K, Köhler J, Paul A, Trarbach T, Schmid KW, Schuler M (2013) Oncogenic RAS simultaneously protects against anti-EGFR antibody-dependent cellular cytotoxicity and EGFR signaling blockade. Oncogene 32:2873–2881. doi:10.1038/onc.2012.302

Kim MA, Jung EJ, Lee HS, Lee HE, Jeon YK, Yang HK, Kim WH (2007) Evaluation of HER-2 gene status in gastric carcinoma using immunohistochemistry, fluorescence in situ hybridization, and real-time quantitative polymerase chain reaction. Hum Pathol 38:1386–1393

Kim MA, Lee HS, Lee HE, Jeon YK, Yang HK, Kim WH (2008) EGFR in gastric carcinomas: prognostic significance of protein overexpression and high gene copy number. Histopathology 52:738–746. doi:10.1111/j.1365-2559.2008.03021.x

Kim C, Lee JL, Ryu MH, Chang HM, Kim TW, Lim HY, Kang HJ, Park YS, Ryoo BY, Kang YK (2011) A prospective phase II study of cetuximab in combination with XELOX (capecitabine and oxaliplatin) in patients with metastatic and/or recurrent advanced gastric cancer. Invest New Drugs 29:366–373. doi:10.1007/s10637-009-9363-0

Lee HE, Kim MA, Lee HS, Jung EJ, Yang HK, Lee BL, Bang YJ, Kim WH (2012) MET in gastric carcinomas: comparison between protein expression and gene copy number and impact on clinical outcome. Br J Cancer 107:325–333. doi:10.1038/bjc.2012.237

Lordick F, Luber B, Lorenzen S, Hegewisch-Becker S, Folprecht G, Wöll E, Decker T, Endlicher E, Röthling N, Schuster T, Keller G, Fend F, Peschel C (2010) Cetuximab plus oxaliplatin/leucovorin/5-fluorouracil in first-line metastatic gastric cancer: a phase II study of the Arbeitsgemeinschaft Internistische Onkologie (AIO). Br J Cancer 102:500–505. doi:10.1038/sj.bjc.6605521

Lordick F, Kang YK, Chung HC, Salman P, Oh SC, Bodoky G, Kurteva G, Volovat C, Moiseyenko VM, Gorbunova V, Park JO, Sawaki A, Celik I, Götte H, Melezínková H, Moehler M, Arbeitsgemeinschaft Internistische Onkologie and EXPAND Investigators (2013) Capecitabine and cisplatin with or without cetuximab for patients with previously untreated advanced gastric cancer (EXPAND): a randomised, open-label phase 3 trial. Lancet Oncol 14:490–499

Moehler M, Mueller A, Trarbach T, Lordick F, Seufferlein T, Kubicka S, Geissler M, Schwarz S, Galle PR, Kanzler S, German Arbeitsgemeinschaft Internistische Onkologie (2011) Cetuximab with irinotecan, folinic acid and 5-fluorouracil as first-line treatment in advanced gastroesophageal cancer: a prospective multi-center biomarker-oriented phase II study. Ann Oncol 22:1358–1366

Nakajima M, Sawada H, Yamada Y, Watanabe A, Tatsumi M, Yamashita J, Matsuda M, Sakaguchi T, Hirao T, Nakano H (1999) The prognostic significance of amplification and overexpression of c-met and c-erb B-2 in human gastric carcinomas. Cancer 85:1894–1902

Ohtsu A, Shah MA, Van Cutsem E, Rha SY, Sawaki A, Park SR, Lim HY, Yamada Y, Wu J, Langer B, Starnawski M, Kang YK (2011) Bevacizumab in combination with chemotherapy as first-line therapy in advanced gastric cancer: a randomized, double-blind, placebo-controlled phase III study. J Clin Oncol 29:3968–3976. doi:10.1200/JCO.2011.36.2236

Okines AF, Ashley SE, Cunningham D, Oates J, Turner A, Webb J, Saffery C, Chua YJ, Chau I (2010) Epirubicin, oxaliplatin, and capecitabine with or without panitumumab for advanced esophagogastric cancer: dose-finding study for the prospective multicenter, randomized, phase II/III REAL-3 trial. J Clin Oncol 28:3945–3950. doi:10.1200/JCO.2010.29.2847

Okines A, Cunningham D, Chau I (2011) Targeting the human EGFR family in esophagogastric cancer. Nat Rev Clin Oncol 8:492–503. doi:10.1038/nrclinonc.2011.45. Review

Parkin DM (2004) International variation. Oncogene 23:6329–6340, Review

Peeters M, Price TJ, Cervantes A, Sobrero AF, Ducreux M, Hotko Y, André T, Chan E, Lordick F, Punt CJ, Strickland AH, Wilson G, Ciuleanu TE, Roman L, Van Cutsem E, Tzekova V, Collins S, Oliner KS, Rong A, Gansert J (2010) Randomized phase III study of panitumumab with fluorouracil, leucovorin, and irinotecan (FOLFIRI) compared with FOLFIRI alone as second-line treatment in patients with metastatic colorectal cancer. J Clin Oncol 28:4706–4713. doi:10.1200/JCO.2009.27.6055

Peters S, Adjei AA (2012) MET: a promising anticancer therapeutic target. Nat Rev Clin Oncol 9:314–326. doi:10.1038/nrclinonc.2012.71. Review

Pinto C, Di Fabio F, Siena S, Cascinu S, Rojas Llimpe FL, Ceccarelli C, Mutri V, Giannetta L, Giaquinta S, Funaioli C, Berardi R, Longobardi C, Piana E, Martoni AA (2007) Phase II study of cetuximab in combination with FOLFIRI in patients with untreated advanced gastric or gastroesophageal junction adenocarcinoma (FOLCETUX study). Ann Oncol 18:510–517

Pinto C, Di Fabio F, Barone C, Siena S, Falcone A, Cascinu S, Rojas Llimpe FL, Stella G, Schinzari G, Artale S, Mutri V, Giaquinta S, Giannetta L, Bardelli A, Martoni AA (2009) Phase II study of cetuximab in combination with cisplatin and docetaxel in patients with untreated advanced gastric or gastro-oesophageal junction adenocarcinoma (DOCETUX study). Br J Cancer 101:1261–1268. doi:10.1038/sj.bjc.6605319

Ralph C, Elkord E, Burt DJ, O'Dwyer JF, Austin EB, Stern PL, Hawkins RE, Thistlethwaite FC (2010) Modulation of lymphocyte regulation for cancer therapy: a phase II trial of tremelimumab in advanced gastric and esophageal adenocarcinoma. Clin Cancer Res 16:1662–1672. doi:10.1158/1078-0432.CCR-09-2870

Rao S, Starling N, Cunningham D, Sumpter K, Gilligan D, Ruhstaller T, Valladares-Ayerbes M, Wilke H, Archer C, Kurek R, Beadman C, Oates J (2010) Matuzumab plus epirubicin, cisplatin and capecitabine (ECX) compared with epirubicin, cisplatin and capecitabine alone as first-line treatment in patients with advanced oesophago-gastric cancer: a randomised, multicentre open-label phase II study. Ann Oncol 21:2213–2219. doi:10.1093/annonc/mdq247

Sahin U, Koslowski M, Dhaene K, Usener D, Brandenburg G, Seitz G, Huber C, Türeci O (2008) Claudin-18 splice variant 2 is a pan-cancer target suitable for therapeutic antibody development. Clin Cancer Res 14:7624–7634. doi:10.1158/1078-0432.CCR-08-1547

Sasako M, Sakuramoto S, Katai H, Kinoshita T, Furukawa H, Yamaguchi T, Nashimoto A, Fujii M, Nakajima T, Ohashi Y (2011) Five-year outcomes of a randomized phase III trial comparing adjuvant chemotherapy with S-1 versus surgery alone in stage II or III gastric cancer. J Clin Oncol 29:4387–4393. doi:10.1200/JCO.2011.36.5908

Schuler M, Zvirbule Z, Lordick F, Krilova A, Helbig I, Schulze-Bergkamen H, Thuss-Patience PC, von Wichert G, Schulmann K, Trarbach T, Bauer S, Mueller C, Al-Batran SE, Huber C, Sahin U, Tureci O (2013) Safety, tolerability, and efficacy of the first-in-class antibody IMAB362 targeting claudin 18.2 in patients with metastatic gastroesophageal adenocarcinomas. J Clin Oncol (Meet Abstr) 31(15 Suppl):4080.

Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A et al (1989) Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 244:707–712

Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L (2001) Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 344:783–792

Smalley SR, Benedetti JK, Haller DG, Hundahl SA, Estes NC, Ajani JA, Gunderson LL, Goldman B, Martenson JA, Jessup JM, Stemmermann GN, Blanke CD, Macdonald JS (2012) Updated analysis of SWOG-directed intergroup study 0116: a phase III trial of adjuvant radiochemotherapy versus observation after curative gastric cancer resection. J Clin Oncol 30:2327–2333. doi:10.1200/JCO.2011.36.7136

Smith I, Procter M, Gelber RD, Guillaume S, Feyereislova A, Dowsett M, Goldhirsch A, Untch M, Mariani G, Baselga J, Kaufmann M, Cameron D, Bell R, Bergh J, Coleman R, Wardley A, Harbeck N, Lopez RI, Mallmann P, Gelmon K, Wilcken N, Wist E, Sánchez Rovira P, Piccart-Gebhart MJ (2007) HERA study team. 2-year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: a randomised controlled trial. Lancet 369:29–36

Stahl M, Walz MK, Stuschke M, Lehmann N, Meyer HJ, Riera-Knorrenschild J, Langer P, Engenhart-Cabillic R, Bitzer M, Königsrainer A, Budach W, Wilke H (2009) Phase III comparison of preoperative chemotherapy compared with chemoradiotherapy in patients with locally advanced adenocarcinoma of the esophagogastric junction. J Clin Oncol 27:851–856. doi:10.1200/JCO.2008.17.0506

Tanner M, Hollmén M, Junttila TT, Kapanen AI, Tommola S, Soini Y, Helin H, Salo J, Joensuu H, Sihvo E, Elenius K, Isola J (2005) Amplification of HER-2 in gastric carcinoma: association with Topoisomerase II alpha gene amplification, intestinal type, poor prognosis and sensitivity to trastuzumab. Ann Oncol 16:273–278

Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Horn L, Drake CG, Pardoll DM, Chen L, Sharfman WH, Anders RA, Taube JM, McMiller TL, Xu H, Korman AJ, Jure-Kunkel M, Agrawal S, McDonald D, Kollia GD, Gupta A, Wigginton JM, Sznol M (2012) Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 366:2443–2454. doi:10.1056/NEJMoa1200690

Trarbach T, Przyborek M, Schleucher N, Heeger S, Lüpfert C, Vanhoefer U (2013) Phase I study of matuzumab in combination with 5-fluorouracil, leucovorin and cisplatin (PLF) in patients with advanced gastric and esophagogastric adenocarcinomas. Invest New Drugs 31:642–652. doi:10.1007/s10637-012-9848-0

Van Cutsem E, Moiseyenko VM, Tjulandin S, Majlis A, Constenla M, Boni C, Rodrigues A, Fodor M, Chao Y, Voznyi E, Risse ML, Ajani JA, V325 Study Group (2006) Phase III study of docetaxel and cisplatin plus fluorouracil compared with cisplatin and fluorouracil as first-line therapy for advanced gastric cancer: a report of the V325 Study Group. J Clin Oncol 24:4991–4997

Van Cutsem E, Peeters M, Siena S, Humblet Y, Hendlisz A, Neyns B, Canon JL, Van Laethem JL, Maurel J, Richardson G, Wolf M, Amado RG (2007) Open-label phase III trial of panitumumab plus best supportive care compared with best supportive care alone in patients with chemotherapy-refractory metastatic colorectal cancer. J Clin Oncol 25:1658–1664

Van Cutsem E, Köhne CH, Hitre E, Zaluski J, Chang Chien CR, Makhson A, D'Haens G, Pintér T, Lim R, Bodoky G, Roh JK, Folprecht G, Ruff P, Stroh C, Tejpar S, Schlichting M, Nippgen J, Rougier P (2009) Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. N Engl J Med 360:1408–1417. doi:10.1056/NEJMoa0805019

Van Cutsem E, de Haas S, Kang YK, Ohtsu A, Tebbutt NC, Ming Xu J, Peng Yong W, Langer B, Delmar P, Scherer SJ, Shah MA (2012) Bevacizumab in combination with chemotherapy as first-line therapy in advanced gastric cancer: a biomarker evaluation from the AVAGAST randomized phase III trial. J Clin Oncol 30:2119–2127. doi:10.1200/JCO.2011.39.9824

van Hagen P, Hulshof MC, van Lanschot JJ, Steyerberg EW, van Berge Henegouwen MI, Wijnhoven BP, Richel DJ, Nieuwenhuijzen GA, Hospers GA, Bonenkamp JJ, Cuesta MA, Blaisse RJ, Busch OR, ten Kate FJ, Creemers GJ, Punt CJ, Plukker JT, Verheul HM, Spillenaar Bilgen EJ, van Dekken H, van der Sangen MJ, Rozema T, Biermann K, Beukema JC, Piet AH, van Rij CM, Reinders JG, Tilanus HW, van der Gaast A, CROSS Group (2012) Preoperative chemoradiotherapy for esophageal or junctional cancer. N Engl J Med 366:2074–2084. doi:10.1056/NEJMoa1112088

Verma S, Miles D, Gianni L, Krop IE, Welslau M, Baselga J, Pegram M, Oh DY, Diéras V, Guardino E, Fang L, Lu MW, Olsen S, Blackwell K, EMILIA Study Group (2012) Trastuzumab emtansine for HER2-positive advanced breast cancer. N Engl J Med 367:1783–1791. doi:10.1056/NEJMoa1209124

Vermorken JB, Mesia R, Rivera F, Remenar E, Kawecki A, Rottey S, Erfan J, Zabolotnyy D, Kienzer HR, Cupissol D, Peyrade F, Benasso M, Vynnychenko I, De Raucourt D, Bokemeyer C, Schueler A, Amellal N, Hitt R (2008) Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med 359:1116–1127. doi:10.1056/NEJMoa0802656

Waddell T, Chau I, Cunningham D, Gonzalez D, Okines AF, Okines C, Wotherspoon A, Saffery C, Middleton G, Wadsley J, Ferry D, Mansoor W, Crosby T, Coxon F, Smith D, Waters J, Iveson T, Falk S, Slater S, Peckitt C, Barbachano Y (2013) Epirubicin, oxaliplatin, and capecitabine with or without panitumumab for patients with previously untreated advanced oesophagogastric cancer (REAL3): a randomised, open-label phase 3 trial. Lancet Oncol 14:481–489. doi:10.1016/S1470-2045(13)70096-2

Wagner AD, Grothe W, Haerting J, Kleber G, Grothey A, Fleig WE (2006) Chemotherapy in advanced gastric cancer: a systematic review and meta-analysis based on aggregate data. J Clin Oncol 24:2903–2909, Review

Wilke H, Van Cutsem E, Oh SC, Bodoky G, Shimada Y, Hironaka S, Sugimoto N, Oleg N. Lipatov, Tae-You Kim, Cunningham D, Ohtsu A, Rougier P, Emig M, Carlesi R, Chandrawansa K and Muro K. A global, phase III, randomized, double-blind study of ramucirumab plus paclitaxel versus placebo plus paclitaxel in the treatment of metastatic gastroesophageal junction (GEJ) and gastric adenocarcinoma following disease progression on first-line platinum- and fluoropyrimidine-containing combination therapy rainbow IMCL CP12-0922 (I4T-IE-JVBE). J Clin Oncol 2014:32 suppl LBA7

Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, Segal NH, Ariyan CE, Gordon RA, Reed K, Burke MM, Caldwell A, Kronenberg SA, Agunwamba BU, Zhang X, Lowy I, Inzunza HD, Feely W, Horak CE, Hong Q, Korman AJ, Wigginton JM, Gupta A, Sznol M (2013) Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med 369:122–133. doi:10.1056/NEJMoa1302369

Ychou M, Boige V, Pignon JP, Conroy T, Bouché O, Lebreton G, Ducourtieux M, Bedenne L, Fabre JM, Saint-Aubert B, Genève J, Lasser P, Rougier P (2011) Perioperative chemotherapy compared with surgery alone for resectable gastroesophageal adenocarcinoma: an FNCLCC and FFCD multicenter phase III trial. J Clin Oncol 29:1715–1721. doi:10.1200/JCO.2010.33.0597

Non-small Cell Lung Cancer, NSCLC

Jens-Peter Marschner, Sonia Quaratino, and Ulf Forssmann

NSCLC and Immunotherapy

Lung cancer is the most common cause of cancer mortality globally, accounting for 1.2 million deaths per year (Ferlay et al. 2010). The majority of lung cancer patients present with advanced disease (stage IIIb/IV), and despite recent treatment advances, the overall 5-year survival rate is 12–15 %. The development of new therapeutic strategies is therefore essential, and immunotherapy offers promising treatment alternatives that may help fight disease death with minimal impact on normal tissues.

Several approaches to immunotherapy for lung cancer have shown promise in early clinical trials and have advanced to late-phase development which is in the focus of this chapter. These include therapeutic vaccines that target shared or tumor-specific antigens, including the cancer/testis antigens MAGE-A3 and MUC1. Another promising avenue is the use of immune checkpoint modulators. These treatments work by targeting molecules that serve as checks and balances in the regulation of immune responses. By blocking inhibitory molecules or, alternatively, activating stimulatory molecules, these treatments are designed to unleash and/or enhance preexisting anticancer immune responses.

The antitumor immune response can certainly influence the clinical outcome in lung cancer. An increased tumor infiltration with CD4+ and CD8+ T cells has been considered a strong favorable prognostic predictor independently associated with improved survival in lung cancer (Kawai et al. 2008). Similarly, a recently published paper shows that a T-helper cell 1 (Th1) enriched gene signature in the tumor microenvironment may favor the presence of immune effector cells in the tumor of patients who respond to a MAGE-A3 cancer vaccine (Ulloa-Montoya et al. 2013). Conversely, the IL4 gene pathway and other genes associated with a Th2 signature are significantly enriched in the blood of NSCLC patients in tumor progression (Chen et al. 2013).

Like the majority of other tumors, also NSCLC uses different strategies to prevent destruction by effector T cells: firstly by downregulating key molecules such as MHC class I molecules and tumor-associated antigens to avoid immune recognition and secondly preventing T-cell activation by disabling T-cell function or inducing T-cell apoptosis (Schreiber et al. 2011).

In surgically resected specimens, 25–94 % of NSCLCs have downregulated HLA class I expression (So et al. 2005) and abnormal

J.-P. Marschner (✉)
Affimed Therapeutics AG, Technologiepark, Im Neuenheimer Feld 528, 69120 Heidelberg, Germany
e-mail: jens-peter@marschner-mail.com

S. Quaratino
Merck KGaA, Frankfurter Strasse 250, 64293 Darmstadt, Germany

U. Forssmann
Bayer Pharma AG, Müllerstraße 178, 13353 Berlin, Germany

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_18,

expression of the β2-microglobulin (Baba et al. 2007), hampering an efficient antigen presentation of tumor-associated epitopes to T cells. Lung cancer cells also express the programmed death ligand-1 (PD-L1) which has been shown to suppress immune responses through engagement with the PD-1 receptor on activated T cells and B cells (Konishi et al. 2004; Topalian et al. 2012).

Alternatively, tumor escape may result from an immunosuppressive tumor microenvironment via the production of vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), or indoleamine 2,3-dioxygenase (IDO) and/or the recruitment of regulatory immune cells that function as the effectors of immunosuppression (Schreiber et al. 2011).

Several cellular and soluble suppressive mechanisms have been described in NSCLC. The increased number of M2 macrophages, which secrete IL-8 and IL-10 and inhibit Th1 immune response, is associated with poor prognosis and disease recurrence in NSCLC (Suzuki et al. 2011). Similarly, a tumor accumulation of myeloid-derived suppressor cells (MDSC) and regulatory T cells (Tregs) is associated with unfavorable prognosis in NSCLC patients (Diaz-Montero et al. 2009; Woo et al. 2001). MDSC can strongly suppress T-cell function via upregulation of reactive oxygen species (ROS) production (Huang et al. 2013). Tumor-infiltrating Foxp3+ Tregs were positively correlated with intratumoral cyclooxygenase-2 (COX-2) expression and associated with a worse prognosis in resected NSCLC (Shimizu et al. 2010; Hanagiri et al. 2013). Both the inhibition of ROS and COX2 might offer a therapeutic option to counterbalance the effects of these two suppressive regulators.

Understanding of the immune-evasion mechanisms regulated by tumor cells is necessary in developing more effective immunotherapeutic approaches to lung cancer. Ultimately, it is likely that the success of immune therapy in lung cancer will depend on a particular immune biomarker signature and the integration of strategies that aims to boost the immune response while downregulating the cancer-induced immune suppression.

Vaccines

Melanoma-Associated Antigen 3 (MAGE-A3, GSK1572932A)

The MAGE-A3 antigen is expressed by various tumors but not in normal tissue (De Plaen et al. 1994). MAGE-A3 is expressed in 35–50 % of NSCLC tumors (Sienel et al. 2004; Vansteenkiste et al. 2007).

The MAGE-A3 immunotherapeutic GSK1572932A consists of the MAGE-A3 peptide in combination with the adjuvant AS15 (Tyagi and Mirakhur 2009).

Phase II Trial in Adjuvant NSCLC

This was a randomized, double-blind, placebo-controlled trial in completely resected MAGE-A3-positive stage IB or II NSCLC patients (Vansteenkiste et al. 2013). Tumors had to be MAGE-A3 positive; 183 patients were enrolled. The hazard ratio for the primary endpoint disease-free interval (DFI) was HR = 0.78 ($p = 0.259$) in favor of the treatment arm. MAGE-A3 treatment was safe. A prospective investigation of a gene signature (GS; Kruit 2008) in a phase II trial in NSCLC revealed a predictive value for the MAGE-A3 treatment effect (Ulloa-Montoya et al. 2013).

Phase III Trial in NSCLC: MAGRIT

A randomized, double-blind phase III trial in patients with resected stage IB/II/IIIA NSCLC was initiated in 2007. Patients were eligible if they were MAGE-A3 positive and underwent surgery with or without standard adjuvant chemotherapy. The primary endpoint is disease-free survival (DFS), and the secondary endpoint is prospective validation of GS (Tyagi and Mirakhur 2009).

The recruitment of 2,270 patients is finished and final data are expected in January 2014 (Clinicaltrials.gov NCT00455572).

L-BLP25 (Tecemotide, Formerly Stimuvax)

MUC1 has a broad distribution in a variety of normal tissues and tumor tissues (Zotter et al.

1988), but is aberrantly glycosylated in tumors which makes it an interesting target for cancer treatment (Kufe 2009; Hiltbold 1999). MUC1 is expressed in >90 % of early stage NSCLC independent of histology (Mitchell et al. 2013).

Tecemotide is a peptide-based vaccine consisting of BLP25 lipopeptide, immunoadjuvant monophosphoryl lipid A, and three lipids forming a liposomal product (Butts et al. 2005).

Phase II Trials in Stage III/IV NSCLC

One hundred seventy-one patients with stage IIIB and IV NSCLC were accrued into a phase IIb trial (Butts et al. 2005). The median overall survival (OS) time was 17.4 months for patients in the tecemotide arm and 13 months in the best supportive care (BSC) arm with a hazard ratio of HR = 0.739 ($p = 0.112$). In the subgroup of patients with stage IIIB locoregional disease ($n = 65$), the hazard ratio was HR = 0.548 with a median OS of 30.6 months in the tecemotide arm vs. 13.3 months in the BSC arm (Butts et al. 2011).

Phase III Trial START

A double-blind phase III trial was conducted in stage IIIA and IIIB locoregional NSCLC (Butts et al. 2013); 1,513 patients that did not progress after chemoradiotherapy (CRT) were randomized to tecemotide or placebo. Prior CRT was given concurrently or sequentially. Due to a clinical hold, the primary analysis cohort consisted of 1,239 patients only. Median OS was 25.6 months in the tecemotide arm vs. 22.3 months in the placebo arm (HR 0.88, $p = 0.123$). A subgroup analysis for predefined strata revealed a more pronounced treatment effect in patients with prior concurrent CRT ($n = 806$). The median OS in this subgroup was 30.8 months for patients in the tecemotide arm and 20.6 months for patients in the placebo arm (HR 0.78, $p = 0.016$). Tecemotide was well tolerated.

Belagenpumatucel-L (TGF-β Antisense Gene-Modified Allogeneic Tumor Cell Vaccine, Lucanix™)

Belagenpumatucel-L is a cell-based vaccine which consists of four human NSCLC cell lines transfected with an antisense gene for TGF-β (Nemunaitis et al. 2006).

Phase II Trials

In a phase II trial in stage III/IV NSCLC patients, Lucanix induced an immune response in all patients and with strong ELISPOT response in those patients that showed stable disease or better (Nemunaitis et al. 2006). Survival duration increased with higher doses of Lucanix and treatment was safe.

The clinical data were confirmed in two smaller phase II studies (Nemunaitis et al. 2009; Fakhari et al. 2009). According to these data, immune responders survived 32.5 months compared to 11.6 months for nonresponders.

Phase III Trial

An international multicenter, randomized, double-blinded, placebo-controlled study of Lucanix maintenance therapy for stages III/IV NSCLC subjects who have responded to or have stable disease following one regimen of frontline, platinum-based CTX was initiated in 2008 (Clinicaltrials.gov NCT00676507). Primary endpoint is overall survival. The recruitment of 506 patients was finished in May 2012 (Press release NovaRx 2012). Preliminary data revealed that the maintenance treatment with Lucanix did not result in a significant survival benefit for the entire patient population investigated ($n > 450$). However, for a subgroup of patients, which was enrolled within 12 weeks after chemotherapy treatment ($n > 290$), the survival benefit was significant (not published, oral communication at ESMO/ECCO 2013).

TG4010 (MVA-MUC1-IL2)

TG4010 is an antigen-specific vaccine targeting MUC1. TG4010 is based on a viral vector, a modified vaccinia of Ankara (MVA), which, in addition to MUC1, expresses interleukin 2 (Limacher and Quoix 2012).

Phase II Studies

A randomized, open-label phase IIb trial investigated the effect of TG4010 in first-line therapy

for stage IIIB "wet"/IV NSCLC which was tested MUC1 positive (Quoix et al. 2011; Ramlau et al. 2008). One hundred forty-four patients were randomized to cisplatin plus gemcitabine plus TG4010 combination or CTX alone. A benefit of TG4010 treatment in terms of progression-free survival (PFS) at 6 months and response rate (RR) was observed; there was no difference in overall survival. A prespecified analysis revealed that a normal percentage of activated natural killer cells (aNK; $CD16^{+}CD56^{+}CD69^{+}$) had positive predictive value for the clinical effect. Overall TG4010 was well tolerated.

Phase IIb/III Trial TIME

This is a double-blind trial comparing the combination of first-line CTX plus TG4010 vs. placebo in stage IV NSCLC with MUC1-expressing tumors. The phase II part aims at prospectively validating the aNK level as predictive marker with PFS as primary endpoint. The phase III part will assess the overall survival for both arms in the study population identified in the phase IIb part. Two hundred six and 800 patients will be enrolled in parts IIB and III, respectively. The study started in 2012 with an estimated primary completion rate (data for primary outcome measure) end of 2015 (Clinicaltrials.gov NCT01383148).

EGF Vaccine (CimaVax)

The EGF vaccine was developed in Cuba with recombinant human EGF linked to a carrier protein (P64k Neisseria meningitidis recombinant protein).

Phase II Trial

Eighty patients with stage IIIB/IV NSCLC who received first-line treatment were randomized to the vaccine plus BSC vs. BSC (Neninger Vinageras et al. 2008; García et al. 2008). About 30 % of these patients had progressive disease (PD) at the time of randomization. The median OS was 6.47 months in the vaccine arm and 5.33 months in the control arm ($p = 0.098$). This effect was more pronounced in patients younger than 60 years. The treatment with the vaccine was safe.

Phase III Trial

In 2006, an open-label phase III randomized trial in stage IIIB/IV NSCLC patients after CTX was initiated at 18 sites in Cuba which is still ongoing; 579 patients were planned to be randomized (Rodríguez et al. 2010). Preliminary results from 160 patients show a trend towards a delayed separation of the survival curves in favor for the vaccine arm.

Another open-label phase III randomized trial in stage IIIB/IV NSCLC patients investigating the vaccine in first-line vs. best supportive care is ongoing and finished recruitment (Clinicaltrials.gov NCT01444118). Data are expected in 2015.

Immune Checkpoint Blockers

Since the mechanism of action of immune checkpoint blockers is not dependent on the expression of specific antigens in contrast to the vaccination approach, a broad application in different tumor types is conceivable. The strategy to augment antitumor responses through the blockade of immune checkpoint pathways has only recently been started to be explored for lung cancer, especially in NSCLC, and therefore, this part is not reporting the very limited experience in SCLC which has recently been summarized by Spigel and Socinski (2013). Two pathways, the CTLA-4:B7-1/-2 and PD-1:PD-L1/-L2 axes, are clinically investigated and the current status is described below.

CTLA4 Blockade

Positive phase III results in metastatic melanoma (Hodi et al. 2010; Robert et al. 2011) have stimulated the exploration of the anti-CTLA-4 monoclonal antibody ipilimumab in lung cancer. Thus, ipilimumab was evaluated in combination with paclitaxel and carboplatin (PC) in a randomized, double-blind, phase II, first-line clinical study in patients with locally advanced or metastatic

NSCLC or extended SCLC (Clinicaltrials.gov NCT00527735). Since the optimal sequence of chemo- and immunotherapy is challenging, two schedules, i.e., concurrent or phased, were explored. The study met its primary endpoint (defined as significant improvement in immune-related (ir)PFS) for phased treatment vs. control (HR 0.72; $p=0.05$), but not for concurrent treatment (HR 0.81; $p=0.13$). The median irPFS/PFS/OS for phased, concurrent, and control treatments (i.e., PC only) were 5.7/5.1/12.2, 5.5/4.1/9.7, and 4.6/4.2/8.3 months, respectively (Lynch et al. 2010). Observed incidences of grade 3/4 treatment-related adverse events were similar: 39, 41, and 37 % for patients in the phased, concurrent, and control groups, respectively. However, the rate of grade 3/4 immune-related AEs were differing as could be expected: 15 % in the phased, 20 % in the concurrent, and 6 % in the control group patients. In addition, a trend for greater clinical activity was observed in patients with squamous histology (Lynch et al. 2010). Overall, these results triggered the initiation of a phase III trial in patients with stage IV squamous cell NSCLC to investigate ipilimumab plus PC vs. placebo plus PC in August 2011 (Clinicaltrials gov NCT01285609).

Tremelimumab, another monoclonal anti-CTLA-4 antibody, was explored in phase II in the maintenance setting after first-line chemotherapy for advanced NSCLC, where it did not improve PFS (Brahmer 2013). To date, no further trials have been initiated.

PD-1/PD-L1 Blockade

While CTLA-4 primarily regulates early stages of T-cell activation at their initial response to antigen as a signal dampener, the role of PD-1 is to limit the activity of T cells in peripheral tissues, especially in inflammatory situations (Pardoll 2012).

The first trial using a blocker of the PD-1/PD-L1 pathway, i.e., nivolumab/BMS-936558 an anti-PD-1 antibody, was a first-in-man single-agent dose-escalation trial. In this trial, one durable complete response (CR) (colorectal carcinoma (CRC)) and two partial responses (PR) (melanoma, renal cell carcinoma (RCC)) were observed in 39 patients. Two additional patients (melanoma, NSCLC) had significant lesional tumor regressions not meeting PR criteria. The initial safety profile was favorable in comparison to ipilimumab (Brahmer et al. 2010). These promising results boosted the clinical development activities on this specific immune checkpoint. The consequent multiple dose-escalation trial with nivolumab containing several expansion cohorts recruited 296 patients overall (Topalian et al. 2012). Remarkably, 14 of 76 (18 %) advanced NSCLC patients evaluable for efficacy displayed an objective response (OR) and five additional patients a stable disease (SD) for more than 24 weeks. The RR was higher in the squamous (6/18, 33 %) compared to the non-squamous subtype (7/56, 12 %). Again the safety profile in the overall study population was favorable with a 14 % rate of grade 3/4 treatment-related adverse events, while 3 deaths from pulmonary toxicity were reported. Nivolumab monotherapy follow-up data presented at ASCO 2013 reported an overall RR of 17 % (22 responses in 129 patients; squamous vs. non-squamous: 17 % vs. 18 %), a median PFS of 2.3 months, and a median OS of 9.6 months (Brahmer et al. 2013).

BMS-936559, an anti-PD-L1 antibody, was directly explored in a multiple dose-escalation phase I trial in 207 patients covering 49 advanced NSCLC patients evaluable for efficacy (Brahmer et al. 2012). RR for squamous and non-squamous subtypes were similar (1/13, 8 % vs. 4/36, 11 %; all patients 5/49, 10 %) and not that impressive. However, as for nivolumab, a dose dependency in NSCLC patients could clearly be observed showing activity at 3 and 10 mg/kg. Grade 3/4 treatment-related adverse events were observed in only 9 % of the overall trial population.

Another anti-PD-L1 antibody, MPDL3280A is also explored in phase I (Spigel et al. 2013a, b). The NSCLC expansion cohort (locally advanced or metastatic disease) was reported to display an impressive overall RR of 24 % (9 of 37 patients with both squamous and non-squamous histology). The incidence of grade 3/4 treatment-emergent adverse events in the NSCLC safety cohort was

34 %. Interestingly no grades 3–5 pneumonitis or diarrhea was reported. Biomarker analyses from archival tumor showed a correlation between PD-L1 status and efficacy. Latest analyses revealed that patients with PD-L1-positive tumors showed an ORR of 100 % (4/4), while patients who were PD-L1 tumor status-negative had an ORR of 15 % (4/26). Further, it was concluded that MPDL3280A is probably the first targeted agent showing more activity in smoking patients than in never smokers. Moreover, the 24-week PFS was reported to be 46 % (Soria et al. 2013).

Several trials have recently been initiated with nivolumab in NSCLC: two open-label randomized phase III trials comparing nivolumab vs. docetaxel in previously treated advanced or metastatic NSCLC, one trial in squamous, and the other trial in non-squamous histology (Clinicaltrials.gov NCT01642004; Clinicaltrials.gov NCT01673867). A phase I trial in stage IIIB/IV NSCLC patients is exploring different combinations of nivolumab with (a) gemcitabine/cisplatin, (b) pemetrexed/cisplatin, (c) carboplatin/paclitaxel, (d) erlotinib, (e) ipilimumab, (f) bevacizumab maintenance, (g) switch maintenance, or (h) as monotherapy in first-line patients with brain metastases (Clinicaltrials.gov NCT01454102). In addition, a randomized phase II trial in subjects with recurrent metastatic NSCLC exploring epigenetic priming with azacitidine and entinostat or oral azacitidine alone prior to nivolumab treatment has been initiated (Clinicaltrials.gov NCT01928576). Priming with these methylation blockers holds promise as DNA demethylation may contribute to PD-1 overexpression. Another anti-PD-1 antibody, lambrolizumab/MK-3475, is also put forward to phase II/III: A randomized trial is exploring its efficacy and safety vs. docetaxel in previously treated subjects with NSCLC (Clinicaltrials.gov NCT01905657). A phase I study of lambrolizumab is investigating the combination with cisplatin/pemetrexed or carboplatin/paclitaxel in patients with advanced NSCLC (Clinicaltrials.gov NCT01840579). The anti-PD-L1 antibody MPDL3280A is also further explored in two phase II trials in advanced NSCLC (Clinicaltrials.gov NCT01846416; Clinicaltrials.gov NCT01903993).

Conclusion

Lung cancer has for a long time not been considered to be a very immunogenic tumor type such as melanoma or renal cancer. This perception has nowadays changed. Several vaccines are in phase III clinical development in NSCLC. Only few data are available so far, and no evidence for the clinical efficacy of vaccines could be demonstrated yet. Recently, a phase III trial of talactoferrin alfa did not meet the primary endpoint (Press release Agennix 2012, not described here because the development was stopped). Also, for tecemotide and Lucanix, a clinical benefit in terms of overall survival could not be demonstrated in large phase III trials. However, for both trials, subgroups of patients have been identified which seem to benefit from the vaccinations. It is remarkable that both subgroups are characterized by a specific timely correlation to the prior chemotherapy. Over the next few years, we will get a clearer picture about the role of vaccines in the treatment of NSCLC. In addition to vaccines, promising results have been observed in early clinical trials using immune checkpoint inhibitors which led to an accelerated clinical development. If the promises of the initial results prove true, the first approval of an immune checkpoint blocker for NSCLC can be expected around 2016.

References

Baba T et al (2007) Lack and restoration of sensitivity of lung cancer cells to cellular attack with special reference to expression of human leukocyte antigen class I and/or major histocompatibility complex class I chain related molecules A/B. Cancer Sci 98:1795–1802

Brahmer J (2013) Harnessing the immune system for the treatment of non-small-cell-lung cancer. J Clin Oncol 31:1021–1028

Brahmer J et al (2010) Phase I study of single-agent anti–programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. J Clin Oncol 28:3167–3175

Brahmer J et al (2012) Safety and activity of anti-PD-L1antibody in patients with advanced cancer. N Engl J Med 366:2455–2465

Brahmer J et al (2013) Survival and long-term follow-up of the phase I trial of nivolumab (Anti-PD-1; BMS-936558; ONO-4538) in patients (pts) with previously treated advanced non-small cell lung cancer (NSCLC). J Clin Oncol 31(suppl):8030

Butts C et al (2005) Randomized phase IIB trial of BLP25 liposome vaccine in stage IIIB and IV non-small cell lung cancer. J Clin Oncol 23:6674–6681

Butts C et al (2011) Updated survival analysis in patients with stage IIIB or IV non-small-cell lung cancer receiving BLP25 liposomal vaccine (L-BLP25): phase IIB randomized, multicenter, open-label trial. J Cancer Res Clin Oncol 137:1337–1342

Butts C et al (2013) START: a phase III study of L-BLP25 cancer immunotherapy for unresectable stage III non-small cell lung cancer. J Clin Oncol 31(suppl):7500

Chen YC et al (2013) Peripheral immune cell gene expression changes in advanced non-small cell lung cancer patients treated with first line combination chemotherapy. PLoS One 8:e57053

Clinicaltrials.gov NCT.01846416 A study of MPDL3280A in patients with PD-L1-positive locally advanced or metastatic non-small cell lung cancer. http://www.clinicaltrials.gov/ct2/show/NCT01846416

Clinicaltrials.gov NCT00455572. Cancer immunotherapy GSK1572932A as adjuvant therapy for patients with tumor-antigen-positive non-small cell lung cancer. http://clinicaltrials.gov/show/NCT00455572

Clinicaltrials.gov NCT01285609. Trial in squamous non small cell lung cancer subjects comparing ipilimumab plus paclitaxel and carboplatin versus placebo plus paclitaxel and carboplatin. http://www.clinicaltrials.gov/ct2/show/NCT01285609

Clinicaltrials.gov NCT01673867. Study of BMS-936558 compared to docetaxel in previously treated advanced or metastatic non-squamous NSCLC. http://www.clinicaltrials.gov/ct2/show/NCT01673867

Clinicaltrials.gov NCT01903993. A study of MPDL3280A compared with docetaxel in patients with non-small cell lung cancer after platinum failure. http://www.clinicaltrials.gov/ct2/show/NCT01903993

Clinicaltrials.gov NCT00527735. Phase II study for previously untreated subjects with non small cell lung cancer (NSCLC) or small cell lung cancer (SCLC). http://www.clinicaltrials.gov/ct2/show/NCT00527735

Clinicaltrials.gov NCT00676507. Phase III Lucanix™ vaccine therapy in advanced non-small cell lung cancer (NSCLC) following front-line chemotherapy (STOP). http://www.clinicaltrials.gov/ct2/show/NCT00676507

Clinicaltrials.gov NCT01383148. Phase IIB/III of TG4010 immunotherapy in patients with stage IV non-small cell lung cancer (TIME). http://www.clinicaltrials.gov/ct2/show/NCT01383148

Clinicaltrials.gov NCT01444118. A randomized trial to study the safety and efficacy of EGF cancer vaccination in late-stage (IIIB/IV) non-small cell lung cancer patients (NSCLC). http://www.clinicaltrials.gov/ct2/show/NCT01444118

Clinicaltrials.gov NCT01454102. Study of nivolumab (BMS-936558) in combination with gemcitabine/cisplatin, pemetrexed/cisplatin, carboplatin/paclitaxel, bevacizumab maintenance, erlotinib, ipilimumab or as monotherapy in first-line or in switch maintenance in subjects with stage IIIB/IV non-small cell lung cancer (NSCLC) (CheckMate 012). http://www.clinicaltrials.gov/ct2/show/NCT01454102

Clinicaltrials.gov NCT01642004. Study of BMS-936558 compared to docetaxel in previously treated advanced or metastatic squamous cell non-small cell lung cancer (NSCLC). http://www.clinicaltrials.gov/ct2/show/NCT01642004

Clinicaltrials.gov NCT01840579. Study of MK-3475 monotherapy in participants with advanced solid tumors and MK-3475 combination therapy in participants with advanced non-small cell lung cancer (MK-3475-011 AM2). http://www.clinicaltrials.gov/ct2/show/NCT01840579

Clinicaltrials.gov NCT01905657. Study of two doses of MK-3475 versus docetaxel in previously-treated participants with non-small cell lung cancer (MK-3475-010). http://www.clinicaltrials.gov/ct2/show/NCT01905657

Clinicaltrials.gov NCT01928576 Phase II anti-PD1 epigenetic priming study in NSCLC. (NA_00084192). http://www.clinicaltrials.gov/ct2/show/NCT01928576

De Plaen E et al (1994) Structure, chromosomal location, and expression of 12 genes of the MAGE family. Immunogenetics 40:360–369

Diaz-Montero CM et al (2009) Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. Cancer Immunol Immunother 58:49–59

Fakhari H et al (2009) Correlation of immune responses and survival in a phase II study of belagenpumatucel-L in non-small cell lung cancer. J Clin Oncol 27(suppl):3013

Ferlay J et al (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 127:2893–2917

García B et al (2008) Effective inhibition of the epidermal growth factor/epidermal growth factor receptor binding by anti-epidermal growth factor antibodies is related to better survival in advanced non-small cell lung cancer patients treated with epidermal growth factor cancer vaccine. Clin Cancer Res 14:840–846

Hanagiri T et al (2013) Clinical significance of the frequency of regulatory T cells in regional lymph node lymphocytes as a prognostic factor for non-small-cell lung cancer. Lung Cancer 81:475–479

Hiltbold EM (1999) Presentation of MUC1 tumor antigen by class I MHC and CTL function correlate with the glycosylation state of the protein taken up by dendritic cells. Cell Immunol 194:143–149

Hodi SF et al (2010) Ipilimumab plus dacarbacine for previously untreated metastatic melanoma. N Engl J Med 363:711–723

Huang A et al (2013) Increased CD14+ HLA-DR-/low myeloid-derived suppressor cells correlate with extrathoracic metastasis and poor response to

chemotherapy in non-small cell lung cancer patients. Cancer Immunol Immunother 62:1439–1451
Kawai O et al (2008) Predominant infiltration of macrophages and CD8(+) T cells in cancer nests is a significant predictor of survival in stage IV non-small cell lung cancer. Cancer 113:1387–1395
Konishi J et al (2004) B7-H1 expression on non small cell lung cancer cells and its relationship with tumor infiltrating lymphocytes and their PD-1 expression. Clin Cancer Res 10:5094–5100
Kruit WHJ (2008) Immunization with recombinant MAGE-A3 protein combined with adjuvant systems AS15 or AS 02B in patients with unresectable and progressive metastatic cutaneous melanoma: a randomized open-label Phase II study of the EORTC Melanoma Group (16032–18031). J Clin Oncol 26(suppl):9065
Kufe DW (2009) Mucins in cancer: function, prognosis and therapy. Nat Rev 9:874–885
Limacher J-M, Quoix E (2012) TG4010. A therapeutic vaccine against MUC1 expressing tumors. Oncoimmunology 1:791–792
Lynch TJ et al (2010) Phase II trial of ipilimumab (IPI) and paclitaxel/carboplatin (P/C) in first line stage IIIb/IV non-small cell lung cancer (NSCLC). J Clin Oncol 28(suppl):7531
Mitchell PL et al (2013) Mucin 1 (MUC1) expression in patients (pts) with early stage non-small cell lung cancer (NSCLC): relationship between immunohistochemistry (IHC), tumor characteristics, and survival. J Clin Oncol 31(suppl):3011
Nemunaitis J et al (2006) Phase II study of belagenpumatucel-L, a transforming growth factor beta-2 antisense gene-modified allogeneic tumor cell vaccine in non-small cell lung cancer. J Clin Oncol 24:4721–4730
Nemunaitis J et al (2009) Phase II trial of belagenpumatucel-L, a TGF-beta2 antisense gene modified allogeneic tumor vaccine in advanced non small cell lung cancer (NSCLC) patients. Cancer Gene Ther 16:620–624
Neninger Vinageras E et al (2008) Phase II randomized controlled trial of an epidermal growth factor vaccine in advanced non-small cell lung cancer. J Clin Oncol 26:1452–1458
Pardoll DM (2012) The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 12:252–264
Press release. Agennix reports results of FORTIS-M phase III trial with talactoferrin alfa in non-small cell lung cancer (2012) http://agennix.com/index.php?option=com_content&view=article&id=227%3Aagennix-reports-results-of-fortis-m-phase-iii-trial-with-talactoferrin-alfa-in-non-small-cell-lung-cancer&catid=23%3Apress-releases-2012&Itemid=56&lang=en
Press release. NovaRx completes enrollment in lucanix pivotal phase III therapeutic vaccine trial for post frontline maintenance therapy in non small cell lung cancer. Press release NovaRX (2012) http://www.prnewswire.com/news-releases/novarx-completes-enrollment-in-lucanix-pivitol-phase-iii-therapeutic-vaccine-trial-for-post-frontline-maintenace-therapy-in-non-small-cell-lung-cancer-159688685.html
Quoix E et al (2011) Therapeutic vaccination with TG4010 and first-line chemotherapy in advanced non-small cell lung cancer: a controlled phase IIb trial. Lancet Oncol 12:1125–1133
Ramlau R et al (2008) Randomized phase IIB trial evaluating the therapeutic vaccine TG4010 (MVA-MUC1-IL2) as an adjunct to chemotherapy in patients with advanced non-small cell lung cancer (NSCLC). J Clin Oncol 26(suppl):8023
Robert C et al (2011) Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 364:2517–2526
Rodríguez PC et al (2010) Clinical development and perspectives of CIMAvax EGF. Cuban vaccine for non-small cell lung cancer therapy. MEDICC Rev 12:17–23
Schreiber RD et al (2011) Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science 331:1565–1570
Shimizu K et al (2010) Tumor infiltrating Foxp3+ regulatory T cells are correlated with cyclooxygenase-2 expression and are associated with recurrence in resected non-small cell lung cancer. J Thorac Oncol 5:585–590
Sienel W et al (2004) Melanoma associated antigen (MAGE)-A3 expression in stages I and II non-small cell lung cancer: results of a multicenter study. Eur J Cardiothorac Surg 25:131–134
So T et al (2005) Haplotype loss of HLA class I antigen as an escape mechanism from immune attack in lung cancer. Cancer Res 65:5945–5952
Soria JC et al (2013) Clinical activity, safety and biomarkers of PD-L1 blockade in non-small cell lung cancer (NSCLC): Additional analyses from a clinical study of the engineered antibody MPDL3280A (anti-PDL1). European Cancer Congress 2013 (ECCO-ESMO-ESTRO), Abstract 3408. http://eccamsterdam2013.ecco-org.eu/Scientific-Programme/Abstract-search.aspx#
Spigel DR, Socinski MA (2013) Rationale for chemotherapy, immunotherapy, and checkpoint blockade in SCLC: beyond traditional treatment approaches. J Thorac Oncol 8:587–598. doi: 10.1097/JTO.0b013e318286cf88
Spigel DR et al (2013a) Rationale for chemotherapy, immunotherapy, and checkpoint blockade in SCLC. J Thorac Oncol 8:587–589
Spigel DR et al (2013b) Clinical activity, safety, and biomarkers of MPDL3280A, an engineered PD-L1 antibody in patients with locally advanced or metastatic lung cancer (NSCLC). J Clin Oncol 31(suppl):8008
Suzuki K et al (2011) Prognostic immune markers in non-small cell lung cancer. Clin Cancer Res 17:5247–5256
Topalian SL et al (2012) Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 366:2443–2454
Tyagi P, Mirakhur B (2009) MAGRIT: the largest-ever phase III lung cancer trial aims to establish a novel tumor-specific approach to therapy. Clin Lung Cancer 10:371–374

Ulloa-Montoya F et al (2013) Predictive gene signature in MAGE-A3 antigen-specific cancer immunotherapy. J Clin Oncol 31:2388–2395

Vansteenkiste J et al (2007) Final results of a multicenter, double blind, randomized placebo controlled phase II study to assess the efficacy of MAGE-A3 immunotherapeutics as adjuvant therapy in stage IB/II non-small cell lung cancer (NSCLC). J Clin Oncol 25(suppl):7554

Vansteenkiste J et al (2013) Adjuvant MAGE-A3 immunotherapy in resected non-small cell lung cancer: phase II randomized study results. J Clin Oncol 31:2396–2403

Woo EY et al (2001) Regulatory CD4+ CD25+ T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. Cancer Res 61:4766–4772

Zotter S et al (1988) Tissue and tumor distribution of human polymorphic epithelial mucin. Cancer Rev 11–12:55–101

Towards More Specificity and Effectivity in the Antileukemia Immune Response

Udo F. Hartwig, Ralf G. Meyer, and Wolfgang Herr

Myeloid Leukemia Vaccines

Introduction

Acute and chronic leukemias are frequently not cured despite several elaborate treatment approaches, including cytotoxic drugs, tyrosine kinase inhibitors, immunomodulatory agents, as well as autologous and allogeneic hematopoietic stem cell transplantation (HSCT). The rationale and aim of using leukemia vaccines in these patients is to eradicate minimal residual disease (MRD) and prolong leukemia remission duration by antigen-specific immune responses. The vaccines can be applied after conventional induction therapy or even after HSCT. This section focuses on myeloid leukemia vaccines, because available preclinical and clinical data demonstrate strong evidence that they are immunogenic in acute and chronic myeloid leukemias.

Ideal myeloid leukemia vaccines are thought to include leukemia-specific antigens that are exclusively expressed by leukemia cells, are absent in normal tissues, and can induce powerful B- and T-cell responses against leukemia. However, except for BCR-ABL and PML-RARα fusion proteins in chronic myeloid leukemia (CML) and acute promyelocytic leukemia (APL), respectively, such leukemia-specific fusion proteins are uncommon in other myeloid leukemias. Moreover, leukemia-associated antigens (LAA) derived from Wilms' tumor 1 (WT1), proteinase 3, preferentially expressed antigen of melanoma (PRAME), and receptor for hyaluronic acid-mediated motility (RHAMM) proteins are predominantly expressed by leukemia cells but are also expressed by normal tissues, albeit to a lesser degree.

Leukemia-Specific Antigen Vaccines

Peptides derived from the BCR-ABL fusion protein p210 have been explored as a leukemia-specific vaccine in CML patients in several clinical pilot studies (Cathcart et al. 2004; Bocchia et al. 2005). The vaccines were usually well tolerated and could be safely applied in patients receiving HSCT, interferon, or imatinib. Most notably, the vaccines elicited BCR-ABL-specific immune responses in a significant proportion of patients. The investigators observed in single patients that successful boosting of BCR-ABL immunity was associated with decrease in MRD levels and allowed for reduction of the imatinib dose. Another potent leukemia-specific

U.F. Hartwig • R.G. Meyer
III. Medizinische Klinik und Poliklinik,
Universitätsmedizin Mainz,
Johannes Gutenberg-Universität Mainz,
Langenbeckstr. 1, 55131 Mainz, Germany
e-mail: udo.hartwig@unimedizin-mainz.de;
ralf.meyer@unimedizin-mainz.de

W. Herr (✉)
Klinik und Poliklinik für Innere Medizin III,
Universitätsklinikum Regensburg,
Franz-Josef-Strauss-Allee 11,
93053 Regensburg, Germany
e-mail: wolfgang.herr@ukr.de

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_19,

antigen is the PML-RARα fusion protein in APL. There is convincing evidence of the usefulness of PML-RARα vaccines from mouse models (Padua et al. 2003). However, the infrequence of APL disease as well as the high efficacy of current conventional treatment options including all-trans-retinoic acid and arsenic trioxide impeded so far intensive exploration in clinical studies.

Leukemia-Associated Antigen Vaccines

WT1 is an oncogenic protein that is overexpressed in most acute myeloid leukemias (AML) and CML. Since its expression is very low or absent in hematopoietic progenitor cells, WT1-derived T-cell epitopes have been chosen by many groups as vaccines in myeloid leukemias (Chaise et al. 2008; Rezvani et al. 2008; Keilholz et al. 2009). In phase I/II studies WT1 vaccinations using peptides, proteins, or nucleic acids as vaccine format proved to be safe and were found to be associated with a reduction in MRD load in single patients. The investigators also reported that WT1 vaccines reliably stimulated antigen-specific T-cell responses in myeloid leukemia patients, suggesting that the frequency of WT1-specific T cells is a suitable surrogate biomarker for vaccine efficacy.

Potential other LAA vaccine candidates that have been investigated in preclinical as well as early clinical studies are PRAME (Quintarelli et al. 2011), proteinase 3 (Rezvani et al. 2008) and RHAMM (Greiner et al. 2010), and autologous leukocyte-derived heat shock proteins (Li et al. 2005). All these trials demonstrated feasibility and safety as well as a positive correlation between vaccine-induced immunity and clinical responses in single indicator patients.

T-Cell Depletion in Allogeneic Stem Cell Transplantation

Introduction

Allogeneic HSCT relies on three modes of action: (1) high-dose (radio-) chemotherapy as part of the conditioning regimen, (2) replacement of a diseased by a healthy donor hematopoiesis, and (3) immune-mediated "graft-versus-leukemia (GvL)" effect. Each of these beneficial effects is associated with significant treatment-related morbidity and mortality: (1) conditioning-mediated organ damage, (2) cytopenia associated with the risk of bleeding and infection, and (3) graft-versus-host disease (GvHD). Considering the risks of individual patients and their diseases, modern transplant strategies can be adjusted to balance the risk of conditioning and its toxicity against GvL and GvHD. GvHD not only depends on the immunologic differences between donor and recipient but is also triggered by the inflammatory status of the recipient following the conditioning regimen. Therefore, strategies combining T-cell-depleted transplantation in the first place with the application of a delayed donor lymphocyte infusion (DLI) are capable of reducing the risk of GvHD while preserving significant GvL and anti-infectious immunity.

T-Cell Depletion from the Graft

The backbone of these strategies is T-cell depletion (TCD) from the graft. In the 1980s and 1990s, when bone marrow was still the main source of hematopoietic stem cells, T cells were depleted by the use of anti-CD6 antibodies and complement-mediated lysis (Soiffer et al. 1990). Positive selection of CD34-positive hematopoietic stem cells was introduced later to reduce the comparably high T-cell content of G-CSF-mobilized peripheral stem cell grafts. However, the nonselective removal of T cells was associated with a higher risk of relapse and infectious complications. In addition, a significant increase of engraftment failures was observed especially following transplantation from HLA-mismatched donors. Therefore, more selective methods like CD3/CD19 depletion have been developed especially in haploidentical transplantation (Bethge et al. 2006). By adding CD19-mediated B-cell depletion to TCD, the incidence of posttransplantation lymphoproliferative disease was markedly reduced. Recently, this strategy has been further modified in that CD3-mediated T-cell depletion was replaced by

αβT-cell depletion (Handgretinger et al. 2012). This strategy preserves γδT cells that have been shown to mediate direct antileukemic immunity without the risk of GvHD.

Depleting T-Cell Subsets from DLI Grafts

Depletion of CD8 T cells from DLI efficiently reduces the incidence and severity of GvHD while preserving at least partly the GvL effect (Giralt et al. 1995). Even more stringent CD8 depletion by immunomagnetic beads has been proven feasible and effective in clinical endpoints (Meyer et al. 2007; Orti et al. 2009). Relying on CD4 rather than CD8 T-cell-containing DLI experienced further support by the finding that HLA-DP-directed CD4 T cells target leukemia cells in HLA-DP-mismatched transplantation (Rutten et al. 2008). This is of relevance, since HLA-DP matching is not part of the donor-search routine, wherefore HLA-DP mismatches are frequent in "HLA-matched" transplantation.

Depleting Naive T Cells

Alloreactivity of donor T cells mainly derives from naive rather than memory T cells (Foster et al. 2004). Depleting naive T cells therefore is a means of preventing GvHD while preserving T-cell memory, e.g., against infectious agents. Different strategies including CD62L (L-selectin) and CCR7 have been used for depletion. However, depleting CD45RA seems to be most efficient and applicable for clinical use (Distler et al. 2011). Clinical application of CD45-RA-depleted DLI is currently under way (Teschner et al. 2014).

Selective Allodepletion

Introduction

The considerable high content of immunocompetent T cells in apheresis products exposes recipients of allogeneic peripheral blood stem cells to an elevated risk of acute and chronic GvHD. Depleting alloreactive T-cell specificities either from the graft in vitro or in vivo by the administration of antibodies reactive to lymphocytes such as alemtuzumab provides an effective strategy to prevent the development of GvHD following allogeneic HSCT. However, this approach is often associated with emerging opportunistic infections, in particular the reactivation of latent Cytomegalovirus, Epstein-Barr virus, and Varicella-Zoster virus infections. Moreover, higher relapse rates are observed due to reduced GvL reactivity primarily mediated by donor T lymphocytes.

Although GvL and graft-versus-host (GvH) reactivity are very closely linked, ample clinical and experimental evidence suggests that antileukemic immune responses can be separated from alloreactivity as, e.g., specific GvL immunity to hematopoiesis-specific minor histocompatibility antigens (mHAg) or de novo expressed leukemia-specific antigens such as peptides derived from the BCR-ABL fusion product exist (Bleakley and Riddell 2004). These observations have formed the basis of selective allodepletion (SD) strategies to harness GvL immunity while minimizing the risk of alloreactivity upon adoptive transfer of donor T lymphocytes, and numerous conceptually different experimental approaches and clinical protocols have been described in the last years to explore SD in vitro as well as in vivo (Tsirigitis et al. 2012). At least all ex vivo strategies have in common that donor T lymphocytes are first stimulated in vitro by coculture with patient-derived stimulators followed by depletion of activated alloreactive specificities using different means as outlined more detailed in the following sections.

Selective Allodepletion Using T-Cell Activation Markers and Immunotoxins

Among all different SD approaches reported, most studies have examined the elimination of alloreactive donor lymphocytes using T-cell activation markers expressed after allostimulation. T cells derived from a healthy HLA-matched or HLA-mismatched donor are stimulated with

patient-derived (allogeneic) stimulator cells such as peripheral blood mononuclear cells, B lymphocyte lines, dendritic cells, or activated T cells in mixed lymphocyte cultures (MLC) (Fig. 1). Alternatively, keratinocytes and fibroblasts have been tested as non-hematopoietic stimulators to stimulate alloreactive specificities directed to ubiquitously but not hematopoiesis-specific expressed mHAg (Nonn et al. 2008). Upon binding of monoclonal antibodies (mAb) directed to markers expressed on activated T cells such as, e.g., CD25, CD69, CD71, or CD137 or combinations thereof that are coupled with magnetic microbeads, activated alloreactive T cells are labeled and can then be separated from nonreactive lymphocytes using immunomagnetic cell separation (Wehler et al. 2007). Using this approach significant reduction of alloreactivity while harnessing antiviral and LAA specificities as shown for the WT1 antigen could be demonstrated in several human in vitro systems and preclinical models using CD69, CD137, HLA-DR, or a combination of CD25 and CD71 as targets for T-cell activation marker-based SD (for review see Tsirigitis et al. 2012).

Accordingly, anti-CD25-specific mAb conjugated to the immunotoxin Ricin A was successfully applied in SD. As shown in pediatric patients who predominantly received stem cell grafts from haploidentical donors but also in HLA-identical HSCT in adults, the administration of CD25-immunotoxin pretreated DLI resulted in clearly reduced incidences of GvHD (André-Schmutz et al. 2002; Salomon et al. 2005).

Selective Allodepletion by Extracorporal Photodepletion

The photodepletion technique represents an appealing alternative to selectively deplete anti-host immunity in vitro (Mielke et al. 2008). Upon stimulation, alloreactive T cells are exposed to the photosensitizing rhodamine-based dye TH9402. TH9402 enters all cells but is only successfully extruded by resting T cells, whereas activated T lymphocytes are impaired to efflux the dye due to deactivation of the protein pump multidrug resistance p-glycoprotein. TH9402 is an inert molecule under normal conditions, but becomes extremely toxic after exposure to light thereby providing the basis for selective killing. In contrast to the CD25-mediated SD, this approach additionally retains donor $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells (Tregs) with the potential to induce tolerance to alloantigens.

A GMP-grade clinical-scale protocol for the treatment of both HLA-mismatched and HLA-matched donor-recipient pairs has been investigated using patient-derived T lymphocytes as antigen-presenting cells, and data from a phase I trial suggested that selectively photodepleted allografts in matched sibling transplantations followed by low-dose immunosuppression may protect against severe acute GvHD but are associated with delayed immune recovery (Mielke et al. 2011).

Further Ex Vivo Based Selective Allodepletion Approaches

In addition to the approaches described above, more experimental procedures include SD by inducing apoptotic signals or negative selection of naive donor lymphocytes. Comparative studies on CD95-induced apoptosis and CD69-based immunomagnetic depletion revealed that stimulating allogeneic T cells in the presence of agonistic anti-CD95 mAb resulted in programmed cell death of alloreactive lymphocytes with superior retainment of antiviral and LAA specificities as well as Tregs when compared to CD69-mediated allodepletion (Hartwig et al. 2008). Another method to perform SD is by simply removing naive precursors from the graft before infusion. Preclinical studies showed that the vast majority of alloreactivity is present among the naive T-cell population, while memory T cells appear to be less potent in causing alloreactivity (Distler et al. 2011). Removal of naive T cells can be easily accomplished by using immunomagnetic beads specific to markers present on the surface of naive cells such as CD45RA, CCR7, and CD62L. Moreover, blockade of important costimulatory signals such as the CD28–CD80/CD86 interaction essential for full T-cell activation can result

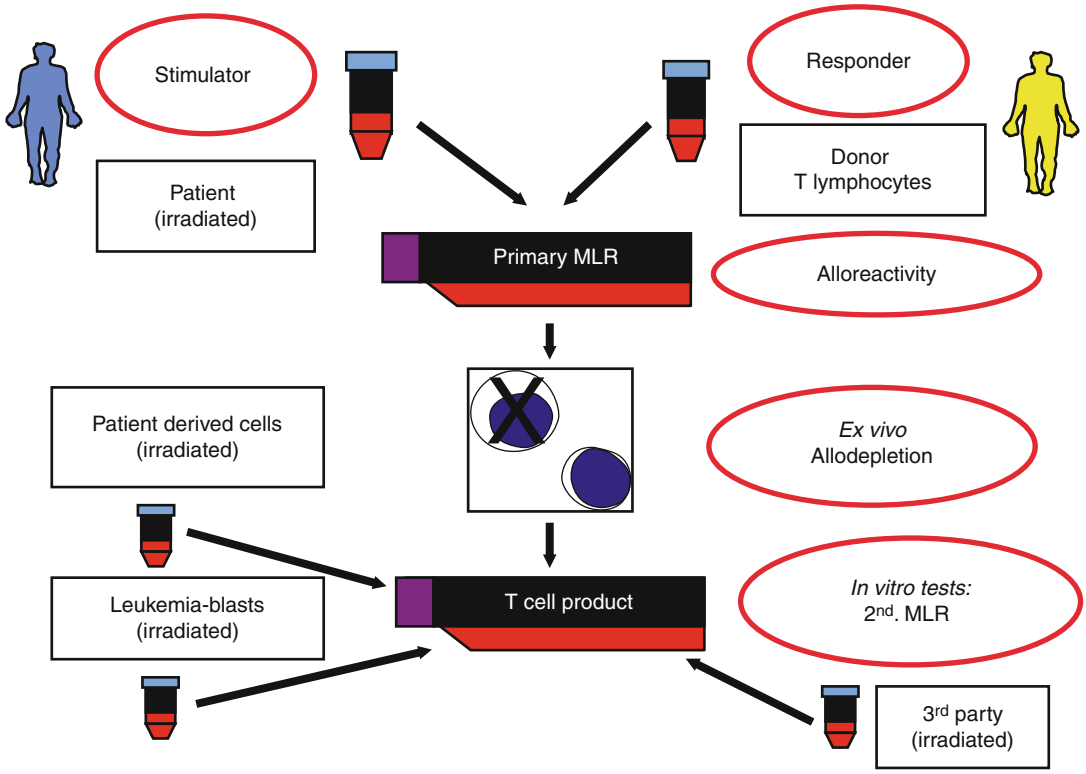

Fig. 1 Procedure of selective allodepletion. Irradiated patient-derived stimulator cells are cocultured with nonirradiated donor lymphocytes in MLC. Following stimulation, activated alloreactive T lymphocytes are then selectively depleted ex vivo using different approaches as described in the text. The modified T-cell product can then be tested in a second mixed lymphocyte reaction (MLR) for residual alloreactivity directed to the patient as well as for T cells recognizing patient-derived leukemia and third-party stimulators (i.e., irrelevant HLA molecules, viral antigens, etc.)

in efficient induction of anergy in alloreactive donor lymphocytes as previously demonstrated in a phase I trial (Guinan et al. 1999).

Selective Allodepletion In Vivo Using Suicide Genes

Transduction of T lymphocytes with genes encoding for proteins that can convert drugs into toxic metabolites or induce apoptotic pathways and normally do not occur in T cells renders these cells sensitive to drugs or other factors. These genes have been named "suicide genes." The herpes simplex virus (HSV) thymidine kinase (TK) gene represents the most well-known suicide gene and encodes for the enzyme TK normally absent in eukaryotic cells. TK converts nucleoside analogs such as ganciclovir into monophosphate and triphosphate forms which interfere with DNA replication resulting in apoptosis induction of dividing cells. Upon retroviral transduction and selection of stable transfectants using additionally expressed cell surface markers, adoptively transferred donor T lymphocytes that become alloreactive in the patient can be eliminated by ganciclovir. Multicenter phase I–II trials performed have successfully demonstrated that this in vivo SD approach is safe and can effectively control the development of GvHD (Ciceri et al. 2009).

Conclusion and Outlook

Early clinical studies have already demonstrated the safety and feasibility of immunotherapeutic strategies aiming to enhance the specificity and effectivity of antileukemia immune responses.

Several immune-modulating agents such as myeloid leukemia vaccines have been investigated in patients before and after allogeneic HSCT with promising clinical results in single patients. Of them, leukemia-specific antigens (e.g., mutated FLT3 (Graf et al. 2007), mutated nucleophosmin 1 (Greiner et al. 2012)) might represent more effective second-generation vaccines than currently used LAA because they are tumor-specific target structures. The SD of alloreactive T cells or even entire T-cell subsets and their add-back as DLI after TCD HSCT has been shown not only to reduce the risk of GvHD but also specifically to improve reconstitution of T cells recognizing infectious agents and leukemia cells. Moreover, overcoming leukemia-induced as well as HSCT-associated immunosuppression could be accomplished by optimizing adjuvant delivery systems or by coadministration of immunostimulatory cytokines or agents blocking negative immunoregulatory checkpoints (e.g., CTLA4, PD-1). Finally, randomized prospective clinical trials are essentially needed to define the role of these individual or combined immunotherapeutic strategies in prophylactic, preemptive, and therapeutic disease settings.

References

André-Schmutz I et al (2002) Immune reconstitution without graft-versus-host disease after hemopoietic stem-cell transplantation: a Phase 1/2 study. Lancet 360:130–137

Bethge WA et al (2006) Haploidentical allogeneic hematopoietic cell transplantation in adults with reduced-intensity conditioning and CD3/CD19 depletion: fast engraftment and low toxicity. Exp Hematol 34: 1746–1752

Bleakley M, Riddell SR (2004) Molecules and mechanisms of the graft-versus-leukaemia effect. Nat Rev Cancer 4:371–380

Bocchia M et al (2005) Effect of a p210 multipeptide vaccine associated with imatinib or interferon in patients with chronic myeloid leukaemia and persistent residual disease: a multicentre observational trial. Lancet 365:657–662

Cathcart K et al (2004) A multivalent bcr-abl fusion peptide vaccination trial in patients with chronic myeloid leukemia. Blood 103:1037–1042

Chaise C et al (2008) DNA vaccination induces WT1-specific T-cell responses with potential clinical relevance. Blood 112:2956–2964

Ciceri F et al (2009) Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical hemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised Phase 1–2 study. Lancet Oncol 10:489–500

Distler E et al (2011) Alloreactive and leukemia-reactive T cells are preferentially derived from naive precursors in healthy donors: implications for immunotherapy with memory T cells. Haematologica 96: 1024–1032

Foster AE et al (2004) Human CD62L- memory T cells are less responsive to alloantigen stimulation than CD62L + naive T cells: potential for adoptive immunotherapy and allodepletion. Blood 104:2403–2409

Giralt S et al (1995) CD8-depleted donor lymphocyte infusion as treatment for relapsed chronic myelogenous leukemia after allogeneic bone marrow transplantation. Blood 86:4337–4343

Graf C et al (2007) A neoepitope generated by an FLT3 internal tandem duplication (FLT3-ITD) is recognized by leukemia-reactive autologous CD8+ T cells. Blood 109:2985–2988

Greiner J et al (2010) High-dose RHAMM-R3 peptide vaccination for patients with acute myeloid leukemia, myelodysplastic syndrome and multiple myeloma. Haematologica 95:1191–1197

Greiner J et al (2012) Mutated nucleophosmin 1 elicit both CD4+ and CD8+ T-cell responses in patients with acute myeloid leukemia. Blood 120:1282–1289

Guinan EC et al (1999) Transplantation of anergic histoincompatible bone marrow allografts. N Engl J Med 340:1704–1714

Handgretinger R et al (2012) Negative depletion of CD3(+) and TcRαβ(+) T cells. Curr Opin Hematol 19:434–439

Hartwig UF et al (2008) Depletion of alloreactive donor T lymphocytes by CD95-mediated activation-induced cell death retains antileukemic, antiviral, and immunoregulatory T cell immunity. Biol Blood Marrow Transplant 14:99–109

Keilholz U et al (2009) A clinical and immunologic phase 2 trial of Wilms tumor gene product 1 (WT1) peptide vaccination in patients with AML and MDS. Blood 113:6541–6548

Li Z et al (2005) Combination of imatinib mesylate with autologous leukocyte-derived heat shock protein and chronic myelogenous leukemia. Clin Cancer Res 11:4460–4468

Meyer RG et al (2007) Prophylactic transfer of CD8-depleted donor lymphocytes after T-cell-depleted reduced-intensity transplantation. Blood 109: 374–382

Mielke S et al (2008) A clinical-scale selective allodepletion approach for the treatment of HLA-mismatched and matched donor-recipient pairs using expanded T lymphocytes as antigen-presenting cells and a TH9402-based photodepletion technique. Blood 111:4392–4402

Mielke S et al (2011) Selectively T cell-depleted allografts from HLA-matched sibling donors followed by low-dose posttransplantation immunosuppression

to improve transplantation outcome in patients with hematologic malignancies. Biol Blood Marrow Transplant 17:1855–1861

Nonn M et al (2008) Selective depletion of alloreactive T lymphocytes using patient-derived nonhematopoietic stimulator cells in allograft engineering. Transplantation 86:1427–1435

Orti G et al (2009) Phase I study of high-stringency CD8 depletion of donor leukocyte infusions after allogeneic hematopoietic stem cell transplantation. Transplantation 88:1312–1318

Padua RA et al (2003) PML-RARA-targeted DNA vaccine induces protective immunity in a mouse model of leukemia. Nat Med 9:1413–1417

Quintarelli C et al (2011) High-avidity cytotoxic T lymphocytes specific for a new PRAME-derived peptide can target leukemic and leukemic-precursor cells. Blood 117:3353–3362

Rezvani K et al (2008) Leukemia-associated antigen-specific T-cell responses following combined PR1 and WT1 peptide vaccination in patients with myeloid malignancies. Blood 111:236–242

Rutten CE et al (2008) HLA-DP as specific target for cellular immunotherapy in HLA class II-expressing B-cell leukemia. Leukemia 22:1387–1394

Salomon SR et al (2005) Selective Depletion of donor lymphocytes: a novel method to reduce the severity of graft-versus-host disease in older patients undergoing matched sibling donor stem cell transplantation. Blood 106:1123–1129

Soiffer RJ et al (1990) Reconstitution of T-cell function after CD6-depleted allogeneic bone marrow transplantation. Blood 75:2076–2084

Teschner D et al (2014) Depletion of naive T cells using clinical grade magnetic CD45RA beads: a new approach for GVHD prophylaxis. Bone Marrow Transplant 49(1):138–144

Tsirigitis P et al (2012) Immunotherapeutic approaches to improve graft-versus-tumor effect and reduce graft-versus-host disease. Immunotherapy 4:407–424

Wehler TC et al (2007) Targeting the activation-induced antigen CD137 can selectively deplete alloreactive T cells from antileukemic and antitumor donor T-cell lines. Blood 109:365–373

Part IV

Biomarkers

Molecular and Metabolic Cues of the Key-Lock Paradigm Dictating Immunogenic Cell Death

Jonathan M. Pitt, Marie Vetizou, Oliver Kepp, Guido Kroemer, and Laurence Zitvogel

A collection of studies over the past decade has brought about a shift in how we understand the interaction between the therapeutic killing of cancer cells and the host antitumour immune response. Rather than a tolerogenic or zero-impact event, the induced death of cancer cells has been shown under certain conditions and circumstances to actually incite an immune response against dead cancer cell antigens. This phenomenon is referred to as 'immunogenic cell death (ICD)', which has been strongly associated with the actions and therapeutic value of certain antineoplastic agents. In itself, ICD induced by such agents has challenged the theory that these therapies simply kill or target proliferation of malignant cells in a cell-autonomous disease. The evidence presented in this chapter points towards an additional aspect to cytotoxic cancer therapies, in that they also induce (or reinstate) an extrinsic control exerted by the host immune system. We will discuss how a precise combination of molecular cues and metabolic variations is induced through ICD to form a key, with the ability to 'unlock' an otherwise restrained immune response against the cancer.

J.M. Pitt • M. Vetizou • O. Kepp
Gustave Roussy Cancer Campus, Villejuif, France

INSERM U1015, Villejuif, France

Faculté de Médecine, Université Paris Sud-XI, Le Kremlin Bicêtre, France

G. Kroemer
Gustave Roussy Cancer Campus, Villejuif, France

INSERM U848, Villejuif, France

Metabolomics Platform, Institut Gustave Roussy, Villejuif, France

Equipe 11 labellisée Ligue contre le Cancer, Centre de Recherche des Cordeliers, Paris, France

Pôle de Biologie, Hôpital Européen Georges Pompidou, AP-HP, Paris, France

Université Paris Descartes-V, Sorbonne Paris Cité, Paris, France

L. Zitvogel (✉)
Gustave Roussy Cancer Campus, Villejuif, France

INSERM U1015, Villejuif, France

Faculté de Médecine, Université Paris Sud-XI, Le Kremlin Bicêtre, France

Center of Clinical Investigations in Biotherapies of Cancer (CICBT) 507, Villejuif, France
e-mail: zitvogel@igr.fr

Control of Cancer Through Immune Cells and Cytokines Post-ICD

It is now well established through both murine (Apetoh et al. 2007; Michaud et al. 2011) and clinical studies (Ray-Coquard et al. 2009) that responses to chemotherapy are more efficient in immunocompetent as opposed to immunodeficient hosts. Indeed, antineoplastic agents cause changes in the immune infiltrate of tumours, with the characteristics of these alterations often associated

This chapter is dedicated to an exemplary scientist, a pioneering immunotherapist and a long-standing friend, Christoph Huber.

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_20,

with the outcome of therapy. For example, an induced increase in the ratio of cytotoxic $CD8^+$ T lymphocyte (CTL) to $Foxp3^+$ regulatory T cells (Treg) is correlated with favourable therapeutic responses in certain cancers (Zitvogel et al. 2013). Therapeutic settings that induce tumour antigen-specific T-cell responses, particularly CTL and T helper (Th)1 responses with their characteristic production of interferon (IFN)-γ, can effectively eliminate tumour cells. The extent to which interleukin (IL)-17-producing T cells (e.g. Th17) contribute post-chemotherapy is less well understood. IL-17A production by γδ T cells has however been shown to be indispensable for optimal anticancer responses following ICD-inducing chemotherapy and radiotherapy (Ma et al. 2011). Early tumour infiltration by this innate lymphocyte population is an essential prerequisite to the later accumulation of IFN-γ-producing $CD8^+$ αβ T cells for effective tumour control. To trigger this immune response, γδ T cells must first be activated by IL-1β-IL-1 receptor signalling, the source of IL-1β being dendritic cells (DCs; the professional antigen-presenting cells of the immune system). Later studies went on to reveal the origins of these DCs in the tumour bed following ICD-inducing chemotherapy. Treatment of previously established murine tumours with anthracyclines resulted in the accumulation of $CD11c^+CD11b^+Ly6C^{hi}$ cells, characteristic of inflammatory monocytes, which were particularly efficient at engulfing tumour antigens and presenting these to $CD8^+$ T cells (Ma et al. 2013a). The tumour infiltration of this myeloid population was dependent upon ATP, as this process failed to occur in murine tumours engineered to overexpress the ecto-ATPase CD39.

Thus, a cascade of events occurs post-chemotherapy-induced ICD, where ATP released by dying cancer cells recruits myeloid precursors able to differentiate into DCs that (1) produce the necessary IL-1β to drive T-cell recruitment via IL-17 from γδ T cells and (2) present tumour antigens to activate these recruited CTL and $CD4^+$ helper T cells that subsequently fight the growing tumour (e.g. through production of IFN-γ). The essential role of the cytokines that mediate this cascade following ICD has been elegantly confirmed in mouse models with neutralising antibodies or genetic deletion of cytokines (*Il1b*, *Il17a*, *Ifng*) and their receptors (*Il1r*, *Il17r*, *Ifngr*) (Ma et al. 2011, 2013a; Michaud et al. 2011; Obeid et al. 2007; Ghiringhelli et al. 2009). Somewhat surprisingly, given the key role of the proinflammatory cytokine IL-1β, TNF-α signalling does not contribute to the antineoplastic effects of anthracycline chemotherapies in various tested murine tumour models (Ma et al. 2013b). Finally, the key role and mechanism of accumulation of Th1 cells in tumour beds has been recently unravelled. Hence, the contribution of gut microbiota through cyclophosphamide-induced permeabilisation of the intestinal barrier became clear when comparing untreated and antibiotic-treated animals or animals reared in SPF and germ-free conditions (Viaud et al. 2013).

The contributions of other immune cell types to anticancer responses post-ICD induction in tumours have been addressed in other studies. Although cancer progression often associates with high titres of tumour-specific antibodies, B cells and the humoral immune response appear to be dispensable in the immune-dependent therapeutic effect of anthracyclines (Hannani et al. 2013).

Subtle Molecular and Metabolic Signals During ICD Unlock the Immune Response

Initiation of host innate immune responses begins with pattern recognition of danger-associated molecules, for example, those that make up a pathogen. Recognition of such molecules is performed by a variety of germline-encoded receptors, many of which are only present on immune cells, termed pattern recognition receptors (PRRs) (Janeway and Medzhitov 2002). Over the past decade, we have shown that subtle biochemical changes in the plasma membrane and microenvironment of dying cells, as only seen with ICD-promoting compounds, also stimulate PRRs of the immune system.

ER Stress and Calreticulin Exposure

The first of these necessary for immune stimulation following cell death is the exposure of calreticulin on dying cells. Calreticulin (CRT)

is the most abundant protein present on the endoplasmic reticulum (ER) lumen, though it is also present in other subcellular compartments. Following administration of ICD inducers, a small proportion of CRT translocates from the ER to the plasma membrane of stressed or dying cancer cells very early in the death process (Kroemer et al. 2013). This exposure of CRT is activated by an ER stress response involving phosphorylation of the eukaryotic translation initiation factor eIF2α by the PKR-like ER kinase (PERK). Downstream activation of pro-apoptotic proteins BAX and BAK occurs subsequently, with anterograde transport of CRT from the ER to the Golgi apparatus allowing exocytosis of CRT-containing vesicles to the cellular plasma membrane. Notably, this external cell membrane exposure of CRT does not occur with anticancer therapies that do not induce ICD. CRT exposure couples to induction of an anti-tumour immune response by acting as an 'eat-me' signal to DCs, which go on to phagocytose tumour cells and present their antigens (Obeid et al. 2007). Any inhibition of CRT exposure through blocking antibodies or CRT transcript knockdown abrogates anthracycline immunogenicity, highlighting the key requirement of this process to ICD (Fig. 1).

The perception of CRT and engulfment of CRT-exposing cells by antigen-presenting cells (APCs, i.e. DCs and macrophages) may occur through the transmembrane receptor CD91. CRT signals through CD91 on APCs to stimulate the production of proinflammatory cytokines such as TNF-α and IL-6, which together with dead cell antigen presentation by DCs can help drive T-cell responses. In a similar scenario, it has also been identified that the proteasome inhibitor bortezomib facilitates immunogenic death of human tumours. Bortezomib induced premortem stress of cancer cells and the surface exposure of heat-shock protein 90 (Hsp90), which like CRT enables phagocytosis by DCs (Spisek et al. 2007). Other membrane molecules externally co-expressed on cancer cells may however inhibit DC phagocytosis, such as CD47 that acts as a 'don't eat me' signal. Indeed, anti-CD47 antibody treatment increases cancer cell phagocytosis by APCs, initiating antitumour cytotoxic T-cell immune responses (Tseng et al. 2013). Finally, clinical evidence supports cancer cell CRT exposure (and the balance of CRT to CD47 exposure) to patient survival in various human cancers, including acute myeloid leukaemia, non-Hodgkin lymphoma and colorectal cancer (Kroemer et al. 2013).

HMGB1 and TLR4

Toll-like receptor (TLR) 4 is a key member of the TLR family of PRRs, best characterised for its sensing of lipopolysaccharide (LPS) present in Gram-negative bacteria and downstream activation of immune cell production of proinflammatory cytokines. TLR4 has been strongly associated with ICD since a landmark study showing that (1) TLR4 expression by DCs is required for the immune response against dying cells in vivo; (2) HMGB1, a nonhistone chromatin protein, binds TLR4 following its release by dying cells during anthracycline treatment; and (3) a TLR4 polymorphism that inhibits HMGB1 binding is negatively associated with the efficacy of antitumour therapy in humans (Apetoh et al. 2007). Accordingly, tumours established in mice deficient for the genes encoding TLR4 or its downstream adaptor molecule MyD88 respond inefficiently to ICD-inducing chemotherapy and radiotherapy. The HMGB1-TLR4 axis in ICD is also essential for the cross-presentation of tumour antigens by DCs, since TLR4 depletion or the depletion or antibody-mediated neutralisation of HMGB1 abrogated this process in vitro (Apetoh et al. 2007). Finally and supporting these findings, it has recently been observed that a high-potency and exclusive TLR4 agonist improves the immunogenicity and efficacy of chemotherapy against tumours that exhibit weak expression of HMGB1 or in tumours that have had HMGB1 depleted by RNA interference (Yamazaki et al. 2013). The role of TLR4 in the efficacy of oxaliplatin has been recently corroborated by another group showing that gut-derived commensals are also a source of TLR4 ligands indispensable for the activation of intratumoural myeloid cells (Iida et al. 2013).

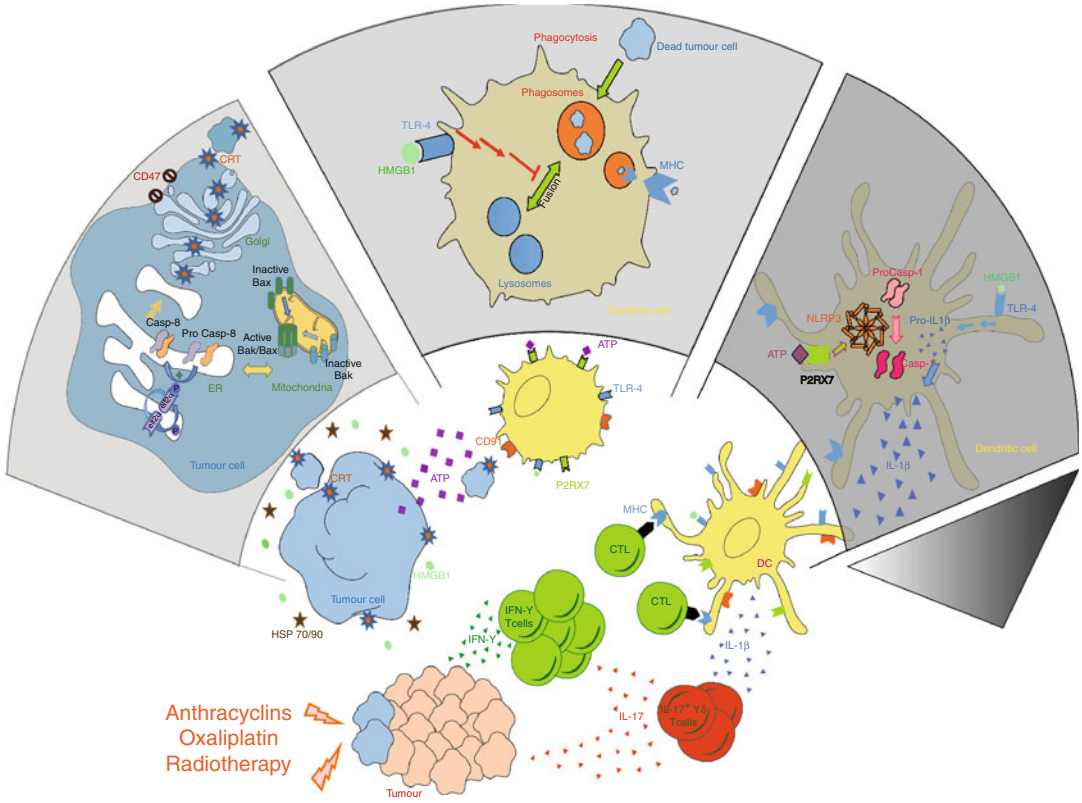

Fig. 1 Molecular and cellular mechanisms dictating ICD. Chemotherapy induces early exposure of calreticulin (*CRT*), which acts as an 'eat-me' signal and enhances the recognition and uptake of tumour cells by dendritic cells (*DC*). Endoplasmic reticulum (*ER*) stress is required for CRT exposure. ICD also requires an intact apoptotic signal that involves caspase-8, BAX and BAK. The release of HMGB1 and ATP by dying tumour cells acts through TLR4 and P2RX7, respectively, as immunogenic signals promoting enhanced antigen processing and presentation and production of IL-1β by DC. The recognition of ATP by its receptor leads to activation of the NLRP3 inflammasome and cleavage of pro-caspase-1 into caspase-1. The ligation of HMGB1 with TLR4 leads to synthesis of pro-IL1β that is cleaved by active caspase-1, culminating in the secretion of IL-1β. This sequence of events allows the polarisation of $CD8^+$ T cells towards IFN-γ production, leading to tumour eradication

Autophagy and the Release of ATP

As discussed, IL-1β is a critical mediator of ICD post-anthracycline chemotherapy. The formation of biologically active IL-1β requires activation of the intracellular danger-sensing apparatus known as the inflammasome. The NLRP3 inflammasome senses pathogen or damage-associated molecular patterns and in response to these interacts with various adaptor molecules to activate caspase-1, which in turn proteolytically matures pro-IL-1β to active IL-1β. Activation of the NLRP3 inflammasome is decisive for an immune response to dying cancer cells, since mice with genetic deletion of the inflammasome component genes *Nlrp3* and *Casp1* fail to respond to oxaliplatin chemotherapy (Ghiringhelli et al. 2009). The release of ATP from dying or stressed cells, a major trigger for inflammasome activation, was critical for immunogenicity via activation of P2RX7 receptors on DCs. This enabled DCs to release IL-1β, which was necessary for the priming of IFN-γ-producing tumour-specific $CD8^+$ T cells. Of note, it is probable that activation of other PRRs is a co-requirement for this immune response, an example being the previously discussed TLR4 activation by HMGB1.

Later studies identified that the release of ATP from dying cancer cells in response to

chemotherapy was largely controlled by autophagy. Autophagy-deficient cancer cells had inhibited release of ATP when undergoing cell death and also failed to recruit T cells and DCs into the tumour bed (Michaud et al. 2011). Both of these deficiencies can however be reversed by increasing ATP in the tumour microenvironment through the pharmacological inhibition of extracellular ATP-degrading enzymes, emphasising the importance of local ATP concentration. Extracellular ATP released by dying cells is a potent chemoattractant for DCs and scavenging macrophages, able to stimulate these myeloid immune cells via P2RY2 and P2RX7 receptors. Indeed, the early tumour infiltration of $CD11c^{+}CD11b^{+}Ly6C^{hi}$ myeloid precursor cells post-chemotherapy is abolished when a broad-spectrum purinergic receptor inhibitor is administered. The same effect is seen when the extracellular concentration of ATP local to tumours is decreased by overexpression of the ecto-ATPase CD39 (Ma et al. 2013a). Importantly, it was shown that ATP concentration (and presumably its signalling through purinergic receptors) might also dictate whether myeloid precursors preferentially differentiate towards DCs (able to drive T-cell responses) as opposed to granulocytes (that may be detrimental to tumour control) (Ma et al. 2013a). Taken together, these studies also highlight a potential immunosurveillance-escape strategy for cancer cells that are able to negatively regulate intrinsic autophagic processes. In accordance with this, autophagy is often disabled during early oncogenesis.

ICD-Inducing Therapies

Not all chemotherapeutic agents employed in the fight against cancer induce ICD. In a screen of 24 distinct cytotoxic chemotherapies, only 4 were observed to induce protective anticancer immune responses in vivo, whereas all agents resulted in equivalent apoptosis of target cells (Obeid et al. 2007). These immunogenic agents were the three anthracyclines doxorubicin, idarubicin and mitoxantrone, plus the platinum compound oxaliplatin. Notably, the structurally related platinum compound cisplatin is unable to induce ER stress and the resulting CRT exposure as seen in oxaliplatin-mediated ICD (Kroemer et al. 2013).

The four identified agents each result in the key hallmarks of ICD following their administration (i.e. CRT and other ER protein exposure and release of ATP and HMGB1 from dying cancer cells). The measurement of these parameters in a screening platform has been used to show that several FDA-approved drugs are also able to induce ICD and as such may prove to be promising adjunctive therapies in cases where standard chemotherapy fails to be sufficiently immunogenic (Menger et al. 2012). Cardiac glycosides (e.g. digoxin, digitoxin) are an example of a drug class found to be particularly efficient at inducing ICD (Menger et al. 2012), this effect observed to occur through their inhibition of Na^{+},K^{+}-ATPase pumps and consequent Ca^{2+} influx into cancer cells.

Future Perspectives

ICD may be considered to operate similarly to vaccination, where the patient's dying cancer cells provide both the tumour cell antigens and the necessary molecular and metabolic 'adjuvants' to activate and facilitate cross-presentation by DCs. An important remaining question is whether ICD-inducing chemotherapeutic regimes elicit a de novo T-cell priming, reactivate existing local effector and memory T cells or result in both phenomena. The described mechanisms of ICD have mostly been validated in transplantable models of mouse sarcoma, carcinoma and lymphoma. Studies in the MMTV-*NeutT* model however do also reveal that immunosurveillance controls breast carcinogenesis and contributes to the efficacy of anticancer chemotherapy. Prendergast and colleagues reported as early as 2005 that the combination of paclitaxel and 1-methyl-DL-tryptophan (an inhibitor of the immunosuppressive enzyme indoleamine 2,3 dioxygenase) exhibited synergistic anticancer effects that were lost upon depletion of $CD4^{+}$ T cells with a specific monoclonal antibody (Muller et al. 2005). Concomitant depletion of $CD4^{+}$ and $CD8^{+}$ T cells accelerated oncogenesis of MMTV-NeuT mice, while blockade of the immunosuppressive

cytokine interleukin-13 postponed oncogenesis in this model (Park et al. 2008). It is difficult to understand why depletion of T cells (by simultaneous injection of antibodies specific for CD4 and CD8) would increase oncogenesis (Park et al. 2008). In another model of transgene-induced breast cancer (MMTV-rtTA,TetO-PyMT:IRES-Luc), which is driven by the tetracycline-inducible expression of the polyoma middle T (PyMT) oncogene, depletion of regulatory T cells resulted in significant inhibition of primary and metastatic cancer progression (Bos et al. 2013), again underscoring the probable importance of immunosurveillance in the pathogenesis of breast cancer. In addition Wolfgang Doppler and colleagues recently reported that treatment of MMTV-*NeutT* mice with doxorubicin or lapatinib (or their combination) mediated antitumour effects that disappeared upon depletion of $CD8^+$ (but not $CD4^+$) T lymphocytes (Hannesdottir et al. 2013). Interestingly, knockout of *Stat1* led to complete failure of chemotherapy with doxorubicin or targeted therapy of MMTV-*NeuT* breast cancers with lapatinib. *Stat1* knockout also abolished the infiltration of tumours with T lymphocytes that is usually observed post-chemotherapy (Hannesdottir et al. 2013). Thus, the pharmacological inhibition of HER2/Neu by lapatinib triggered immune-dependent anticancer effects (Hannesdottir et al. 2013), confirming previous results obtained with therapeutic anti-HER2/Neu antibodies (Park et al. 2010; Stagg et al. 2011).

This demonstration of a critical role of the immune system in the response to cytotoxic agents can be extended to oncogene-targeting therapies. In two independent genetically modified PTEN-deficient breast cancers resistant to trastuzumab (one presenting with an ERBB2 knock-in mutation and the other with an NIC deficiency), Wang and colleagues reported that the combination of an HER2/Neu antibody and an Akt inhibitor triciribine effectively inhibited tumour growth in a T-cell-dependent manner. Indeed, in addition to blocking PI3K/AKT and MAPK signalling pathways, the combination treatment enhanced $CD4^+$ and $CD8^+$ T-cell infiltration into the tumour microenvironment that was geared towards a Th1-polarisation. Neutralising anti-IFN-γ antibodies compromised the synergistic antitumour effects of HER2/Neu antibody and an Akt inhibitor triciribine (Wang et al. 2012). Moreover, the combined targeted therapy increased the expression of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4, also known as CD152), a counter-inhibitory receptor of CD28. The authors additionally showed that boosting T-cell responses by blockade of CTLA4 could further improve the antitumour activity of HER2/Neu antibody and triciribine combination treatment (Wang et al. 2012).

Such results appear incompatible with those reported by Karine de Visser and colleagues (Ciampricotti et al. 2012), where it was shown that spontaneous mouse breast carcinomas respond equally to oxaliplatin or doxorubicin in the absence or the presence of Rag1 and Rag2 recombinases (required for T- and B-cell generation). However, one possibility to explain this discrepancy resides in the fact that Doppler and colleagues treated breast cancers from MMTV-NeutT mice only once with doxorubicin (Hannesdottir et al. 2013), while de Visser and colleagues applied at least three cycles of chemotherapy (Ciampricotti et al. 2012), which might have induced a severe state of immunodepression.

These preclinical data may have consequences for clinical management. There is ample evidence that the density, composition and function of the T-cell infiltrate have a major impact on the prognosis and therapeutic response of human breast cancers of different subtypes (Denkert et al. 2010; DeNardo et al. 2011; Senovilla et al. 2012; Loi 2013; Zitvogel et al. 2013).

ICD Mechanisms as Biomarkers and Compensatory Therapies

A promising future aspect in the study of the mechanisms dictating ICD is whether they can themselves be used as biomarkers that can predict a patient's therapeutic response. This will require several subtle measurements: (1) the monitoring of the ER stress response through measurement of CRT and Hsp90 (versus CD47) exposure on cancer cells, (2) measurement of cancer cell autophagy and (3) investigating the subcellular localisation of HMGB1. Each of

these measurements would be required pre- and post-chemotherapy and would be ideally completed beside identification of intratumoural immune cells such as the different T-cell populations and DCs and DC precursors.

Many currently used anticancer therapies fail to promote ICD (Obeid et al. 2007). Strategies to restore ICD with adjunctive therapies therefore have potential in cases where ICD is absent. This would first require a detailed confirmation of which factors of ICD and the resulting immune response are suboptimal, so to enable a targeted strategy. Examples of conceivable compensatory strategies include intratumoural administration of ER-stressing agents (e.g. GADD34 inhibitors) or recombinant CRT. Also, the administration of TLR4 agonists may prove to be a useful adjunctive compensation therapy in cases with poor HMGB1 release features (Yamazaki et al. 2013). Feasible strategies to increase local concentrations of ATP (e.g. ectonucleotidase inhibitors) or signalling through purinergic receptors (i.e. P2RX7 agonists) could also potentially restore a lack of immunogenicity by a given therapeutic (Michaud et al. 2011). Intratumoural therapies of recombinant cytokines such as IL-1β or IL-17 may be effective if their production by immune cells within the tumour is low or absent. Finally, following the discovery that cardiac glycosides induce ICD and improve responses to non-ICD-inducing drugs in vivo, the administration of cardiac glycosides may prove a beneficial and reachable future strategy (Menger et al. 2012).

References

Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, Mignot G, Maiuri MC, Ullrich E, Saulnier P, Yang H, Amigorena S, Ryffel B, Barrat FJ, Saftig P, Levi F, Lidereau R, Nogues C, Mira JP, Chompret A, Joulin V, Clavel-Chapelon F, Bourhis J, Andre F, Delaloge S, Tursz T, Kroemer G, Zitvogel L (2007) Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med 13(9):1050–1059

Bos PD, Plitas G, Rudra D, Lee SY, Rudensky AY (2013) Transient regulatory T cell ablation deters oncogene-driven breast cancer and enhances radiotherapy. J Exp Med 210(11):2435–2466

Ciampricotti M, Hau CS, Doornebal CW, Jonkers J, de Visser KE (2012) Chemotherapy response of spontaneous mammary tumors is independent of the adaptive immune system. Nat Med 18(3):344–346; author reply 346

DeNardo DG, Brennan DJ, Rexhepaj E, Ruffell B, Shiao SL, Madden SF, Gallagher WM, Wadhwani N, Keil SD, Junaid SA, Rugo HS, Hwang ES, Jirstrom K, West BL, Coussens LM (2011) Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. Cancer Discov 1(1):54–67

Denkert C, Loibl S, Noske A, Roller M, Muller BM, Komor M, Budczies J, Darb-Esfahani S, Kronenwett R, Hanusch C, von Torne C, Weichert W, Engels K, Solbach C, Schrader I, Dietel M, von Minckwitz G (2010) Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. J Clin Oncol 28(1):105–113

Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C, Vermaelen K, Panaretakis T, Mignot G, Ullrich E, Perfettini JL, Schlemmer F, Tasdemir E, Uhl M, Genin P, Civas A, Ryffel B, Kanellopoulos J, Tschopp J, Andre F, Lidereau R, McLaughlin NM, Haynes NM, Smyth MJ, Kroemer G, Zitvogel L (2009) Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. Nat Med 15(10):1170–1178

Hannani D, Locher C, Yamazaki T, Colin-Minard V, Vetizou M, Aymeric L, Viaud S, Sanchez D, Smyth MJ, Bruhns P, Kroemer G, Zitvogel L (2014) Contribution of humoral immune responses to the antitumor effects mediated by anthracyclines. Cell Death Differ 21:50–58

Hannesdottir L, Tymoszuk P, Parajuli N, Wasmer MH, Philipp S, Daschil N, Datta S, Koller JB, Tripp CH, Stoitzner P, Muller-Holzner E, Wiegers GJ, Sexl V, Villunger A, Doppler W (2013) Lapatinib and doxorubicin enhance the Stat1-dependent antitumor immune response. Eur J Immunol 43(10):2718–2729

Iida N, Dzutsev A, Stewart CA, Smith L, Bouladoux N, Weingarten RA, Molina DA, Salcedo R, Back T, Cramer S, Dai RM, Kiu H, Cardone M, Naik S, Patri AK, Wang E, Marincola FM, Frank KM, Belkaid Y, Trinchieri G, Goldszmid RS (2013) Commensal bacteria control cancer response to therapy by modulating the tumor microenvironment. Science 342(6161):967–970

Janeway CA Jr, Medzhitov R (2002) Innate immune recognition. Annu Rev Immunol 20:197–216

Kroemer G, Galluzzi L, Kepp O, Zitvogel L (2013) Immunogenic cell death in cancer therapy. Annu Rev Immunol 31:51–72

Loi S (2013) Tumor-infiltrating lymphocytes, breast cancer subtypes and therapeutic efficacy. Oncoimmunology 2(7):e24720

Ma Y, Aymeric L, Locher C, Mattarollo SR, Delahaye NF, Pereira P, Boucontet L, Apetoh L, Ghiringhelli F, Casares N, Lasarte JJ, Matsuzaki G, Ikuta K, Ryffel B, Benlagha K, Tesniere A, Ibrahim N, Dechanet-Merville J, Chaput N, Smyth MJ, Kroemer G, Zitvogel L (2011) Contribution of IL-17-producing

gamma delta T cells to the efficacy of anticancer chemotherapy. J Exp Med 208(3):491–503

Ma Y, Adjemian S, Mattarollo SR, Yamazaki T, Aymeric L, Yang H, Portela Catani JP, Hannani D, Duret H, Steegh K, Martins I, Schlemmer F, Michaud M, Kepp O, Sukkurwala AQ, Menger L, Vacchelli E, Droin N, Galluzzi L, Krzysiek R, Gordon S, Taylor PR, Van Endert P, Solary E, Smyth MJ, Zitvogel L, Kroemer G (2013a) Anticancer chemotherapy-induced intratumoral recruitment and differentiation of antigen-presenting cells. Immunity 38(4):729–741

Ma Y, Yamazaki T, Yang H, Kepp O, Galluzzi L, Zitvogel L, Smyth MJ, Kroemer G (2013b) Tumor necrosis factor is dispensable for the success of immunogenic anticancer chemotherapy. Oncoimmunology 2(6):e24786

Menger L, Vacchelli E, Adjemian S, Martins I, Ma Y, Shen S, Yamazaki T, Sukkurwala AQ, Michaud M, Mignot G, Schlemmer F, Sulpice E, Locher C, Gidrol X, Ghiringhelli F, Modjtahedi N, Galluzzi L, Andre F, Zitvogel L, Kepp O, Kroemer G (2012) Cardiac glycosides exert anticancer effects by inducing immunogenic cell death. Sci Transl Med 4(143):143ra99

Michaud M, Martins I, Sukkurwala AQ, Adjemian S, Ma Y, Pellegatti P, Shen S, Kepp O, Scoazec M, Mignot G, Rello-Varona S, Tailler M, Menger L, Vacchelli E, Galluzzi L, Ghiringhelli F, di Virgilio F, Zitvogel L, Kroemer G (2011) Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. Science 334(6062):1573–1577

Muller AJ, DuHadaway JB, Donover PS, Sutanto-Ward E, Prendergast GC (2005) Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene Bin1, potentiates cancer chemotherapy. Nat Med 11(3):312–319

Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, Castedo M, Mignot G, Panaretakis T, Casares N, Metivier D, Larochette N, van Endert P, Ciccosanti F, Piacentini M, Zitvogel L, Kroemer G (2007) Calreticulin exposure dictates the immunogenicity of cancer cell death. Nat Med 13(1):54–61

Park JM, Terabe M, Donaldson DD, Forni G, Berzofsky JA (2008) Natural immunosurveillance against spontaneous, autochthonous breast cancers revealed and enhanced by blockade of IL-13-mediated negative regulation. Cancer Immunol Immunother 57(6):907–912

Park S, Jiang Z, Mortenson ED, Deng L, Radkevich-Brown O, Yang X, Sattar H, Wang Y, Brown NK, Greene M, Liu Y, Tang J, Wang S, Fu YX (2010) The therapeutic effect of anti-HER2/neu antibody depends on both innate and adaptive immunity. Cancer Cell 18(2):160–170

Ray-Coquard I, Cropet C, Van Glabbeke M, Sebban C, Le Cesne A, Judson I, Tredan O, Verweij J, Biron P, Labidi I, Guastalla JP, Bachelot T, Perol D, Chabaud S, Hogendoorn PC, Cassier P, Dufresne A, Blay JY, European Organization for R, Treatment of Cancer Soft T, Bone Sarcoma G. (2009) Lymphopenia as a prognostic factor for overall survival in advanced carcinomas, sarcomas, and lymphomas. Cancer Res 69(13):5383–5391

Senovilla L, Vitale I, Martins I, Tailler M, Pailleret C, Michaud M, Galluzzi L, Adjemian S, Kepp O, Niso-Santano M, Shen S, Marino G, Criollo A, Boileve A, Job B, Ladoire S, Ghiringhelli F, Sistigu A, Yamazaki T, Rello-Varona S, Locher C, Poirier-Colame V, Talbot M, Valent A, Berardinelli F, Antoccia A, Ciccosanti F, Fimia GM, Piacentini M, Fueyo A, Messina NL, Li M, Chan CJ, Sigl V, Pourcher G, Ruckenstuhl C, Carmona-Gutierrez D, Lazar V, Penninger JM, Madeo F, Lopez-Otin C, Smyth MJ, Zitvogel L, Castedo M, Kroemer G (2012) An immunosurveillance mechanism controls cancer cell ploidy. Science 337(6102):1678–1684

Spisek R, Charalambous A, Mazumder A, Vesole DH, Jagannath S, Dhodapkar MV (2007) Bortezomib enhances dendritic cell (DC)-mediated induction of immunity to human myeloma via exposure of cell surface heat shock protein 90 on dying tumor cells: therapeutic implications. Blood 109(11):4839–4845

Stagg J, Loi S, Divisekera U, Ngiow SF, Duret H, Yagita H, Teng MW, Smyth MJ (2011) Anti-ErbB-2 mAb therapy requires type I and II interferons and synergizes with anti-PD-1 or anti-CD137 mAb therapy. Proc Natl Acad Sci U S A 108(17):7142–7147

Tseng D, Volkmer JP, Willingham SB, Contreras-Trujillo H, Fathman JW, Fernhoff NB, Seita J, Inlay MA, Weiskopf K, Miyanishi M, Weissman IL (2013) Anti-CD47 antibody-mediated phagocytosis of cancer by macrophages primes an effective antitumor T-cell response. Proc Natl Acad Sci U S A 110(27):11103–11108

Viaud S, Saccheri F, Mignot G, Yamazaki T, Daillere R, Hannani D, Enot DP, Pfirschke C, Engblom C, Pittet MJ, Schlitzer A, Ginhoux F, Apetoh L, Chachaty E, Woerther PL, Eberl G, Berard M, Ecobichon C, Clermont D, Bizet C, Gaboriau-Routhiau V, Cerf-Bensussan N, Opolon P, Yessaad N, Vivier E, Ryffel B, Elson CO, Dore J, Kroemer G, Lepage P, Boneca IG, Ghiringhelli F, Zitvogel L (2013) The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. Science 342(6161):971–976

Wang Q, Li SH, Wang H, Xiao Y, Sahin O, Brady SW, Li P, Ge H, Jaffee EM, Muller WJ, Hortobagyi GN, Yu D (2012) Concomitant targeting of tumor cells and induction of T-cell response synergizes to effectively inhibit trastuzumab-resistant breast cancer. Cancer Res 72(17):4417–4428

Yamazaki T, Hannani D, Poirier-Colame V, Ladoire S, Locher C, Sistigu A, Prada N, Adjemian S, Catani JP, Freudenberg M, Galanos C, Andre F, Kroemer G, Zitvogel L (2014) Defective immunogenic cell death of HMGB1-deficient tumors: compensatory therapy with TLR4 agonists. Cell Death Differ 21:69–78

Zitvogel L, Galluzzi L, Smyth MJ, Kroemer G (2013) Mechanism of action of conventional and targeted anticancer therapies: reinstating immunosurveillance. Immunity 39(1):74–88

Pathogenesis of Minimal Residual Disease: CTCs as Novel Biomarkers in Cancer Disease

Tobias M. Gorges and Klaus Pantel

Cancer Progression

Most cancer-related deaths are caused by the dissemination of aggressive cancer cells (metastasis) (Fig. 1). The traditional point of view expects metastasis formation as late-stage event in tumor progression. However, increasing research data suggest that epithelial cells may disseminate even before the development of manifested primary tumors is visible (Kang and Pantel 2013). It is thought that only a small number of cancer cells are able to pass the multistep process of intravasation, survival in the bloodstream, extravasation, and formation of MRD or solid metastasis. The bone marrow (BM) seems to be a common organ to which tumor cells home at an early stage. Disseminated tumor cells (DTCs) that have reached the BM or other secondary organs have been linked to poor prognosis (Pantel and Brakenhoff 2004). DTCs may stay in a dormant state but are also able to proliferate and recirculate into the blood. It was shown that the vascular cell adhesion molecule 1 (VCAM-1) seems to promote the crossover from an indolent MRD to an overt metastasis (Liu et al. 2011). It is assumed that tumor cells which already moved into remote body parts may adopt a niche-specific expression profile. Cancer cells that have withstood the era of dissemination may further re-infiltrate into their tumors of origin ("tumor self-seeding") or novel niches leading to a broad heterogeneity of cells found in individual patients (Alix-Panabières et al. 2012). These insights made CTCs interesting and promising novel biomarkers in cancer research. Current clinical trials indicated that tumor cells found in the bloodstream can be deployed for diagnostic, monitoring, and prognostic purposes (Pantel and Alix-Panabières 2013). However, detection of CTCs still remains an obstacle since CTCs appear in the background of up to 10,000,000 normal blood cells at which the number of CTCs might even be lower in primary disease. In addition, Denève and co-workers could show that the liver seems to filter CTCs, decreasing the chances of CTC identification in peripheral blood samples, e.g., in colorectal cancer (Denève et al. 2013). Hence, accurate detection of CTCs is only achievable by specific and selective enrichment of tumor cells or systematic removal of PBMCs and red blood cells (RBCs).

CTC Detection

CTC Enrichment

The enrichment of tumor cells is feasible through a multitude of different procedures (Alix-Panabières et al. 2012). Many enrichment strategies rely on a positive cell selection targeting the epithelial cell adhesion molecule (EpCAM). EpCAM is a transmembrane glycoprotein

T.M. Gorges • K. Pantel (✉)
Department of Tumor Biology,
University Medical Center Hamburg-Eppendorf,
Martinistrasse 52, 20246 Hamburg, Germany
e-mail: t.gorges@uke.de; pantel@uke.de

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_21,

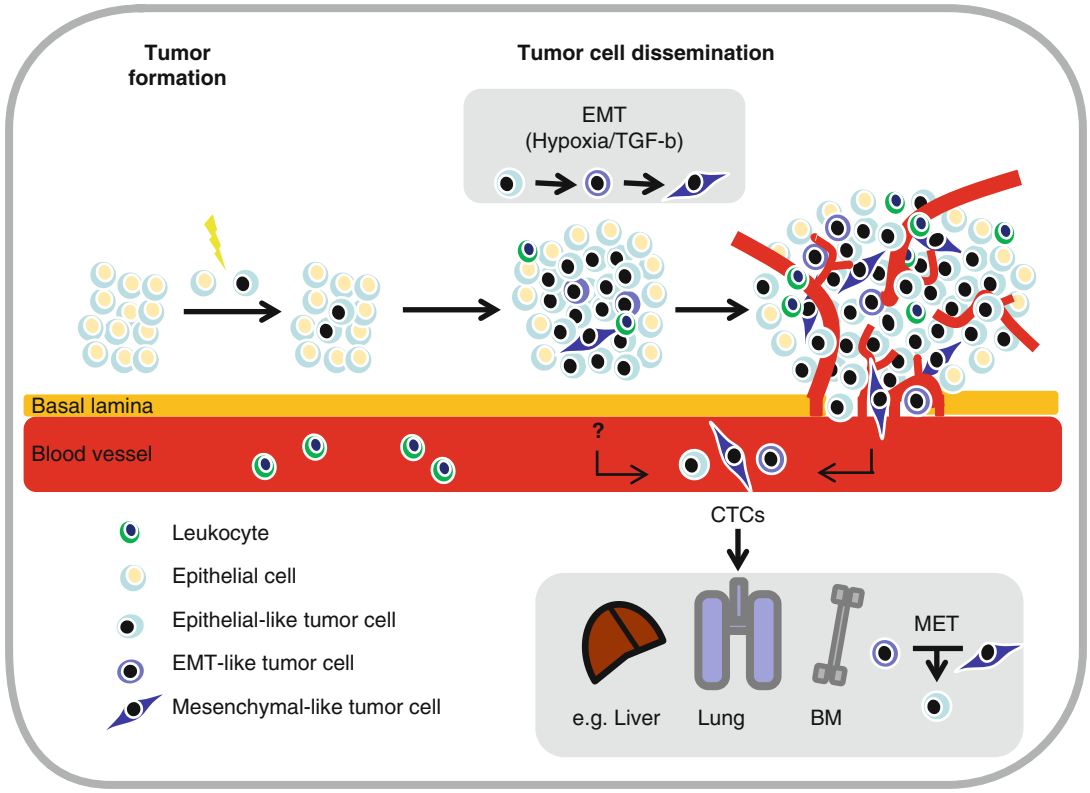

Fig. 1 Aggressive tumor cells of malignant tumors intravasate into the bloodstream and disseminate throughout the body leading to the occurrence of metastasis in secondary organs. It is estimated that EMT-associated changes seem to be required for cancer invasion whereby the reverse molecular process (MET) seems to be needed for the establishment of micro-metastasis after homing of CTCs into novel niches

involved in cell signaling, migration, proliferation, and differentiation (Maetzel et al. 2009; Osta et al. 2004; Münz et al. 2004). Since EpCAM is overexpressed in a huge variety of carcinoma, it has become an attractive target for the positive enrichment of CTCs. The most prominent detection method based on EpCAM enrichment is the CellSearch® system. To date, this FDA-approved technology is the only system which has been used in more than 170 clinical trials. Due to the high reproducibility and the only FDA clearance for a CTC detection system so far, this method should be used as "standard" for all other detection methods appearing on the market. The AdnaTest® is another commercially available research tool for the positive enrichment of CTCs based on epithelial markers. This system has already been used to show efficiency of therapy monitoring in the clinics (Tewes et al. 2009). Microfluidic devices like the IsoFlux approach, the CTC-, or the Herringbone (HB)-Chip have also been used to capture EpCAM-positive tumor cells in blood samples with a relatively high purity (Harb et al. 2013; Nagrath et al. 2007; Maheswaran et al. 2008; Stott et al. 2010). A novel CTC chip combines a size-based filtration with an affinity-based enrichment strategy enhancing the chance of systematic removal of PBMCs and RBCs (Hou et al. 2013). However, most of all chip-based arrays are not commercially available, and larger independent clinical studies have to show the clinical value of the captured tumor cells. Since nearly all in vitro detection methods for CTCs rely on a small sample volume (5–10 ml), novel approaches have to be developed to bypass the problem of sample volume limitations. The GILUPI GmbH has just recently introduced an EpCAM-coated CellCollector to capture CTCs in

vivo. The CellCollector is positioned through a cannula into the arm vein for an intended contact time of 30 min (Saucedo-Zeni et al. 2012; Gorges and Pantel 2013). Using this approach up to 1.5 l of blood may be screened for CTCs increasing the chances of diagnostic sensitivity.

Epithelial to Mesenchymal Transition (EMT): A Handicap for Epithelial Marker-Based Enrichment

EpCAM-positive CTCs have shown clinical relevance in various studies (Cristofanilli et al. 2004; De Bono et al. 2008; Cohen et al. 2009). However, this enrichment strategy disregards the fact that some CTC subpopulations might get lost due to a biological process termed as epithelial to mesenchymal transition (EMT) (Weinberg 2008). EMT-associated changes have been linked to embryogenesis, but current findings in cancer research suggest that EMT might also play a crucial role for malignant tumor progression. EMT-associated changes seem to be required for the gain of stemness features and cancer invasion, and the reverse molecular process (mesenchymal to epithelial transition (MET)) seems to be needed for the establishment of micro-metastasis after homing of CTCs into novel niches (Bednarz-Knoll et al. 2012). Since EMT-associated changes have been discovered on DTCs and CTCs (Bartkowiak et al. 2010; Gorges et al. 2012; Armstrong et al. 2011; Kallergi et al. 2011), current EpCAM-dependent technologies have to be overworked for the detection of CTC subpopulations which underwent an EMT shift.

The removal of $CD45^+$ leukocytes might be an option to capture CTCs having insufficient expression of epithelial surface marker proteins anymore. The PowerMag system is a novel platform for leukocyte depletion and CTC detection. CTCs have been discovered with a recovery rate of 46–62 % (Lin et al. 2013). The EPISPOT assay detects viable CTCs that were enriched after the depletion of $CD45^+$ blood cells. Keeping the viability of CTCs provides a significant advantage that may assist in selecting effective personalized treatment regimens. Removal of $CD45^+$ leukocytes is further combinable with density gradient centrifugation approaches (FICOLL® or OncoQuick®) improving the low purity of this principle alone. However, density gradient centrifugation (combined with or without CD45 depletion) still leads to a high loss of tumor cells conducting to false-negative results in clinical samples (Gorges and Pantel 2013). Size-based filtration devices like the ISET® system or the ScreenCell® approach also seem to detect CTCs with a high recovery rate independently of epithelial markers. However, this principle is hampered by the fact that leukocytes might clog the filter pores, the variable size of different tumor cell subpopulations, and the deformability of CTCs. Unlike other filtration assays, a new spiral biochip separates viable CTCs with a relatively high sensitivity and high throughput (~3 ml/h) (Dean Flow Fractionation (DFF)) (Hou et al. 2013). Here, issues from clogging are eliminated by virtue of large micro-channel dimensions and high flow conditions.

CTC Identification

The FDA-approved CellSearch® system identifies CTCs as $EpCAM^+$; $DAPI^+$ (4,6-diamino-2-phenylindole); KRT-8^+, -18^+, and/or −19^+; and $CD45^-$ using an automated fluorescence scanning system (CellTracks Analyzer II). An additional fluorescence channel might be used for the detection of therapy-relevant markers or the identification of mesenchymal-like CTC populations (Armstrong et al. 2011). Classification of CTCs by fluorescence staining labeling epithelial-specific KRTs and leukocyte-specific CD45 in combination with nuclear counterstain (DAPI) is also applicable for other approaches (microfluidic devices, the GILUPI device, or density centrifugation-based procedures). However, Joosse and co-workers demonstrated that KRT expression may vary on CTCs suggesting to stain additional KRTs which leads to an improved identification (Joosse et al. 2012). Novel proteins like plastin-3 might also help to identify CTCs that have undergone EMT-associated processes since this marker seems to be found on

EpCAM-positive and EpCAM-negative CTC populations (Yokobori et al. 2013). In addition, adenoviruses carrying the green fluorescence protein gene (GFP) under the hTERT promoter (TelomeScan®) can also be used for the identification of epithelial-like and mesenchymal-like cancer cells (Ito et al. 2012). The EPISPOT detects CTCs based on the release of tumor-specific proteins like KRT-19, MUC1 (Mucin-1), PSA (prostate-specific antigen), or the stem cell factor FGF-2 (fibroblast growth factor 2) (Alix-Panabiéres et al. 2012). Further, PCR-based technologies (polymerase chain reaction) verify CTCs on a tumor-specific DNA or multi-marker gene (mRNA) profile. The AdnaTest® system identifies CTCs based on tumor-associated gene transcripts (*EpCAM*, *MUC1*, and *HER2*), EMT-associated markers (*PI3Kα* (*phosphatidylinositol 3-kinase alpha*), *Akt-2*, and *Twist1*), or stemness indicators (*ALDH1* (*aldehyde dehydrogenase 1*)) (Kasimir-Bauer et al. 2012).

Clinical Relevance of CTCs

At present, examination of the success or the failure of cancer therapy requires sophisticated laboratory and imaging technologies. Research on CTCs could already exhibit that CTC counts are a precise indication for disease progression in metastatic disease at any time point during therapy (Hayes et al. 2006). Hence, CTC enumeration during treatment may help to monitor the efficacy of systemic adjuvant therapy in real time. Clinical data already presented that a reduction of CTC numbers during treatment could be related to radiographic response (Maheswaran et al. 2008). The assessment of CTCs even seems to be an earlier and more reproducible indication of the disease status compared to radiologic approaches (Budd et al. 2006). Additionally, CTC counts already provided an earlier assessment of therapy response compared to the commonly used PSA test in prostate cancer (Saad and Pantel 2012; Scher et al. 2011). The auspicious SWOG-S0500 trial will determine whether treatment decision-making based on blood levels of tumor cells (≥5 per 7.5 ml) in women with metastatic breast cancer receiving chemotherapy will derive increased PFS and OS.

A large number of clinical trials quested to answer the prognostic impact of CTCs in cancer disease. Using CellSearch® a poor prognosis for metastatic breast, colon, lung, and prostate cancer patients was already shown. Patients who presented basal CTC counts of ≥3 or ≥5 tumor cells per 7.5 ml of blood demonstrated a significantly shorter progression-free (PFS) and overall survival (OS) (Cristofanilli et al. 2004; De Bono et al. 2008; Cohen et al. 2009). Besides enumeration, molecular characterization of tumor cells also identifies patients at higher risk of metastasis (Wülfing et al. 2006; Bednarz et al. 2010). Recent findings of the German SUCCESS trial indicate a correlation to the appearance of CellSearch®-enumerated CTCs (≥1 per 7.5 ml) with decreased PFS and OS even in cancer patients without visible metastasis (Rack et al. 2010). The detection of CTCs based on *KRT-19* mRNA expression has also been associated with a reduced OS and disease-free survival (DFS) in early breast cancer (Xenidis et al. 2007). Furthermore, Georgoulias and co-workers found that chemotherapy-resistant CTCs with "secondary adjuvant" trastuzumab treatment resulted in a significantly reduced probability of disease relapse and increased disease-free interval compared to early breast cancer patients receiving only standard treatment (Georgoulias et al. 2012). Additional clinical trials like the neoadjuvant GEPARQuattro and GEPARQuinto study will help to answer the question whether observed decreases in CTC detection rates are associated with measurable benefit for the individual cancer patients and will give new insights into the use of CTCs as predictive markers.

CTCs: A Real-Time Liquid Biopsy

Molecular profiling of CTCs offers the opportunity to monitor serial changes of cancer biology on a single cell level ("liquid biopsy"). However, it is still undetermined if a modification

in treatment decisions based on the CTC status will result in a measurable benefit for the individual cancer patient. The amplification or overexpression of the human epidermal growth factor receptor 2 (HER2) plays an important role in the development and progression of carcinoma (Hudis 2007a, b). HER2 has become an eminent target in cancer therapy since the expression has been correlated with response to HER2-targeting drugs like trastuzumab or lapatinib (Arteaga et al. 2011). Several studies have shown discordance between the HER2 status of the primary tumor and CTCs in the same patients (Riethdorf et al. 2010). Just recently, Liu and co-workers could show that HER2-positive breast cancer patients with HER2-positive CTCs have longer PFS after anti-HER2 treatment than HER2-positive patients with HER2-negative CTCs indicating the value of CTCs as liquid biopsy (Liu et al. 2013). Complementarily, the ongoing German multicenter, randomized, phase III study (DETECT III) compares standard therapy alone versus standard therapy combined with lapatinib, randomizing metastatic breast cancer patients with initially HER2-negative primary breast tumors and HER2-positive CTCs.

Therapeutic relevant targets like the epidermal growth factor receptor (EGFR) could also be quantified on CTCs identifying patients who might respond to EGFR inhibitors like erlotinib or gefitinib (Punnoose et al. 2010). However, drug resistance mutations (T790M) were also already found on CTCs matching patients with a reduced potency of any ATP-competitive kinase inhibitor (Stott et al. 2010). A sturdy protocol for the quantitative genomic analysis of CTCs and DTCs has been published by Hannemann and coworkers showing a heterogeneous intra- and inter-patient status of the *EGFR* gene (Hannemann et al. 2011). Current data also demonstrated a considerable intra- and inter-patient heterogeneity of genetic alterations for therapeutic targets like *EGFR*, *KRAS,* or *PIK3CA* (Gasch et al. 2013). These findings might help to explain the variable response rates to EGFR-inhibition-based therapies. Genomic profiling of CTCs by array comparative genomic hybridization (CGH) and next-generation sequencing (NGS) showed that most mutations initially found in CTCs were also present at subclonal level in the primary tumors and metastases indicating the relevance of CTCs as liquid biopsy (Heitzer et al. 2013). Furthermore, multicolor fluorescence in situ hybridization (FISH) on CTCs of the ERG, AR, and PTEN gene loci showed a genetic heterogeneity for PTEN and AR in patients with castration-resistant prostate cancer (CRPC) (Attard et al. 2009).

Azab and co-workers could demonstrate that hypoxic conditions in solid tumors promote metastasis through the activation of proteins involved in the EMT process (Azab et al. 2012). EMT-associated changes are also thought to induce stem cell properties, making EMT markers up-and-coming targets for the treatment decisions based on a CTC profile. EMT- and stemness-associated markers like ALDH1, CXCR4, EGFR, FOXC2, N-Cadherin, Snail1, Twist1, ZEB2 or vimentin have already been described to be upregulated on DTCs or CTCs in animal models as well as in patient-derived samples (Gorges et al. 2012; Kasimir-Bauer et al. 2012; Mani et al. 2008; Mego et al. 2012). However, the clinical relevance of these CTCs has to be cleared.

Summary

The molecular analysis of CTCs as novel biomarkers in cancer research has gained more and more attention during the last years. The enumeration of CTCs provides promising information in the use as stratification markers estimating the risk for metastatic relapse and as monitoring markers evaluating a change in therapy years before the appearance of disease progression is visible using current imaging approaches. However, the biological and clinical value of CTCs exceed their mere enumeration since CTCs are also thought to be suitable as "liquid biopsy." Ongoing and future studies will answer the key question whether the modification in treatment decisions based on the CTC number and/or profile will lead to a measurable benefit in clinical outcome for cancer patients.

References

Alix-Panabiéres C (2012) EPISPOT assay: detection of viable DTCs/CTCs in solid tumor patients. Recent Results Cancer Res 195:69–76. doi:10.1007/978-3-642-28160-0_6

Alix-Panabières C, Schwarzenbach H, Pantel K (2012) Circulating tumor cells and circulating tumor DNA. Annu Rev Med 63:199–215. doi:10.1146/annurev-med-062310-094219

Armstrong AJ, Marengo MS, Oltean S, Kemeny G, Bitting RL, Turnbull JD, Herold CI, Marcom PK, George DJ, Garcia-Blanco MA (2011) Circulating tumor cells from patients with advanced prostate and breast cancer display both epithelial and mesenchymal markers. Mol Cancer Res 9(8):997–1007

Arteaga CL, Sliwkowski MX, Osborne CK, Perez EA, Puglisi F, Gianni L (2011) Treatment of HER2-positive breast cancer: current status and future perspectives. Nat Rev Clin Oncol 9(1):16–32. doi:10.1038/nrclinonc

Attard G, Swennenhuis JF, Olmos D, Reid AH, Vickers E, A'Hern R, Levink R, Coumans F, Moreira J, Riisnaes R, Oommen NB, Hawche G, Jameson C, Thompson E, Sipkema R, Carden CP, Parker C, Dearnaley D, Kaye SB, Cooper CS, Molina A, Cox ME, Terstappen LW, de Bono JS (2009) Characterization of ERG, AR and PTEN gene status in circulating tumor cells from patients with castration-resistant prostate cancer. Cancer Res 69(7):2912–2918

Azab AK, Hu J, Quang P, Azab F, Pitsillides C, Awwad R, Thompson B, Maiso P, Sun JD, Hart CP, Roccaro AM, Sacco A, Ngo HT, Lin CP, Kung AL, Carrasco RD, Vanderkerken K, Ghobrial IM (2012) Hypoxia promotes dissemination of multiple myeloma through acquisition of epithelial to mesenchymal transition-like features. Blood 119(24):5782–5794

Bartkowiak K, Effenberger KE, Harder S, Andreas A, Buck F, Peter-Katalinic J, Pantel K, Brandt BH (2010) Discovery of a novel unfolded protein response phenotype of cancer stem/progenitor cells from the bone marrow of breast cancer patients. J Proteome Res 9(6):3158–3168

Bednarz N, Eltze E, Semjonow A, Rink M, Andreas A, Mulder L, Hannemann J, Fisch M, Pantel K, Weier HU, Bielawski KP, Brandt B (2010) BRCA1 loss preexisting in small subpopulations of prostate cancer is associated with advanced disease and metastatic spread to lymph nodes and peripheral blood. Clin Cancer Res 16(13):3340–3348

Bednarz-Knoll N, Alix-Panabières C, Pantel K (2012) Plasticity of disseminating cancer cells in patients with epithelial malignancies. Cancer Metastasis doi:. doi:10.1007/s10555-012-9370-z

Budd GT, Cristofanilli M, Ellis MJ, Stopeck A, Borden E, Miller MC, Matera J, Repollet M, Doyle GV, Terstappen LW, Hayes DF (2006) Circulating tumor cells versus imaging – predicting overall survival in metastatic breast cancer. Clin Cancer Res 12(21):6403–6409

Cohen SJ, Punt CJ, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY, Picus J, Morse MA, Mitchell E, Miller MC, Doyle GV, Tissing H, Terstappen LW, Meropol NJ (2009) Prognostic significance of circulating tumour cells in patients with metastatic colorectal cancer. Ann Oncol 20:1223–1229

Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben M, Doyle GV, Allard WJ, Terstappen LW, Hayes DF (2004) Circulating tumour cells, disease progression and survival in metastatic breast cancer. N Engl J Med 351:781–791

De Bono JS, Scher HI, Montgomery RB, Parker C, Miller MC, Tissing H, Doyle GV, Terstappen LW, Pienta KJ, Raghavan D (2008) Circulating tumour cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. Clin Cancer Res 14:6302–6309

Denève E, Riethdorf S, Ramos J, Nocca D, Coffy A, Daurès JP, Maudelonde T, Fabre JM, Pantel K, Alix-Panabières C (2013) Capture of viable circulating tumor cells in the liver of colorectal cancer patients. Clin Chem 59(9):1384–1392. doi:10.1373/clinchem.2013.202846

Gasch C, Bauernhofer T, Pichler M, Langer-Freitag S, Reeh M, Seifert AM, Mauermann O, Izbicki JR, Pantel K, Riethdorf S (2013) Heterogeneity of epidermal growth factor receptor status and mutations of KRAS/PIK3CA in circulating tumor cells of patients with colorectal cancer. Clin Chem 59(1):252–260. doi:10.1373/clinchem.2012.188557

Georgoulias V, Bozionelou V, Agelaki S, Perraki M, Apostolaki S, Kallergi G, Kalbakis K, Xyrafas A, Mavroudis D (2012) Trastuzumab decreases the incidence of clinical relapses in patients with early breast cancer presenting chemotherapy-resistant CK-19mRNA-positive circulating tumor cells: results of a randomized phase II study. Ann Oncol 23(7):1744–1750

Gorges TM, Pantel K (2013) Circulating tumor cells as therapy-related biomarkers in cancer patients. Cancer Immunol Immunother 62(5):931–939. doi:10.1007/s00262-012-1387-1

Gorges TM, Tinhofer I, Drosch M, Roese L, Zollner TM, Krahn T, von Ahsen O (2012) Circulating tumour cells escape from EpCAM-based detection due to epithelial-to-mesenchymal transition. BMC Cancer 12(1):178

Hannemann J, Meyer-Staeckling S, Kemming D, Alpers I, Joosse SA, Pospisil H, Kurtz S, Görndt J, Püschel K, Riethdorf S, Pantel K, Brandt B (2011) Quantitative high-resolution genomic analysis of single cancer cells. PLoS One 6(11):e26362

Harb W, Fan A, Tran T, Danila DC, Keys D, Schwartz M, Ionescu-Zanetti C (2013) Mutational analysis of circulating tumor cells using a novel microfluidic collection device and qPCR assay. Transl Oncol 6(5):528–538

Hayes DF, Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Miller MC, Matera J, Allard WJ, Doyle GV, Terstappen LW (2006) Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. Clin Cancer Res 12(14 Pt 1):4218–4224

Heitzer E, Auer M, Gasch C, Pichler M, Ulz P, Hoffmann EM, Lax S, Waldispuehl-Geigl J, Mauermann O, Lackner C, Höfler G, Eisner F, Sill H, Samonigg H, Pantel K, Riethdorf S, Bauernhofer T, Geigl JB, Speicher MR (2013) Complex tumor genomes inferred from single circulating tumor cells by array-CGH and next-generation sequencing. Cancer Res 73(10):2965–2975. doi:10.1158/0008-5472.CAN-12-4140

Hou HW, Warkiani ME, Khoo BL, Li ZR, Soo RA, Tan DS, Lim WT, Han J, Bhagat AA, Lim CT (2013) Isolation and retrieval of circulating tumor cells using centrifugal forces. Sci Rep 3:1259. doi:10.1038/srep01259

Hudis CA (2007a) Current status and future directions in breast cancer therapy. Clin Breast Cancer 4(Suppl 2):S70–S75

Hudis CA (2007b) Trastuzumab–mechanism of action and use in clinical practice. N Engl J Med 357(1):39–51

Ito H, Inoue H, Sando N, Kimura S, Gohda K, Sato J, Murakami K, Ito S, Odaka N, Satodate H, Kudo SE (2012) Prognostic impact of detecting viable circulating tumour cells in gastric cancer patients using a telomerase-specific viral agent: a prospective study. BMC Cancer 12:346. doi:10.1186/1471-2407-12-346

Joosse SA, Hannemann J, Spötter J, Bauche A, Andreas A, Müller V, Pantel K (2012) Changes in keratin expression during metastatic progression of breast cancer: impact on the detection of circulating tumor cells. Clin Cancer Res 18(4):993–1003

Kallergi G, Papadaki MA, Politaki E, Mavroudis D, Georgoulias V, Agelaki S (2011) Epithelial to mesenchymal transition markers expressed in circulating tumour cells of early and metastatic breast cancer patients. Breast Cancer Res 13(3):R59

Kang Y, Pantel K (2013) Tumor cell dissemination: emerging biological insights from animal models and cancer patients. Cancer Cell 23(5):573–581. doi:10.1016/j.ccr.2013.04.017

Kasimir-Bauer S, Hoffmann O, Wallwiener D, Kimmig R, Fehm T (2012) Expression of stem cell and epithelial-mesenchymal transition markers in primary breast cancer patients with circulating tumor cells. Breast Cancer Res 14(1):R15

Lin HC, Hsu HC, Hsieh CH, Wang HM, Huang CY, Wu MH, Tseng CP (2013) A negative selection system PowerMag for effective leukocyte depletion and enhanced detection of EpCAM positive and negative circulating tumor cells. Clin Chim Acta 419:77–84. doi:10.1016/j.cca.2013.01.018

Liu Z, Fusi A, Klopocki E, Schmittel A, Tinhofer I, Nonnenmacher A, Keilholz U (2011) Negative enrichment by immunomagnetic nanobeads for unbiased characterization of circulating tumor cells from peripheral blood of cancer patients. J Transl Med 9:70

Liu Y, Liu Q, Wang T, Bian L, Zhang S, Hu H, Li S, Hu Z, Wu S, Liu B, Jiang Z (2013) Circulating tumor cells in HER2-positive metastatic breast cancer patients: a valuable prognostic and predictive biomarker. BMC Cancer 13:202. doi:10.1186/1471-2407-13-202

Maetzel D, Denzel S, Mack B, Canis M, Went P, Benk M, Kieu C, Papior P, Baeuerle PA, Munz M, Gires O (2009) Nuclear signalling by tumour-associated antigen EpCAM. Nat Cell Biol 11(2):162–171. doi:10.1038/ncb1824

Maheswaran S, Sequist LV, Nagrath S, Ulkus L, Brannigan B, Collura CV, Inserra E, Diederichs S, Iafrate AJ, Bell DW, Digumarthy S, Muzikansky A, Irimia D, Settleman J, Tompkins RG, Lynch TJ, Toner M, Haber DA (2008) Detection of mutations in EGFR in circulating lung-cancer cells. N Engl J Med 359(4):366–377

Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Brisken C, Yang J, Weinberg RA (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 133(4):704–715

Mego M, Mani SA, Lee BN, Li C, Evans KW, Cohen EN, Gao H, Jackson SA, Giordano A, Hortobagyi GN, Cristofanilli M, Lucci A, Reuben JM (2012) Expression of epithelial-mesenchymal transition-inducing transcription factors in primary breast cancer: The effect of neoadjuvant therapy. Int J Cancer 130(4):808–816. doi:10.1002/ijc.26037

Münz M, Kieu C, Mack B, Schmitt B, Zeidler R, Gires O (2004) The carcinoma-associated antigen EpCAM upregulates c-myc and induces cell proliferation. Oncogene 23(34):5748–5758

Nagrath S, Sequist LV, Maheswaran S, Bell DW, Irimia D, Ulkus L, Smith MR, Kwak EL, Digumarthy S, Muzikansky A, Ryan P, Balis UJ, Tompkins RG, Haber DA, Toner M (2007) Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature 450(7173):1235–1239

Osta WA, Chen Y, Mikhitarian K, Mitas M, Salem M, Hannun YA, Cole DJ, Gillanders WE (2004) EpCAM is overexpressed in breast cancer and is a potential target for breast cancer gene therapy. Cancer Res 64(16):5818–5824

Pantel K, Alix-Panabières C (2013) Real-time liquid biopsy in cancer patients: fact or fiction? Cancer Res 73(21):6384–6388. doi:10.1158/0008-5472.CAN-13-2030

Pantel K, Brakenhoff RH (2004) Dissecting the metastatic cascade. Nat Rev Cancer 4(6):448–456. doi:10.1038/nrc1370

Punnoose EA, Atwal SK, Spoerke JM, Savage H, Pandita A, Yeh RF, Pirzkall A, Fine BM, Amler LC, Chen DS, Lackner MR (2010) Molecular biomarker analyses using circulating tumor cells. PLoS One 5(9):e12517

Rack B, Schindlbeck C, Andergassen U, Lorenz R, Zwingers T, Schneeweiss A, Lichtenegger W, Beckmann MW, Sommer H, Pantel K, Friese K, Janni W (2010) Use of circulating tumor cells (CTCs) in peripheral blood of breast cancer patients before and after adjuvant chemotherapy to predict risk for relapse: the SUCCESS Trial 7. Clin Oncol 28:7s

Riethdorf S, Müller V, Zhang L, Rau T, Loibl S, Komor M, Roller M, Huober J, Fehm T, Schrader I, Hilfrich J,

Holms F, Tesch H, Eidtmann H, Untch M, von Minckwitz G, Pantel K (2010) Detection and HER2 expression of circulating tumor cells: prospective monitoring in breast cancer patients treated in the neoadjuvant GeparQuattro trial. Clin Cancer Res 16(9):2634–2645

Saad F, Pantel K (2012) The current role of circulating tumor cells in the diagnosis and management of bone metastases in advanced prostate cancer. Future Oncol 8(3):321–331

Saucedo-Zeni N, Mewes S, Niestroj R, Gasiorowski L, Murawa D, Nowaczyk P, Tomasi T, Weber E, Dworacki G, Morgenthaler NG, Jansen H, Propping C, Sterzynska K, Dyszkiewicz W, Zabel M, Kiechle M, Reuning U, Schmitt M, Lücke K (2012) A novel method for the in vivo isolation of circulating tumor cells from peripheral blood of cancer patients using a functionalized and structured medical wire. Int J Oncol 41(4):1241–1250. doi:10.3892/ijo.2012.1557

Scher HI, Morris MJ, Basch E, Heller G (2011) End points and outcomes in castration-resistant prostate cancer: from clinical trials to clinical practice. J Clin Oncol 29(27):3695–3704

Stott SL, Hsu CH, Tsukrov DI, Yu M, Miyamoto DT, Waltman BA, Rothenberg SM, Shah AM, Smas ME, Korir GK, Floyd FP Jr, Gilman AJ, Lord JB, Winokur D, Springer S, Irimia D, Nagrath S, Sequist LV, Lee RJ, Isselbacher KJ, Maheswaran S, Haber DA, Toner M (2010) Isolation of circulating tumor cells using a microvortex-generating herringbone-chip. Proc Natl Acad Sci U S A 107(43):18392–18397

Tewes M, Aktas B, Welt A, Mueller S, Hauch S, Kimmig R, Kasimir-Bauer S (2009) Molecular profiling and predictive value of circulating tumor cells in patients with metastatic breast cancer: an option for monitoring response to breast cancer related therapies. Breast Cancer Res Treat 115(3):581–590. doi:10.1007/s10549-008-0143-x

Weinberg RA (2008) Twisted epithelial–mesenchymal transition blocks senescence. Nat Cell Biol 10:1021–1023. doi:10.1038/ncb0908-1021

Wülfing P, Borchard J, Buerger H, Heidl S, Zänker KS, Kiesel L, Brandt B (2006) HER2-positive circulating tumor cells indicate poor clinical outcome in stage I to III breast cancer patients. Clin Cancer Res 12(6):1715–1720

Xenidis N, Markos V, Apostolaki S, Perraki M, Pallis A, Sfakiotaki G, Papadatos-Pastos D, Kalmanti L, Kafousi M, Stathopoulos E, Kakolyris S, Mavroudis D, Georgoulias V (2007) Clinical relevance of circulating CK-19 mRNA-positive cells detected during the adjuvant tamoxifen treatment in patients with early breast cancer. Ann Oncol 18(10):1623–1631

Yokobori T, Iinuma H, Shimamura T, Imoto S, Sugimachi K, Ishii H, Iwatsuki M, Ota D, Ohkuma M, Iwaya T, Nishida N, Kogo R, Sudo T, Tanaka F, Shibata K, Toh H, Sato T, Barnard GF, Fukagawa T, Yamamoto S, Nakanishi H, Sasaki S, Miyano S, Watanabe T, Kuwano H, Mimori K, Pantel K, Mori M (2013) Plastin3 is a novel marker for circulating tumor cells undergoing the epithelial-mesenchymal transition and is associated with colorectal cancer prognosis. Cancer Res 73(7):2059–2069. doi:10.1158/0008-5472.CAN-12-0326

Genomics Meets Cancer Immunotherapy

John C. Castle, Sebastian Boegel, Thomas Bukur, Valesca Boisguerin, and Martin Loewer

The NGS Technology Platform

The use of polymerase chain reaction (PCR) and reverse transcriptase (RT) transformed molecular biology: Sanger-based chain-terminating dideoxynucleotide sequencing was established in the 1970s and was subsequently used for the human genome project (Lander et al. 2001; Venter et al. 2001). Completed in 2000, the assembly of the first human genome took over 10 years, cost over one billion dollars, and required over 20 laboratories. Today, we can sequence a human genome – healthy or tumor – in less than 1 week, in one lab, for less than 10,000 euros. This decrease of 4.2 orders of magnitude (a 16,350-fold decrease) over the past decade is the result of a revolutionary technology platform, namely, "next-generation sequencing" (NGS) (Wetterstrand 2013).

By sequencing nucleic acids, NGS can be used to define genomes and, by counting the number of reads associated with each transcript, to determine gene expression profiles. NGS platforms generate nucleotide sequences similar to those from Sanger sequencing. A lab technician extracts nucleic acids, DNA or RNA, from a sample; prepares a library; and places the library in the NGS instrument which sequences the molecules in the library and outputs computer files that are converted into nucleotide sequence reads of A, C, G, and T. Unlike Sanger sequencing, a single 1-week run of an NGS instrument can generate billions of sequence reads representing multiple genomes and transcriptomes. This cornucopia of data – multiple terabytes (TB) – requires extensive IT infrastructures, bioinformatics processing, and biostatistical analyses for the subsequent data interpretation and generation of actionable knowledge.

There are several types of NGS instruments, each using a different flavor of sequencing (Metzker 2010). Sequencing by synthesis is similar to that of Sanger sequencing and is used in instruments from Illumina. The Illumina Hiseq 2500 sequences molecules from the 5′ and 3′ ends, resulting in a 150 nt paired-end sequence read (300 nt total length) for each input molecule. In 10 days, one HiSeq flow-cell run can generate 3 billion sequence reads comprising 600 billion nucleotides. The desktop-sized Illumina MiSeq instrument is faster and generates fewer but longer sequence reads. Pyrosequencing, used by Roche 454 instruments, sequences by monitoring pyrophosphate release upon incorporation of a nucleotide into a growing DNA strand, which is detected by a coupled enzymatic reaction involving ATP sulfurylase. Sequencing by ligation is used by the SOLiD instruments from Life Technologies. Similar to the 454 platform, DNA fragments are ligated to adaptors and immobilized onto beads contained within the emulsified

J.C. Castle (✉) • S. Boegel • T. Bukur
V. Boisguerin • M. Loewer
TRON gGmbH–Translational Oncology,
Johannes Gutenberg-University Medical Center gGmbH,
Johannes Gutenberg University of Mainz Medicine,
Langenbeckstr. 1, Building 708, Mainz 55131, Germany
e-mail: john.castle@tron-mainz.de

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_22,

droplets followed by PCR. Sequencing by monitoring pH is used by the Ion Torrent Personal Genome Machine (PGM) desktop sequencers from Life Technologies: the instruments integrate semiconductor technology to sensitively detect pH changes. During DNA replication, individual nucleotides (A, C, G, and T) are flooded into the chamber and incorporated into a growing DNA strand. Nucleotide incorporation releases a hydrogen ion and a pyrophosphate; the PGM semiconductor chip detects the positively charged hydrogen release.

Third-generation sequencing instruments in development are designed to increase speed, read lengths, and accuracy while decreasing costs and simplifying workflows. The Pacific Biosciences RS II instrument, which is commercially available, sequences single molecules using a DNA polymerase that is confined into a small volume. As replication takes place, fluorescently labeled nucleotides are incorporated by the polymerase into the growing DNA strand and imaged by optics. The resulting sequence reads have a median length of over 8,000 nt, enabling easier computational assembly of a genome. Oxford Nanopore Technologies has announced plans to sell miniaturized sequencing instruments the size of a USB stick. The device detects electric charges as DNA molecules are pulled through nanopores in a graphene sheet, which should enable long sequence reads and high throughput.

Mutation Detection for Cancer Applications

In addition to improved application in research settings, a goal for the technology is to create NGS platforms – machines and software – approved for standard clinical use. However, while NGS has revolutionized our ability to identify somatic mutations, the platform is not error-free, with some studies reporting validation rates of as low as 54 % (Yoshida et al. 2011). Multiple experimental and algorithmic factors contribute to the false detections. Process-related error sources include PCR artifacts, biases in priming and targeted enrichment resulting in uneven coverage, instrument base-calling errors, and misaligned sequence reads. Biological challenges include tumor heterogeneity, low tumor purity, extensive copy number variations, and mutation heterozygosity. Further, while we can determine false-positive rates (i.e., specificity) by validating detections with low-throughput assays, determining false-negative rates (i.e., sensitivity) is more problematic as every genomic location could be a potential undetected mutation.

Given the large discrepancies, one is left wondering which mutations to select for clinical decision-making and for follow-up experiments. Researchers often rely on personal experience, arbitrary filtering thresholds, and ad hoc filtering to select mutations. A statistical value would be useful, such as a *p*-value reflecting the confidence that the mutation is found in the tumor sample and not in the patient. We have developed a methodology to assign a confidence value – a false discovery rate (FDR) – to individual identified mutations (Lower et al. 2012). The method is applicable both to the selection and prioritization of mutations and for the development and optimization of mutation detection algorithms and methods. In a proof of concept experiment, we sequenced the B16F10 melanoma genome using an Illumina Hiseq 2000 and prioritized 4,078 identified somatic point mutations. We assigned an FDR to each mutation and showed that 50 of the 50 selected mutations with low FDRs (high confidence) validated as tumor-specific mutations while 0 of the 44 identified mutations with high FDRs (low confidence) validated. Further efforts by us and others are incorporating advanced statistical frameworks to optimize mutation detection in impure samples with heterogeneous subclonal populations (Cibulskis et al. 2013; Koboldt et al. 2012).

Profiling Gene Expression from FFPE Samples

NGS can be used to accurately determine RNA gene expression profiles by sequencing RNA-derived cDNA libraries ("RNA-Seq"). Clinical samples preserved as formalin-fixed,

paraffin-embedded (FFPE) samples, often with extensive phenotypic and outcome annotation, would be an immense resource for biomarker and therapy research and development. However, gene expression profiling from FFPE samples has been hampered by the extensive RNA degradation and modifications that occur during the fixation, embedding, and long-term storage.

Many groups have worked to create workflows enabling gene expression profiling of these samples (e.g., Duenwald et al. 2009; Adiconis et al. 2013). We tested multiple RNA extraction kits and RNA-Seq library preparation methods with FFPE samples. We performed and analyzed a total of 141 different RNA extractions and 208 RNA-Seq libraries, including optimization of lab steps and the computational data processing. Using the best method, we benchmarked (1) the reproducibility by sequencing several intra- and inter-day replicates of the same samples and (2) the sensitivity of methods with matching FFPE and fresh frozen breast cancer tumor samples. Our results show that the optimized platform we developed is able to effectively profile FFPE samples up to 20 years old when multiple criteria are met, including procedures for FFPE block handling and cutting, RNA extraction, quality control, NGS library construction, and data processing.

Public Cancer Datasets for Expression and Mutations

Using similar methods for mutation detection and gene expression, thousands of tumors have been sequenced by the Cancer Genome Association (TCGA) and International Cancer Genome Consortium (ICGC) (e.g., Kandoth et al. 2013), and the resulting datasets have been placed into the public domain. For example, over 1,000 breast tumor mutation and expression profiles are now easily available for analysis and download from websites such as the UCSC Cancer Genome Browser (Cline et al. 2013). The breast cancer datasets include not only genomic profiling results but also clinical data, including age, gender, IHC, PAM50 classifications, and outcome, enabling extensive data mining for candidate targets and biomarkers, tumor subclassification, and delineation of cancer pathways. For instance, RNA expression of ER, PR, and HER2 and a proliferation marker such as KI-67 clearly correspond to breast cancer subtypes, and the subtypes have unmistakably different mutation patterns (Fig. 1a). Basal-like tumors have high KI-67 expression, frequent TP53 mutations, and rare PIK3CA mutation. Luminal A tumors have low KI-67 expression, few TP53 mutations, and frequent PIK3CA mutations.

Determining HLA Type and Expression

Human leukocyte antigen (HLA) class I and class II molecules display peptide antigens that are derived from intracellular and extracellular proteins on the surface of nucleated cells, respectively (Fig. 1b). The HLA loci are highly polymorphic, with three major gene loci for class I (A, B, and C) and three major loci for class II (DP, DQ, DR). Over 7,500 major class I alleles and over 2,200 major class II have been reported (Robinson et al. 2013). Exons 2 and 3 (class I) and exon 2 (class II) encode for the peptide binding groove and contain most of the polymorphisms (Fig. 1b). Existing HLA typing techniques utilize labor and time-intensive methods, such as serological antibody-based histocompatibility testing, sequence-specific oligonucleotide (SSO) hybridization, PCR amplification with sequence-specific primers (SSP), and sequence-based typing (SBT). Recent studies described development of high-throughput HLA genotyping assays using NGS and genomic DNA (e.g., Wang et al. 2012).

The NGS assays exome-seq and RNA-Seq rapidly generate billions of short nucleic acid sequence reads that unbiasedly cover the entire exome and transcriptome. Adoption of the assays has been rapid: clinical and research labs worldwide have deposited >11,700 human RNA-Seq sample profiles into public repositories (NCBI SRA, November 04, 2013), plus several thousand additional tumor profiles from ICGC and

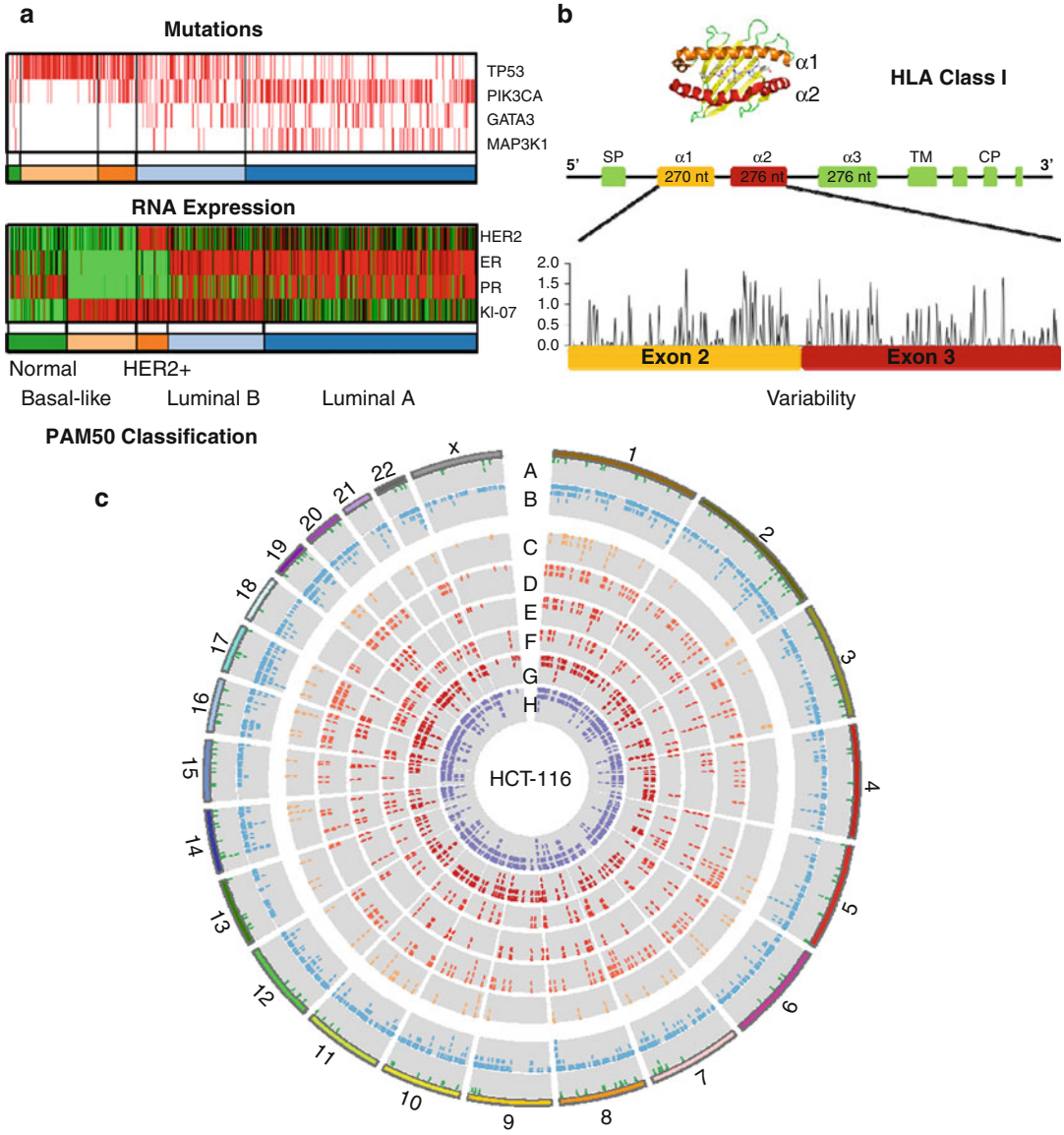

Fig. 1 *Top left*: RNA-Seq gene expression and mutations from the TCGA Breast Tumor cohort, accessed through the UCSC Cancer Genomics Browser. Gene expression values are normalized by subtracting the mean from all samples. Values greater than the mean are *red*; values less than the mean are *green*. *Top right*: the gene structure of the human leukocyte antigen (*HLA*) class I molecule, including the HLA crystal structure 3OXR from PDB, showing the helix chains α1 (*orange*) and α2 (*red*) that bind peptide; the eight HLA exons that encode for signal peptide (*SP*), α1, α2, α3, transmembrane domain (*TM*), and cytoplasmic tail (*CP*) domains; and the highly polymorphic exons 2 and 3. Variation is defined as (2 – information content). *Bottom*: predicted immunogenic single nucleotide variants (*SNVs*) in HCT116 human colorectal cells. (**a**, **b**): non-synonymous mutations in HCT116 from CCLE and COSMIC, respectively. (**c–h**)Mutations predicted to bind (<500 nM) to HCT116 HLA alleles A*01:01, A*02:01, B*18:01, B*45:01, C*05:01, and C*07:01

TCGA studies. While many of these patient samples have valuable clinical annotation, the HLA types and expression have not been determined.

The main challenge to determining the HLA type from NGS reads is the polymorphic nature of the HLA loci. Four algorithms have been developed to determine the HLA type directly

from standard NGS data. HLAminer (Warren et al. 2012) uses a targeted de novo assembly technique to reconstruct the HLA genes and subsequent alleles from the reads and an alignment-based approach using a reciprocal BLAST against a reference database containing all known HLA class I and II alleles. The result is a list of candidate alleles for each target HLA gene. This method can also be applied to whole genome and whole exome sequencing.

ATHLATES (Liu et al. 2013) focuses on whole exome sequencing reads to determine the HLA genotype. The first step is an alignment of the exome-seq reads against the nucleotide sequences of all known HLA alleles followed by the recovery of exon sequences via an assembly of the mapped reads. The algorithm then automatically infers the homozygous or heterozygous allelic pair that best explains the read data.

HLAforest (Kim and Pourmand 2013) uses an alignment-based approach exploiting the hierarchical structure of the HLA nomenclature by building a tree for each read based on the set of possible alignments followed by an assignment of weights for each node based on the quality scores of mismatched positions. In an iterative process, the HLA haplotypes with the highest probabilities are reported.

We developed seq2HLA (Boegel et al. 2013) specifically to use with RNA-Seq NGS sequence reads. It utilizes an alignment-based approach to determine the HLA type, confidence score, zygosity, and locus-specific expression levels.

An advantage of these tools is that they can be applied to existing standard NGS datasets without the requirement for a change in the laboratory protocols. For example, we are mining public RNA-Seq data repositories to determine the HLA types of human cell lines, to search for possible risk biomarkers, and for our efforts to make individualized cancer immunotherapies.

Integrated Mutation, Expression, and HLA

With the identified tumor mutations, the gene expression, and HLA types in a tumor, one can predict expressed, non-synonymous mutations that may be presented on the tumor HLA molecules and thus potentially part of the T-cell druggable genome and immunotherapy targets (Diekmann et al. 2012). We used the CCLE (Barretina et al. 2012) and COSMIC (Forbes et al. 2011) databases to identify non-synonymous mutations in HCT116 human colorectal cells; RNA-Seq reads and seq2HLA to HLA type HCT116 as A*01:01, A*02:01, B*18:01, B*45:01, C*05:01, and C*07:01; and the IEDB consensus algorithm (Kim et al. 2011) to predict which mutations will be in an MHC-presented peptide (Fig. 1c).

NGS Identification and Analysis of Viruses in Cancer

Cancer immunotherapies can vaccinate against tumor-associated viruses. Over 100 years ago, Ellermann and Bang documented viral transmission of cancer (Ellermann and Bang 1908). More than 60 years later, Epstein and Barr published the discovery of the first human cancer-related virus particles in Burkitt's lymphoma cell lines (Epstein et al. 1964).

Nevertheless, the detection of viruses in biological samples remains laborious. ELISA and electron microscopy can fail to identify latent viruses in cancer samples. Sequence-based technologies like PCR and microarrays detect only known viruses. NGS, however, can be used to unbiasedly determine all nucleic acids comprising a biological sample, enabling not only the analysis of the host nucleic acids but also identification of foreign infectious agents (Westermann et al. 2012).

Today, seven human viruses are known to have oncogenic potential and are associated with 10–15 % of all human cancers (Moore and Chang 2010). These include single- and double-stranded DNA viruses and positive-stranded RNA viruses from several families (Table 1). However, almost all of these viruses have closely related viruses that are not oncogenic. It is assumed that almost every virus has oncogenic potential, although only few are oncogenic (Moore and Chang 2010). Recent oncovirus detections include human papillomavirus (HPV) with head and neck cancers

Table 1 Human oncogenic viral agents

Viral agent	Virus genome	Associated cancers
Epstein-Barr virus (EBV), also known as human herpes virus 4 (HHV4)	Double-stranded DNA herpes virus	Nasopharyngeal carcinoma, Burkitt's lymphoma, some non-Hodgkin's lymphomas, Hodgkin's lymphoma, some gastrointestinal lymphomas, extranodal NK/T-cell lymphoma
Merkel cell polyomavirus (MCV)	Double-stranded DNA polyomavirus	Merkel cell carcinoma
Hepatitis B virus (HBV)	Single-stranded and double-stranded DNA hepadenovirus	Hepatocellular carcinoma
Hepatitis C virus (HCV)	Positive-strand single-stranded RNA flavivirus	Hepatocellular carcinoma, some non-Hodgkin's lymphomas
Kaposi's sarcoma herpes virus (KSHV), also known as human herpes virus 8 (HHV8)	Double-stranded DNA herpes virus	Kaposi's sarcoma, primary effusion lymphoma, some multicentric Castleman's disease
Human papillomavirus type 16 (HPV-16)	Double-stranded DNA papillomavirus	Carcinomas of the cervix, vulva, vagina, penis, anus, oral cavity, oropharynx, tonsil, head, and neck
Human T-lymphotropic virus type 1 (HTLV-1)	Positive-strand single-stranded RNA retrovirus	Adult T-cell leukemia and lymphoma
Cytomegalovirus (CMV), also known as human herpes virus 5 (HHV5)	Double-stranded DNA herpes virus	Glioblastoma
Human immunodeficiency virus type 1 (HIV-1)	Positive-strand single-stranded RNA retrovirus	Immunosuppression promotes different types of cancers through other viruses

and the human cytomegalovirus (CMV), also known as human herpes virus 5 (HHV5), with glioblastoma (Soderberg-Naucler et al. 2013). With the development of more sensitive detection methods, the number of known cancer-related viral agents will likely grow.

Further, NGS enables scientists to detect virus integration sites (Sung et al. 2012), distinguishing between different strains of viruses, and analyze the host immune gene expression in response to virus infection (Peng et al. 2010). Analysis algorithms start with the NGS reads, remove sequences associated with the host, and compare the remaining fragments compared to a database of known virus genomes, followed by a virus genome assembly (Li et al. 2013; Chen et al. 2013; Wang et al. 2013). The algorithms have been applied to TCGA tumor NGS RNA-Seq reads to detect viruses (Khoury et al. 2013) and identify host gene expression response to the virus infections (Tang et al. 2013). Analyzing over 4,000 cancer profiles, the authors demonstrated that the virus RNA and a virus-specific response pattern could be detected, suggesting different therapy selection for virus-associated and virus-free tumors.

Conclusion and Outlook

Here, we have highlighted applications where the NGS platform is both rapidly evolving and already having an impact: tumor mutation detection, tumor gene expression profiling, HLA typing and expression profiling, immunogenic mutation predictions, and oncovirus detection. These cutting-edge workflows have already entered clinical trials, such as for the design of patient-specific, individualized therapeutic cancer vaccines (APVACs) (Castle et al. 2012; Britten et al. 2013). The field is rapidly finding novel uses for the NGS platform that will be enabling for cancer immunotherapies, such as a patient "liquid biopsy" that

incorporates an NGS profile of patient blood to longitudinally follow T-cell receptor (TCR) repertoires (clinical trial NCT01306188) and mutations in CTCs and cell-free DNA (Dawson et al. 2013; Heitzer et al. 2013).

Acknowledgment We warmly thank Christoph Huber for his medical and scientific forethought, advice, guidance, mentorship, and friendship.

References

Adiconis X et al (2013) Comparative analysis of RNA sequencing methods for degraded or low-input samples. Nat Methods 10(7):623–629. doi:10.1038/nmeth.2483

Barretina J et al (2012) The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature 483(7391):603–607. doi:10.1038/nature11003

Boegel S et al (2013) HLA typing from RNA-Seq sequence reads. Genome Med 4(12):102. doi:10.1186/gm403

Britten CM et al (2013) The regulatory landscape for actively personalized cancer immunotherapies. Nat Biotechnol 31(10):880–882. doi:10.1038/nbt.2708

Castle JC et al (2012) Exploiting the mutanome for tumor vaccination. Cancer Res 72(5):1081–1091. doi:10.1158/0008-5472.CAN-11-3722

Chen Y et al (2013) VirusSeq: software to identify viruses and their integration sites using next-generation sequencing of human cancer tissue. Bioinformatics 29(2):266–267. doi:10.1093/bioinformatics/bts665

Cibulskis K et al (2013) Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. Nat Biotechnol 31(3):213–219. doi:10.1038/nbt.2514

Cline MS et al (2013) Exploring TCGA Pan-Cancer data at the UCSC Cancer Genomics Browser. Sci Rep 3:2652. doi:10.1038/srep02652

Dawson SJ et al (2013) Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med 368(13):1199–1209. doi:10.1056/NEJMoa1213261

Diekmann J et al (2012) The T cell druggable genome. Eur Pharm Rev 17(4):16–19

Duenwald S et al (2009) Development of a microarray platform for FFPET profiling: application to the classification of human tumors. J Transl Med 7:65. doi:10.1186/1479–5876–7–65, 1479-5876-7-65 [pii]

Ellermann V, Bang O (1908) Experimentelle Leukämie bei Hühnern. Zentralbl Bakterielle Parasitkunde Abt I 46:595–609

Epstein MA et al (1964) Virus particles in cultured lymphoblasts from Burkitt's lymphoma. Lancet 1(7335):702–703

Forbes SA et al (2011) COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. Nucleic Acids Res 39(Database issue):D945–D950. doi:10.1093/nar/gkq929

Heitzer E et al (2013) Complex tumor genomes inferred from single circulating tumor cells by array-CGH and next-generation sequencing. Cancer Res 73(10):2965–2975. doi:10.1158/0008-5472.CAN-12-4140

Kandoth C et al (2013) Mutational landscape and significance across 12 major cancer types. Nature 502(7471):333–339. doi:10.1038/nature12634

Khoury JD et al (2013) Landscape of DNA virus associations across human malignant cancers: analysis of 3,775 cases using RNA-Seq. J Virol 87(16):8916–8926. doi:10.1128/JVI.00340-13

Kim HJ, Pourmand N (2013) HLA haplotyping from RNA-seq data using hierarchical read weighting. PLoS One 8(6):e67885. doi:10.1371/journal.pone.0067885

Kim Y et al (2011) Applications for T-cell epitope queries and tools in the Immune Epitope Database and Analysis Resource. J Immunol Methods 374(1–2):62–69. doi:10.1016/j.jim.2010.10.010

Koboldt DC et al (2012) VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome Res 22(3):568–576. doi:10.1101/gr.129684.111

Lander ES et al (2001) Initial sequencing and analysis of the human genome. Nature 409(6822):860–921

Li JW et al (2013) ViralFusionSeq: accurately discover viral integration events and reconstruct fusion transcripts at single-base resolution. Bioinformatics 29(5):649–651. doi:10.1093/bioinformatics/btt011

Liu C et al (2013) ATHLATES: accurate typing of human leukocyte antigen through exome sequencing. Nucleic Acids Res 41(14):e142. doi:10.1093/nar/gkt481

Lower M et al (2012) Confidence-based somatic mutation evaluation and prioritization. PLoS Comput Biol 8(9):e1002714. doi:10.1371/journal.pcbi.1002714

Metzker ML (2010) Sequencing technologies – the next generation. Nat Rev Genet 11(1):31–46. doi:10.1038/nrg2626

Moore PS, Chang Y (2010) Why do viruses cause cancer? Highlights of the first century of human tumour virology. Nat Rev Cancer 10(12):878–889. doi:10.1038/nrc2961

Peng X et al (2010) Unique signatures of long noncoding RNA expression in response to virus infection and altered innate immune signaling. MBio 1(5):e00206–10. doi:10.1128/mBio.00206-10

Robinson J et al (2013) The IMGT/HLA database. Nucleic Acids Res 41(Database issue):D1222–D1227. doi:10.1093/nar/gks949

Soderberg-Naucler C et al (2013) Survival in patients with glioblastoma receiving valganciclovir. N Engl J Med 369(10):985–986. doi:10.1056/NEJMc1302145

Sung WK et al (2012) Genome-wide survey of recurrent HBV integration in hepatocellular carcinoma. Nat Genet 44(7):765–769. doi:10.1038/ng.2295

Tang KW et al (2013) The landscape of viral expression and host gene fusion and adaptation in human cancer. Nat Commun 4:2513. doi:10.1038/ncomms3513

Venter JC et al (2001) The sequence of the human genome. Science 291(5507):1304–1351. doi:10.1126/science.1058040

Wang C et al (2012) High-throughput, high-fidelity HLA genotyping with deep sequencing. Proc Natl Acad Sci U S A 109(22):8676–8681. doi:10.1073/pnas.1206614109

Wang Q et al (2013) VirusFinder: software for efficient and accurate detection of viruses and their integration sites in host genomes through next generation sequencing data. PLoS One 8(5):e64465. doi:10.1371/journal.pone.0064465

Warren RL et al (2012) Derivation of HLA types from shotgun sequence datasets. Genome Med 4(12):95. doi:10.1186/gm396

Westermann AJ et al (2012) Dual RNA-seq of pathogen and host. Nat Rev Microbiol 10(9):618–630. doi:10.1038/nrmicro2852

Wetterstrand KA (2013) DNA sequencing costs: data from the NHGRI Genome Sequencing Program (GSP). Available at: www.genome.gov/sequencingcosts. Accessed 28 Oct 2013

Yoshida K et al (2011) Frequent pathway mutations of splicing machinery in myelodysplasia. Nature 478(7367):64–69. doi:10.1038/nature10496

Part V

Featured Chapters

Oncolytic Viruses

Michael D. Mühlebach and Stefan Hutzler

Introduction

The concept of using replicating viruses to treat cancer in patients, so-called virotherapy, has been studied for more than 100 years. However, early trials suffered either from lack of efficacy or from putting patients at risk to become severely ill due to viral pathogenesis. Since approximately 20 years, the use of biotechnology that allows the generation of recombinant viruses with adapted biological properties fostered the analysis of these oncolytic viruses (OVs) as an additional treatment modality in oncology with considerable progress (Cattaneo et al. 2008).

The basic concept of OV therapy relies on preferred replication and spread of viruses in tumor cells and thereby selective killing of infected cells and destruction of tumors. Anecdotal, but well-documented, case reports have been describing tumor patients benefitting from viral infections, e.g., measles virus (Bluming and Ziegler 1971) or vaccinia virus (Hansen and Libnoch 1978). Unfortunately, there are quite some obstacles to eliminate the full tumor burden within a patient solely by viral infection. Natural resistance to viral infection, structure of the tumor tissue with stromal barriers for viral dissemination, preformed neutralizing antibodies or innate immunity inhibiting systemic spread of OV, premature onset of antiviral immunity, and other mechanisms have to be overcome for the OV to reach infection of all tumor cells. OVs are engineered on multiple layers to master to these challenges.

Especially the patients' adaptive immune system, if not suppressed by the underlying disease or the treatment modalities used otherwise (e.g., chemotherapy), is very powerful in eliminating viral infections, both systemically and locally. Fortunately, this is just one side of the story as it has turned out in recent clinical trials, especially when developing recombinant viruses built to synergize with the immune system by inducing antitumoral immunity.

Oncolytic Viruses and the Immune System

As summarized by Russell and colleagues, these results let to a change in the oncolytic paradigm: Starting with the selective viral infection of tumor cells and the subsequent lysis of those believed to be OV's mode of action (Russell et al. 2012), it has become evident that not only the direct killing but rather the stimulation of the immune system plays a critical role for the current success of an OV in treating cancer malignancies (Bartlett et al. 2013). The mode of action of OVs is now hypothesized as a two-step process: (1) debulking of the tumor mass by viral replication and

M.D. Mühlebach (✉) • S. Hutzler
Section Oncolytic Measles Viruses
and Vaccine Vectors, Paul-Ehrlich-Institut,
Paul-Ehrlich-Str. 51-59, Langen 63225, Germany
e-mail: michael.muehlebach@pei.de

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_23,

direct cytolysis and (2) induction of antitumoral immunity by *in situ* vaccination via the release of danger signals due to viral replication and destruction of tumor cells. However, the immune system does not only support the efficacy of OVs by the induction of an antitumoral immunity, but also limits viral spread and therefore the largest possible number of infected tumor cells. Furthermore the immunosuppressive tumor microenvironment (TME) (Zou 2005) has to be overturned by OVs to induce an antitumoral immunity for successful tumor destruction. These different levels of OVs as interaction partners of the immune system and their thereby associated activity as immunotherapeutic agents are presented in Fig. 1 and will be discussed in detail in the following paragraphs.

Impairment of Oncolytic Viruses by Antiviral Immune Reaction

Clinical trials which show promising results so far mostly deliver the OV by direct intra-tumoral application to achieve efficient local delivery of the virus, thus evading neutralization by serum factors (e.g., complement or antibodies), or sequestration by the mononuclear phagocytic system in the liver and spleen. In contrast, especially for the better treatment of metastatic cancers, a systemic approach would be desirable. Therefore, great effort is made to circumvent those drawbacks of systemic application. One possibility in the case of neutralizing antibodies is to hide the OV in primary cells, which are able to home to tumor beds, like dendritic cells (DCs)

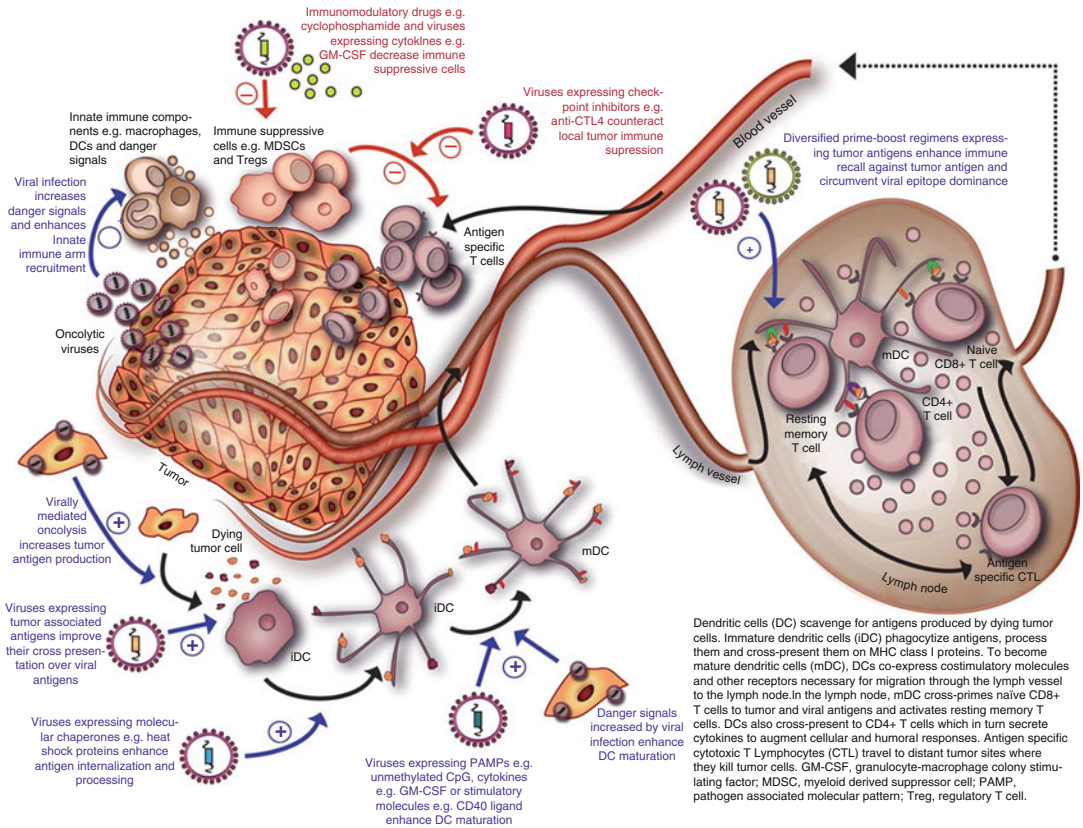

Fig. 1 Interactions of oncolytic viruses with the immune system boosting antitumoral immune responses (Reproduced from Elsedawy and Russell (2013), copyright © 2013)

or T cells, which was shown for oncolytic reovirus (Ilett et al. 2009, 2011). Furthermore mesenchymal stem cells, which preferentially engraft in solid tumors (Ling et al. 2010), have been studied to deliver OV to tumor beds (Mader et al. 2009). During sequestration, OVs are coated by different serum factors and subsequently phagocytized by, e.g., splenic macrophages (Underhill and Ozinsky 2002) or hepatic Kupffer cells (Haisma et al. 2009). A potential route to avoid sequestration is to coat viral particles with polymers like polyethylene glycol (Tesfay et al. 2013) and *N*-[2-hydroxypropyl]methacrylamide (Fisher and Seymour 2010), which are already clinically used and have shown to prolong circulation times of proteins (Duncan 2006). Moreover, pretreatment with immunosuppressive drugs, like cyclophosphamide (Peng et al. 2013) or rapamycin (Meng et al. 2013), reveals direct antitumor effects and has been demonstrated to significantly reduce neutralizing antibodies and to increase viral progeny.

The Immune System can Promote the Efficacy of OV

Contrasting the inhibitory effects of the immune system on viruses, OVs themselves have the capacity to invert the immunosuppressive TME and thus initiate an antitumoral immune response, which increases the efficacy of oncolytic virotherapy (Bartlett et al. 2013). This stimulation is mainly triggered by the release of danger-associated molecular patterns (DAMPs) as well as pathogen-associated molecular patterns (PAMPs) by the lysis of virus-infected cells or during viral infection, respectively (Matzinger 2002; Medzhitov and Janeway 2002). Especially the infection of cancer cells and the ensuing different types of cell death including late apoptosis, necrosis, pyroptosis, and autophagic cell death are considered to be "immunogenic cell deaths" (ICDs) (Green et al. 2009). Within those ICDs, DAMPs (mainly surface-exposed calreticulin, secreted ATP, and HMGB1) and PAMPs as well as a natural repertoire of tumor-associated antigens (TAAs) are primarily presented to DCs to elicit an antitumoral and antiviral immune response (Aymeric et al. 2010). This antitumoral immune response includes the activation of cytotoxic T lymphocytes (CTLs) as well as $CD4^+$ T cells, which promotes the eradication of the tumor mass and is further able to lead in a systemic antitumor immunity (Gauvrit et al. 2008). Moreover, certain OVs are able to inherently stimulate cells of the innate immune system, which are critical for induction of a powerful adaptive immune response. This has been demonstrated for reovirus that is naturally activating DCs (Errington et al. 2008). To further enhance this elicited antitumoral immune response, OVs can be armed by different strategies, which will be discussed in the next section.

Arming of Oncolytic Viruses

To enhance the therapeutic efficacy of OVs, these have been armed with several different effector gene classes using recombinant DNA technology. Apart from arming with suicide genes like yeast cytosine deaminase, marker/effector genes such as the NIS gene, or proapoptotic factors like TRAIL, a number of different cytokine genes have been cloned into the genome of diverse prospective OVs; among these were recombinant vaccinia virus (VV), herpes simplex virus (HSV), measles virus (MV), Semliki Forest virus (SFV), or adenovirus (AdV). These viruses have been constructed to further increase the immunostimulatory properties of *in situ* cytolysis caused by OVs after infection of tumor nodules to achieve even enhanced induction of antitumoral immunity. Examples of cytokines expressed by OVs include IL-2, IL-12, IL-15, IL-18, or IL-28; chemokines such as IFNβ and CCL5; inhibitory antibodies specific for antigens on immunosuppressive cells (e.g., regulatory T cells) such as CTLA-4; or costimulatory molecules, e.g., B7.1. One of the most widely studied cytokines used in this concept has been GM-CSF, which revealed successful induction of antitumoral

immunity when being expressed in the backbones of VV (Mastrangelo et al. 1999), HSV (Liu et al. 2003), MV (Grote et al. 2003), or AdV (Kanerva et al. 2013). Indeed, with the exception of MV-GMCSF, which due to lack of adequate animal models only has been shown quite recently to possess enhanced stimulatory properties for adaptive immunity (Grossardt et al. 2013), all of these GM-CSF-armed viruses have been shown exceptional promise in the treatment of human cancer patients in clinical trial phases I–III (Elsedawy and Russell 2013; Bartlett et al. 2013) or during fast access program on the hospital exemption basis (Tong et al. 2012).

Oncolytic Viruses as Carriers of Tumor-Associated Antigens

Besides arming OVs with immunostimulatory molecules, OVs can also be engineered in such a way that they express TAA, thus increasing the amount of TAA in the immunostimulatory context of viral tumor infection, thereby enhancing the antitumoral immunity and treatment efficacy. An oncolytic Newcastle disease virus was engineered to express β-galactosidase as model TAA and induced significant tumor regression in immunocompetent animals (Vigil et al. 2008). Vesicular stomatitis virus (VSV) engineered to express chicken ovalbumin (OVA) as model antigen was also able to induce a robust anti-OVA immunity in B16-ova tumor-bearing mice and lead to complete regression of many tumors (Diaz et al. 2007; Wongthida et al. 2011). Engineered to express an entire cDNA library of normal prostate antigens as clinically more relevant antigens, VSV caused striking results in tumor regression of cancers with the same histological type, without signs of autoimmunity (Kottke et al. 2011). In another report, a similar cDNA library approach helped to identify three TAA cDNA clones out of the VSV library, which are in combination as efficacious as the complete cDNA library (Pulido et al. 2012).

Moreover, the combination of different OVs carrying the same TAA was shown to boost the oncolytic efficacy of the regimen. A prime/boost regime with VV and SFV, both expressing OVA as model antigen, showed increased efficacy compared to single administration in an ovarian cancer model (Zhang et al. 2010). AdV and VSV were engineered to encode human dopachrome tautomerase (hDCT) as TAA in a B16 melanoma model that expresses endogenously DCT. By priming with Ad-hDCT and subsequent boosting with VSV-hDCT, the immune response could be shifted from a prevalent antiviral immune reaction to a predominant anti-TAA response accompanied by durable cures (Bridle et al. 2009, 2010).

Clinical Data of OV-Induced Antitumoral Immunity

So far, no viruses engineered to express and thereby present TAAs to the patients' immune systems have been introduced into clinical trials. In contrast, one of the clinically most advanced OV systems is an oncolytic HSV-1 armed with GM-CSF, talimogene laherparepvec (T-Vec, formerly known as OncoVEX^{GMCSF}). This virus is based on an early-passage clinical isolate deleted of its neurovirulence factor ICP34.5 for safety reasons. In addition, the ICP47 gene has been deleted in this virus, too, with twofold effect: On the one hand, deletion of ICP47 enhances expression of the viral US11 gene, which can compensate for the deletion of ICP34.5 in cancer cells. On the other hand, ICP47 normally suppresses presentation of peptides via MHC-I, thereby inhibiting visibility of the viral infection for the immune system. Thus, the ICP47-deleted version of HSV is more immunogenic. The immunostimulatory properties have further been enhanced by expression of GM-CSF by the recombinant virus. T-Vec has shown efficacy in preclinical animal models already demonstrating an effector role for the immune system (Liu et al. 2003). Safety and first hints for clinical efficacy were demonstrated also in clinical phase I and II studies. Based on these data, a pivotal phase III trial (OPTiM, NCT00769704) was launched (Kaufman and

Bines 2010) treating 436 melanoma patients in stages IIIB–IV randomized 2:1 to receive either T-Vec i.t. or GM-CSF s.c.. The objective overall response rate in the treatment group was 26.4 % including 10.8 % of patients with a complete systemic response, despite that only single tumor lesions had been injected with the active substance. Response rates in the control group were 5.7 % for overall response and 0.7 % with a complete response, only (Bartlett et al. 2013).

Another GM-CSF-armed OV is Pexa-Vec (pexastimogene devacirepvec; JX-594), a recombinant vaccinia virus (Wyeth strain), which showed prolonged median overall survival (14.1 compared to 6.7 month) in the high-dose group of a randomized phase II dose-finding trial (Heo et al. 2013). Thereby, Pexa-Vec demonstrated a direct oncolytic as well as an immunological mode of action with the induction of antiviral CTLs and antibody-mediated complement-dependent cytotoxicity against TAAs. Notably, Pexa-Vec recently failed to reach its primary endpoint in a randomized phase IIB study (TRAVERSE, NCT01387555) for the second-line treatment of advanced liver cancer patients. Nevertheless, the sponsor has announced to move Pexa-Vec into phase III clinical trial for first-line treatment in HCC.

Apart from T-Vec and Pexa-Vec, two other advanced OV have to be discussed, namely, Reolysin and Oncorine (H101). Reolysin is an unmodified reovirus isolate, which has progressed into clinical phase III, also (NCT01166542). While not expressing an immunostimulatory cargo, Reolysin is immunostimulatory by nature, as discussed earlier. Oncorine, an engineered adenovirus deleted in its E1B gene and very similar to another early OV candidate Onyx-O15, is exceptional in more than one circumstance. Worldwide, it has been the first OV, which gained regulatory approval by Chinese authorities in 2005 for the treatment of head and neck cancer (Garber 2006). Unfortunately, data about the efficacy or the mode of action of the clinical Oncorine treatment are hardly available, which otherwise might be helpful in the development of further OVs.

Conclusion and Outlook

As illustrated above, treating cancer patients using replicative OV preparations may currently be interpreted as *in situ* vaccination by triggering an endogenous antitumoral immune response via generation of PAMPs and release of DAMPs due to viral replication, thus representing a truly immunotherapeutic approach. With the first well-documented phase III clinical studies completed, marketing authorization of OVs with clearly immune-mediated mode of action may be expected, in the near future. While showing efficacy as single agent, future studies and applications may incorporate combination therapies either with other immunotherapies (Melcher et al. 2011) or with standard therapies like certain chemotherapeutic treatment options having an immune system-mediated effect, as well. By smart choice and proper timing, potential synergies between different treatment modalities do even stress the promising future of OVs as immunotherapeutic treatment modality. Enhanced clinical efficacy may be expected from new generations of OVs replicating with greater specificity and efficiency in tumors to gain more efficient direct viral oncolysis, as well (Miest and Cattaneo 2014) – a strategy that may very well synergize with the immunotherapeutic approach.

Acknowledgment We thank Roberto Cattaneo (Mayo Clinic, Rochester, MN) for critical reading of the manuscript.

References

Aymeric L et al (2010) Tumor cell death and ATP release prime dendritic cells and efficient anticancer immunity. Cancer Res 70:855–858

Bartlett DL et al (2013) Oncolytic viruses as therapeutic cancer vaccines. Mol Cancer 12:103

Bluming AZ, Ziegler JL (1971) Regression of Burkitt's lymphoma in association with measles infection. Lancet 2:105–106

Bridle BW et al (2009) Vesicular stomatitis virus as a novel cancer vaccine vector to prime antitumor immunity amenable to rapid boosting with adenovirus. Mol Ther 17:1814–1821

Bridle BW et al (2010) Potentiating cancer immunotherapy using an oncolytic virus. Mol Ther 18:1430–1439
Cattaneo R, Miest T, Shashkova EV, Barry MA (2008) Reprogrammed viruses as cancer therapeutics: targeted, armed and shielded. Nat Rev Microbiol 6:529–540
Diaz RM et al (2007) Oncolytic immunovirotherapy for melanoma using vesicular stomatitis virus. Cancer Res 67:2840–2848
Duncan R (2006) Polymer conjugates as anticancer nanomedicines. Nat Rev Cancer 6:688–701
Elsedawy NB, Russell SJ (2013) Oncolytic vaccines. Expert Rev Vaccin 12:1155–1172
Errington F et al (2008) Reovirus activates human dendritic cells to promote innate antitumor immunity. J Immunol 180:6018–6026
Fisher KD, Seymour LW (2010) HPMA copolymers for masking and retargeting of therapeutic viruses. Adv Drug Deliv Rev 62:240–245
Garber K (2006) China approves world's first oncolytic virus therapy for cancer treatment. J Natl Cancer Inst 98:298–300
Gauvrit A et al (2008) Measles virus induces oncolysis of mesothelioma cells and allows dendritic cells to cross-prime tumor-specific CD8 response. Cancer Res 68:4882–4892
Green DR, Ferguson T, Zitvogel L, Kroemer G (2009) Immunogenic and tolerogenic cell death. Nat Rev Immunol 9:353–363
Grossardt C et al (2013) Granulocyte-macrophage colony-stimulating factor-armed oncolytic measles virus is an effective therapeutic cancer vaccine. Hum Gene Ther 24:644–654
Grote D, Cattaneo R, Fielding AK (2003) Neutrophils contribute to the measles virus-induced antitumor effect: enhancement by granulocyte macrophage colony-stimulating factor expression. Cancer Res 63:6463–6468
Haisma HJ et al (2009) Scavenger receptor A: a new route for adenovirus 5. Mol Pharm 6:366–374
Hansen RM, Libnoch JA (1978) Remission of chronic lymphocytic leukemia after smallpox vaccination. Arch Intern Med 138:1137–1138
Heo J et al (2013) Randomized dose-finding clinical trial of oncolytic immunotherapeutic vaccinia JX-594 in liver cancer. Nat Med 19:329–336
Ilett EJ et al (2009) Dendritic cells and T cells deliver oncolytic reovirus for tumour killing despite pre-existing anti-viral immunity. Gene Ther 16:689–699
Ilett EJ et al (2011) Internalization of oncolytic reovirus by human dendritic cell carriers protects the virus from neutralization. Clin Cancer Res 17:2767–2776
Kanerva A et al (2013) Antiviral and antitumor T-cell immunity in patients treated with GM-CSF-coding oncolytic adenovirus. Clin Cancer Res 19:2734–2744
Kaufman HL, Bines SD (2010) OPTIM trial: a Phase III trial of an oncolytic herpes virus encoding GM-CSF for unresectable stage III or IV melanoma. Future Oncol 6:941–949
Kottke T et al (2011) Broad antigenic coverage induced by vaccination with virus-based cDNA libraries cures established tumors. Nat Med 17:854–859
Ling X et al (2010) Mesenchymal stem cells overexpressing IFN-beta inhibit breast cancer growth and metastases through stat3 signaling in a syngeneic tumor model. Cancer Microenviron 3:83–95
Liu BL et al (2003) ICP34.5 deleted herpes simplex virus with enhanced oncolytic, immune stimulating, and anti-tumour properties. Gene Ther 10:292–303
Mader EK et al (2009) Mesenchymal stem cell carriers protect oncolytic measles viruses from antibody neutralization in an orthotopic ovarian cancer therapy model. Clin Cancer Res 15:7246–7255
Mastrangelo MJ et al (1999) Intratumoral recombinant GM-CSF-encoding virus as gene therapy in patients with cutaneous melanoma. Cancer Gene Ther 6:409–422
Matzinger P (2002) The danger model: a renewed sense of self. Science 296:301–305
Medzhitov R, Janeway CA Jr (2002) Decoding the patterns of self and nonself by the innate immune system. Science 296:298–300
Melcher A, Parato K, Rooney CM, Bell JC (2011) Thunder and lightning: immunotherapy and oncolytic viruses collide. Mol Ther 19:1008–1016
Meng S, Xu J, Wu Y, Ding C (2013) Targeting autophagy to enhance oncolytic virus-based cancer therapy. Expert Opin Biol Ther 13:863–873
Miest TS, Cattaneo R (2014) New viruses for cancer therapy: meeting clinical needs. Nat Rev Microbiol 12:23–34
Peng KW et al (2013) Using clinically approved cyclophosphamide regimens to control the humoral immune response to oncolytic viruses. Gene Ther 20: 255–261
Pulido J et al (2012) Using virally expressed melanoma cDNA libraries to identify tumor-associated antigens that cure melanoma. Nat Biotechnol 30:337–343
Russell SJ, Peng KW, Bell JC (2012) Oncolytic virotherapy. Nat Biotechnol 30:658–670
Tesfay MZ et al (2013) PEGylation of vesicular stomatitis virus extends virus persistence in blood circulation of passively immunized mice. J Virol 87:3752–3759
Tong AW et al (2012) Oncolytic viruses for induction of anti-tumor immunity. Curr Pharm Biotechnol 13:1750–1760
Underhill DM, Ozinsky A (2002) Phagocytosis of microbes: complexity in action. Annu Rev Immunol 20:825–852
Vigil A, Martinez O, Chua MA, Garcia-Sastre A (2008) Recombinant Newcastle disease virus as a vaccine vector for cancer therapy. Mol Ther 16:1883–1890
Wongthida P et al (2011) Activating systemic T-cell immunity against self tumor antigens to support oncolytic virotherapy with vesicular stomatitis virus. Hum Gene Ther 22:1343–1353
Zhang YQ, Tsai YC, Monie A, Wu TC, Hung CF (2010) Enhancing the therapeutic effect against ovarian cancer through a combination of viral oncolysis and antigen-specific immunotherapy. Mol Ther 18:692–699
Zou W (2005) Immunosuppressive networks in the tumour environment and their therapeutic relevance. Nat Rev Cancer 5:263–274

Part VI

Final Remarks

Final Words

Cedrik Michael Britten, Mustafa Diken,
Sebastian Kreiter, and Hans-Georg Rammensee

When the idea for a book dedicated to the 70th birthday and the lifetime achievements of Professor Dr. Christoph Huber was born, we immediately considered asking authors that not only are renowned experts in the field but also have a strong personal relation to Christoph Huber and the Association for Cancer Immunotherapy (CIMT). Each author we approached gladly accepted our invitation in less than 12 h. Given the fact that all authors have crowded agendas, this outcome as well as the huge enthusiasm with which everyone immediately started to work was fantastic. Furthermore, the authors and editors have agreed to donate their royalties to the Association for Cancer Immunotherapy (CIMT) to support the non-for-profit aims of CIMT.

This unusual commitment must have a particular reason. We believe that this reason is Christoph Huber himself. Due to the high level of dedication and passion for his work and the respect he has always shown for his colleagues and his patients, Christoph Huber has become a role model for many of us. He makes us strive for higher aims.

We hope that readers of this book will not only be fascinated by its scientific content but also sense the enthusiasm the authors have for their research. We also hope that the book will provide new insights into novel studies and research findings stimulating readers to work towards a better diagnosis and treatment of cancer in the future.

The editors are indebted to the authors for the fruitful cooperation and their scientifically relevant contributions. We also thank Springer, especially our contact person Meike Stoeck, for the help and professional collaboration. This book project would not have been possible without the valuable input and restless work of the CIMT team in Mainz, Germany, namely, Christine Castle and Dr. Stefanie Watzka. We are very grateful to all people who have supported the Association

C.M. Britten (✉)
Immunotherapy Development Center (IDC), TRON-Translational Oncology, University Medical Center, Johannes Gutenberg University, Mainz, Germany

BioNTech RNA Pharmaceuticals, Mainz, Germany
e-mail: cedrik.britten@tron-mainz.de

M. Diken
Department of Immunotherapy Development Center, TRON-Translational Oncology, University Medical Center, Johannes Gutenberg University, Mainz 55131, Germany

S. Kreiter
Department of Immunotherapy Development Center, TRON-Translational Oncology, University Medical Center, Johannes Gutenberg University, Mainz 55131, Germany

BioNTech AG, Mainz 55131, Germany

H.-G. Rammensee
Department of Immunology, University of Tübingen, Tübingen, Germany

C.M. Britten et al. (eds.), *Cancer Immunotherapy Meets Oncology*,
DOI 10.1007/978-3-319-05104-8_24,

for Cancer Immunotherapy (CIMT) through the years. But most of all, we thank Professor Dr. Christoph Huber. We feel honored to know him, to learn from him, and to work with him.

The Editors
Cedrik Michael Britten, Mustafa Diken, Sebastian Kreiter, and Hans-Georg Rammensee
Mainz, May 2014