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Natalia Savelyeva
Christian Ottensmeier *Editors*

Cancer Vaccines

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Natalia Savelyeva · Christian Ottensmeier
Editors

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Responsible Series Editor: Yuri Gleba

Springer

Editors
Natalia Savelyeva
Faculty of Medicine, Cancer Sciences Unit
University of Southampton
Southampton
UK

Christian Ottensmeier
Faculty of Medicine, Cancer Sciences Unit
University of Southampton
Southampton
UK

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Preface

Immunotherapy treatments which harness and enhance the intrinsic powers of the immune system have revolutionised management of solid malignancies. It is extraordinary that over the span of just a few years the way clinicians think about solid tumour management has changed: even in advanced and in rapidly progressive disease like previously fatal cancers such as melanoma, lung cancer or bladder cancers have become treatable. Cautious optimism is emerging that some patients may become long-term disease free and perhaps may be cured.

However, these strategies rely on releasing the pre-existing anti-tumour immune response and only benefit up to 50% of patients. For those patients whose cancers cannot be controlled in this way, induction of immune responses and re-education of T-cells through vaccination can offer a path forward. The concept of boosting insufficient pre-existing responses or priming anti-cancer immune responses using vaccines is gathering pace to provide hope for patients. Developing successful vaccination strategies is crucial and significant advances have already been made in this field.

This book provides an overview of a number of approaches for vaccination, in particular, for lung and head and neck cancers including approaches which are still in preclinical development as well as those which have been already clinically tested. The importance of selection of a vaccine platform to induce successful antigen-specific immune responses has been given attention. The book highlights both laboratory and clinical experience with nucleic acid vaccines including DNA vaccines, which in combination with electroporation have become an effective way of antigen delivery in vivo leading to the successful targeting of the Human Papilloma Virus-driven high-grade cervical dysplasia. In parallel, novel and potent vaccines using mRNA are rapidly being transferred from the laboratory to bedside.

Vaccination approaches for targeting viral oncoantigens in cancers linked to viral aetiology are contrasted with challenges associated with targeting self-cancer antigens in cancer which are not induced by viruses. The need to overcome immunological tolerance to self-cancer antigens by cancer vaccines has been addressed here by two parallel approaches, one based on using chimeric antigens and another on including foreign linked CD4 T-cell help. We share our own clinical

experience of using foreign linked T-cell help in DNA vaccines that have also fuelled the development of new generations of cancer vaccines.

Finally, the authors of this book draw attention to the importance of using costimulatory antibodies in combinational strategies as exemplar for new avenues which are opening up for cancer immunotherapy.

This book is aimed at postgraduate students, non-clinical researchers as well as clinicians and all those who aspire to develop novel vaccination approaches for cancer patients with unmet clinical needs.

Southampton, UK

Natalia Savelyeva
Christian Ottensmeier

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New Approaches in Immunotherapy for the Treatment of Lung Cancer

Sonia Quaratino, Ulf Forssmann and Jens-Peter Marschner

Abstract Despite the several advances in the last few years into treatment of advanced lung cancer, the 5-year survival remains extremely low. New therapeutic strategies are currently under investigation, and immunotherapy seems to offer a promising treatment alternative. In the last decade, therapeutic cancer vaccines in lung cancer have been rather disappointing, mainly due to the lack of efficient predictive biomarkers. A better refinement of the patient population that might respond to treatment might finally lead to a success story. For the first time, the immune checkpoint inhibitors are demonstrating sustained antitumor response and improved survival and they may be the first immunotherapeutics available for patients with lung cancer.

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S. Quaratino (✉)
Kymab Ltd, The Bennet Building (B930), Babraham Research Campus, Cambridge CB22 3AT, UK
e-mail: sonia.quaratino@novartis.com

U. Forssmann
Bayer Pharma AG, Müllerstraße 178, 13353 Berlin, Germany

J.-P. Marschner
Affimed Therapeutics AG, Technologiepark, Im Neuenheimer Feld 528, 69120 Heidelberg, Germany

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1 Introduction—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). Non-small cell lung cancer (NSCLC) accounts for 80–85 % of all cases and small cell lung cancer accounts for the remaining 15–20 % (Peters et al. 2012). Within NSCLC, the squamous cell carcinoma accounts for approximately 30 % and the non-squamous NSCLC for the 70 %: the latter group is mainly formed by adenocarcinomas, but it can also include large cell carcinomas and less well-differentiated variants (Goldstraw et al. 2011) (Fig. 1).

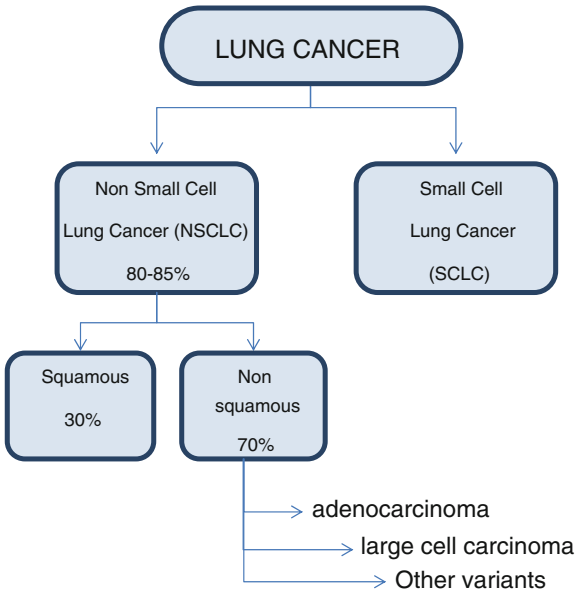

Fig. 1 Cellular classification of lung cancer

The majority of lung cancer patients present with advanced disease (stage IIIb/IV), and despite targeted therapy has increased the treatment options in the latest years, the overall 5-year survival rate is less than 5 % (Detterbeck et al. 2009).

In first line, the standard of care for stage IV patients consists in the combination of cytotoxic chemotherapy (carboplatin or cisplatin in combination with paclitaxel, gemcitabine, docetaxel, vinorelbine, irinotecan, and pemetrexed) (National Comprehensive Cancer Network (NCCN) 2014).

More recently, targeted therapies have increased the treatment options for patients with certain genetic mutations, opening the path for a more personalized treatment (Blackhall et al. 2013). For instance, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) such as gefitinib, erlotinib, and afatinib offer a therapeutic window in patients with identified genetic alterations of a key oncogenic driver, the EGFR (Peters et al. 2012). The EML4-ALK fusion gene, resulting from an inversion in chromosome 2, has also been identified as an oncogenic driver in NSCLC (Kwak et al. 2010). It is encountered more frequently in never-smokers, the adenocarcinoma subtype and in younger patients, representing probably ~5 % of adenocarcinoma (Shaw et al. 2009). Patients with ALK mutation can be efficiently treated by the TKI crizotinib (Shaw et al. 2013).

While targeted therapies against driver oncoproteins such as EGFR and ALK have increased the treatment options for patients with non-squamous NSCLC, there are few therapeutic alternatives against tumors without known-driver mutations, and chemotherapy (carboplatin + paclitaxel or pemetrexed) remains the standard of care for squamous NSCLC patients.

The development and implementation of new therapeutic strategies is therefore essential to improve prognosis in lung cancer, and immunotherapy may offer a promising treatment alternative.

Immunotherapy using therapeutic cancer vaccines has shown promise in early clinical trials and has advanced to late-phase development with disappointing results. These include the therapeutic vaccines that target MAGE-3 and MUC1. A promising approach currently in clinical evaluation in NSCLC is the use of immune checkpoint modulators. By blocking inhibitory molecules or, alternatively, activating stimulatory molecules, these treatments are designed to unleash and/or enhance preexisting anticancer immune responses.

2 The Role of the Immune System in NSCLC

The immune system has a complex interaction with the tumor, as it can have either a tumor-promoting or tumor-inhibitory role, depending on the tissue localization, the cell types of the tumor immune infiltrate and the cytokines they secrete. The nature of the immune cells within the tissue can influence tumor progression and has prognostic significance.

An increased tumor infiltration with CD4+ and CD8+ T-helper (Th) 1 cells has been considered a strong favorable prognostic predictor independently associated

with improved survival in lung cancer (Kawai et al. 2008). Similarly, a Th1-enriched gene signature in the tumor microenvironment may favor the presence of immune effector cells in the tumor of patients who responded to the MAGE-3 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). In NSCLC, higher number of cytotoxic T cells (CTL) and Th1 type cells have been associated with survival benefit regardless of the disease subtype (Bremnes 2011; Dieu-Nosjean 2008), whereas a low CTL/Treg ratio and Th2 type cells are a predictor of recurrence and shorter survival (Petersen et al. 2006).

Like the majority of other tumors, also NSCLC uses different strategies to evade the immune system and prevent destruction by effector T cells. Firstly by down-regulating 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 down-regulated HLA class I expression (So et al. 2005) and abnormal 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 negative regulator PD-1 expressed 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 up-regulation 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.

The immune system plays an active role not only in adenocarcinoma but also in squamous cell carcinoma. For instance, a higher number of infiltrating CD8+ CTL and a significantly lower number of Tregs have been found in squamous than adenocarcinoma (Black 2013; Stinchcombe 2014). However, in squamous cell carcinoma, the favorable CTL/Treg ratio does not correlate with a survival

advantage, possibly due to an immunosuppressive tumor environment and other immune-evading strategies (Suzuki et al. 2011; Stinchcombe 2014).

A major component of the adaptive immune response relies on the specific recognition of T-cell antigens. This starts with the T-cell receptor recognizing the antigenic peptide presented by the major histocompatibility complex (MHC) on the antigen-presenting cells (APCs), leading to T-cell activation. Engagement of the co-receptor CD28 with B7-1 (CD80) and B7-2 (CD86) ligands on the APCs provides a stimulatory signal for T cells, sustaining T-cell activation (Chambers and Allison 1997). Subsequent engagement of the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) with the same ligands on the APCs results in attenuation of the response (Walunas et al. 1994; Chambers and Allison 1997). Tregs are also known to up-regulate CTLA-4, further contributing to suppressed activation and expansion of CTL (Peggs et al. 2009). Another negative regulator expressed on activated T cells is programmed death 1 (PD1), whose binding with its ligands PD-L1 and PD-L2 on APCs and tumor cells results in down-regulation of T-cell activation (Dong et al. 2002). Tumors are able to up-regulate the PD-L1 ligand expression in order to bind to the PD1 expressed on T cells and thus down-regulating the immune response and promoting T-cell apoptosis (Dong et al. 2002). Blocking the negative T-cell regulators has proven to sustain the T-cell immune response and enhanced antitumor immunity (Leach et al.1996; Brahmer 2014). In NSCLC, elevated PD-L1 levels were positively correlated with increased TILs and associated with better outcome in lung carcinomas (Velcheti et al. 2014). Higher PD-L1 levels were detected in squamous cell carcinomas compared to adenocarcinoma (56.7 % vs. 27.5 %; $P = 0.009$) despite having comparable lymphocytic infiltrates (Velcheti et al. 2014).

The potential to identify subsets of NSCLC patients with an inflammatory tumor microenvironment that predicts for longer survival may also predict for response to immunotherapy and checkpoint inhibitor blockade.

Understanding of the immune evasion mechanisms regulated by tumor cells is necessary for 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 down-regulating the cancer-induced immune suppression.

3 Vaccines

Vaccines investigated in NSCLC are usually antigen specific and are based on peptides, viral vectors, cell lines, or anti-idiotypic antibodies. Ideally, antigens selected for the immunological approaches should be highly expressed by the tumor but not by healthy tissues and should be immunogenic (Bradbury and Shepherd 2008).

This article will focus on vaccines in advanced stage of clinical development. Table 1 provides a list of vaccines which are currently investigated in phase III clinical trials.

Table 1 Therapeutic cancer vaccines in phase III clinical development in NSCLC

NSCLC stage	Vaccine (*sponsor*)	Mode of action	Antigen	Adjuvant	Phase III trial		
					Design	No. of patients	Trial duration (START data)
Stage Ib/IIIa, adjuvant	GSK1572932A (*GSK*)	Peptide based	MAGE A3	AS15	Double-blind, placebo-controlled, phase III (**14**); *MAGRIT* MAGE-A3 versus placebo after resection ± adjuvant CTX Prim. EP: DFS	2,270	2007–2014 (data available; **14a**)
Stage III, maintenance	L-BLP25; tecemotide (*Merck KGaA*)	Peptide based	MUC1	MPL	Double-blind, placebo-controlled, phase III (**28**); *START* L-BLP25 maintenance versus placebo after CRT Prim. EP: OS	1,513	2007–2012 (data available; **28a**)
					Double-blind, placebo-controlled, phase III (**28b**); *START2* L-BLP25 maintenance versus placebo after concurrent CRT Prim. EP: OS	1,000	2014–2018
	GV1001; tertomotide (*KAEL-GemVax*)	Peptide based	telomerase (hTERT)	GMCSF	Double-blind, placebo-controlled, phase III (**37**); *LucaVax* GV1001 maintenance versus placebo after CRT Prim EP: OS	600	4 years planned (not open)

(continued)

Table 1 (continued)

NSCLC stage	Vaccine (*sponsor*)	Mode of action	Antigen	Adjuvant	Phase III trial		
					Design	No. of patients	Trial duration (START data)
Stage III/IV	Belagenpumatucel-L; Lucanix *(NovaRx)*	Cell based	4 allogeneic NSCLC cell lines	TGF-β antisense	Double-blind, placebo-controlled, phase III (**42**) Lucanix maintenance versus placebo after first line; *STOP* Prim. EP: OS	532	2008–2013 (data available; **45**)
	Racotumomab (*Recombio SL; CIMAB, Cuba*)	Anti-idiotypic	NGc-containing gangliosides	–	Randomized, open-label, phase III (**49**) Racotumomab+BSC versus BSC after first line Prim. EP: OS	1,082	2011–2015
Stage IV[(1)]	TG4010; MVA-MUC1-IL2 *(transgene)*	Viral vector based	MUC1	IL2	Double-blind, placebo-controlled, phase IIb/III (**58a**); *TIME* MUC1 +, % ANK-cells CTX+TG4010 versus CTX +placebo in first line Phase IIb: prim. EP: validation of biomarker Phase III: prim. EP: OS	210 (ph IIb) 800 (ph III)	2012–2015 (ph IIb data available; **58b**)
	CIMAVax–(*CIMAB, Cuba; Bioven*)	rhEGF linked to carrier protein	EGF	Montanide ISA 51	Randomized, open-label, phase III (**68**) CIMAvax versus best supportive care after CTX Prim. EP: OS	579	2006–?
					Randomized, open-label, phase III (**70**) CIMAvax versus BSC first line	?	2011–2015 (trial terminated; **70**)

(continued)

Table 1 (continued)

NSCLC stage	Vaccine (*sponsor*)	Mode of action	Antigen	Adjuvant	Phase III trial		
					Design	No. of patients	Trial duration (START data)
					Prim. EP: OS		
	Talactoferrin alfa (*aggenix*)	Unspecific immune stimulation	–	–	Double-blind, placebo-controlled, phase III (**77**); *FORTIS-M* Talactoferrin+BSC versus placebo+BSC in 3rd+ line Prim. EP: OS	742	2008–2012 (data available; **78**)
					Double-blind, placebo-controlled, phase III (**79**); *FORTIS-C* Talactoferrin+CTX versus placebo+CTX in first line Prim EP: OS and PFS (co-primary)	94 (1,100)	2009–2012 (trial stopped; **80**)
	Tergenpumatucel-L; HyperAcute (*newlink genetics*)	Cell based	3 allogeneic NSCLC cell lines	αGal	Randomized, open-label, phase IIb/III (**87**) Phase IIb: dose schedule finding (2 doses) Phase III: tergenpumatucel-L versus CTX first line; Prim. EP: OS	240	2013–2015

Prim. EP primary endpoint
CTX chemotherapy
PFS progression-free survival
BSC best supportive care
ANK activated NK-cells
1 Includes stage IIIB (wet)

3.1 Melanoma-Associated Antigen 3 (MAGE-A3, GSK1572932A)

The MAGE-A3 antigen is expressed by various tumors. It is not expressed in normal tissue except testis and placenta; however, testis and placenta lack the presentation by HLA molecules (De Plaen et al. 1994). MAGE-A3 is expressed in 35–50 % of NSCLC tumors (Van den Eynde BJ 1997; Sienel 2004; Vansteenkiste et al. 2007), and its expression seems to be a prognostic factor for the clinical outcome in NSCLC (Brichard 2007).

Consequently, MAGE-A3 is a tumor specific antigen which makes it unique for the development of an antigen-specific cancer immunotherapy (ASCI). The MAGE-A3 immunotherapeutic GSK1572932A consists of the MAGE-A3 peptide in combination with an adjuvant. The peptide used in newer clinical trials is produced by recombinant technology (Tyagi and Mirakhur 2009). In earlier clinical trials, the adjuvant $AS02_B$ was used, including the phase II study in NSCLC. However, compared to $AS02_B$, the adjuvant AS15 resulted in an increased clinical activity as demonstrated in a melanoma trial (Kruit 2008). Therefore, the AS15 immunostimulant was chosen for all further trials (MAGRIT).

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). 33 % of the 1,089 screened patients were MAGE-A3 positive, 183 patients were enrolled, and 122 patients were assigned to MAGE-A3, 60 to placebo.

After a median observation period of 44 months, the hazard ratio for the primary endpoint disease-free interval (DFI) was 0.75 (95 % CI, 0.46–1.23; $p = 0.254$) in favor of the MAGE-A3 treatment. This effect was not statistically significant. Similar results have been observed for the secondary endpoints disease-free survival (DFS) and overall survival (OS). After 70 weeks of median observation, the effect for the primary endpoint DFI was almost the same with $HR = 0.78$ (95 % CI, 0.49–1.24; $p = 0.259$). In addition, this effect was consistent for all stratification factors (disease stage, histology, and resection technique). However, after 70 months of median observation, the treatment effect on the secondary endpoint DFS was weaker and did even no longer exist for OS.

A total of 117 of 119 MAGE-A3-treated patients who were eligible for immune response assessment developed anti-MAGE-A3 IgG antibodies after four doses of the vaccine. The remaining two patients developed an immune response after six and nine doses. No induction of immune response was observed in 57 placebo-treated patients. There was no correlation between immune response and DFI.

MAGE-A3 immunotherapeutic treatment was safe with no relevant difference in terms of grade 3/4 AES compared to placebo. Local reactions were more frequently observed in the MAGE-A3 arm and were all of mild-to-moderate intensity.

Gene signature (GS) was investigated in tumor samples in a phase II trial in melanoma (Kruit et al. 2013). 84 genes, mainly immune related, were identified as potentially predictive for the efficacy of MAGE-A3. This GS was then prospectively investigated in the phase II trial in NSCLC (Ulloa-Montoya et al. 2013). 61 (39 %) of 157 tested NSCLC patients were GS positive. Comparing the effect of the vaccine with placebo for the primary endpoint DFI, hazard ratio was 0.42 (95 % CI, 0.17–1.03; $p = 0.06$) for the GS-positive patients and hazard ratio was 1.17 (95 % CI, 0.59–2.31; $p = 0.65$) for the GS-negative patients. Also, for OS, where no clinical effect was seen after 70 months of median observation in the whole population, hazard ratio was 0.63 (95 % CI, 0.22–1.78; $p = 0.81$) in favor of MAGE-A3 vaccination in patients with positive GS.

Phase III trial in NSCLC: MAGRIT A randomized, double-blind phase III trial in patients with resected stage IB/II/IIIA NSCLC was initiated based on the phase II data. Patients were eligible if they were MAGE-A3 positive, underwent surgery with or without standard adjuvant chemotherapy. 13 intramuscular injections are scheduled over 27 months. The primary endpoint was DFS, and the secondary endpoint was prospective validation of GS (Tyagi and Mirakhur 2009).

MAGRIT started in October 2007 and aimed to recruit 2,270 patients from around 400 sites in 33 countries (clinicaltrials.gov). On April 2, 2014 GlaxoSmithKline announced its decision to stop the MAGRIT trial, after establishing that it will not be possible to identify a subpopulation of gene signature-positive NSCLC patients that may benefit from the treatment (GSK press release). Data showed that MARGIT did not meet its first or second co-primary endpoints as it did not significantly extend disease-free survival when compared to placebo in either the overall MAGE-A3-positive population or in those MAGE-A3-positive patients who did not receive chemotherapy.

In summary, although the phase II trial indicated a clinical benefit of MAGE-A3 immunotherapy in patients with completely resected stage IB/II NSCLC, an effect which was even more pronounced in patients with positive GS, the phase III trial MARGIT failed to demonstrate a clinical benefit for the patients.

3.2 L-BLP25 (Tecemotide, Formerly Stimuvax)

The mucinous glycoprotein MUC1, a member of a family of mucins, is an integral membrane protein with extracellular, transmembrane, and cytoplasmic domains. MUC1 has a broad distribution in a variety of normal tissues and tumor tissues (Zotter et al. 1988; Ho et al. 1993; Kufe 2009). MUC1 has oncogenic potential and is able to confer resistance to genotoxic agents (Agrawal et al. 1998). Further, MUC1 seems to play a role in tumor progression, because it can stimulate cell proliferation through growth factor receptor, β-catenin, and ERα, and also suppresses apoptosis through the regulation of JNK, NF-kB, HSP90, and extrinsic

apoptotic pathways (Bafna et al. 2010). MUC1 also alters various signaling pathways (Zhao et al. 2009; Raijna et al. 2011). In contrast to normal tissue, MUC1 is aberrantly glycosylated in tumors which makes it a unique target for cancer treatment. Furthermore, underglycosylated MUC1 primes class I-restricted CTL more efficiently than glycosylated MUC1 (Hiltbold 1999). MUC1 is expressed in >90 % of early-stage NSCLC independent of histology. MUC1 expression is generally maintained in paired primary/nodal tumor samples (Mitchell et al. 2013).

Tecemotide is a peptide-based vaccine consisting of BLP25 lipopeptide, immunoadjuvant monophosphoryl lipid A (MPL), and three lipids forming a liposomal product (Butts et al. 2005). Three days after a low-dose intravenous cyclophosphamide administration, tecemotide is administered subcutaneously weekly over 8 weeks, followed by administration every 6 weeks until disease progression.

Phase II trials in stage III/IV NSCLC Tecemotide was investigated as maintenance treatment in a randomized phase IIB trial in stage IIIB/IV NSCLC (Butts et al. 2005). Patients were eligible if they did not progress after initial therapy, i.e. chemoradiotherapy or chemotherapy. They were randomly assigned to tecemotide plus best supportive care (BSC) or BSC alone. The primary endpoint was OS. 171 patients were accrued, 65 with stage III disease, and 106 with stage IV disease. The median OS time was 17.4 months for patients in the tecemotide arm and 13 months in the BSC arm with a hazard ratio of 0.739 (95 % CI, 0.509–1.073; $p = 0.112$) in favor of tecemotide. This effect was even more pronounced in the subgroup of patients with stage IIIB locoregional disease; the hazard ratio was 0.524 (95 % CI, 0.261–1.052; $p = 0.069$). An updated survival analysis revealed that patients with stage IIIB locoregional disease had a median OS of 30.6 months in the tecemotide arm versus 13.3 months in the BSC arm with a 45 % reduced risk to die [HR 0.548, 95 % CI 0.301–0.999 (Butts et al. 2011)].

Comparable survival data, observed in a single-arm phase II trial in stage III locoregional NSCLC patients ($n = 22$), provided some consistency (Butts et al. 2010). Long-term observation data of this trial indicated a median OS of 51.9 months (Butts et al. 2012).

Phase III trial START Encouraged by the phase II data, a randomized, double-blind phase III trial was conducted in stage III locoregional NSCLC (Butts et al. 2013, 2014). 1,513 patients that did not progress after chemoradiotherapy were randomized to tecemotide or placebo in a 2:1 manner. Chemoradiotherapy was given concurrently or sequentially and consisted of a platinum-based chemotherapy and at least 50 Gy of radiotherapy. Caused by a clinical hold, the primary analysis cohort consisted of 1,239 patients. Median OS was 25.6 months in the tecemotide arm versus 22.3 months in the placebo arm (HR 0.88, 95 % CI, 0.75–1.03, $p = 0.123$). A subgroup analysis for the strata of START revealed a more pronounced treatment effect in patients with prior concurrent chemoradiotherapy ($n = 806$). 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, 95 % CI, 0.64–0.95, $p = 0.016$). Tecemotide was well tolerated with no safety concerns.

Based on the subgroup finding of START, the START2 trial was initiated. START2 is a phase III, multicenter, 1:1 randomized, double-blind, placebo-controlled clinical trial designed to assess the efficacy, safety, and tolerability of tecemotide in patients suffering from unresectable, locally advanced (stage III) NSCLC who have had a response or stable disease after at least two cycles of platinum-based concurrent chemoradiotherapy (CRT). The study is expected to recruit about 1,000 patients. The study's primary endpoint is OS. Secondary endpoints include time to symptom progression, progression-free survival, and time to progression (Merck KGaA press release, clinicaltrials.gov NCT02049151). The trial has been stopped in September 2014 (press release Merck KGaA).

In summary, the phase III trial could not demonstrate a survival benefit for patients treated with tecemotide. However, subgroup analyses indicate that patients with prior concurrent chemoradiotherapy may benefit from tecemotide treatment. The value of this subgroup finding is currently investigated in another phase III trial.

3.3 GV1001 (Telomerase Vaccine, Tertomotide)

The reverse transcriptase subunit of telomerase (hTERT) is overexpressed in the majority of tumors and, under normal conditions, by embryonic cells and bone marrow stem cells (Kim et al. 1994). It is absent in most adult tissues. Therefore, it is an attractive target for antigen-specific cancer immunotherapy.

GV1001 is a 16-mer peptide vaccine which is injected intradermally 10–15 min after the administration of the adjuvant GM-CSF. It induces both, $CD4^+$ and $CD8^+$, responses and may cause epitope spreading (Inderberg-Suso EM et al. 2012). The administration schedule consists of 3 injections in the first week, followed by injections in weeks 2, 3, 4, 6, 10, and then booster injections every 4 weeks. GV1001 was investigated in several tumor entities such as breast and prostate cancer, melanoma, and other solid tumors; however, most data were generated in pancreatic cancer and NSCLC. Based on phI/II data in a trial in patients with pancreatic cancer (Bernhardt et al. 2006; Kyte et al. 2009), two phase III trials have been initiated in this indication. One was stopped early because a lack of efficacy (press release), the second failed to demonstrate evidence for efficacy (Middleton et al. 2013).

Phase I/II trials in NSCLC In the trial CTN-2006, 23 unresectable stage III NSCLC patients were vaccinated after the initial treatment with radiotherapy plus weekly docetaxel. Specific T-cell response was shown in 16 of 20 eligible patients. Survival was longer in patients with immune response. The treatment was well tolerated (Brunsvig et al. 2011).

A phase I/II trial, CTN 2000, investigated the vaccination in 26 patients, most of them in stage IV disease. Immune responses were detected in 11 out of 24 eligible patients (Brunsvig et al. 2006). An 8-year update of this trial revealed that OS was significantly increased in immune responders compared to non-responders with a

median survival of 19 and 3.5 months, respectively (Brunsvig et al. 2011). Four patients were long-term survivors, two of them received vaccinations still 9 years after start of vaccination. All four long-term survivors exhibited strong and durable T-cell responses.

Phase III trial LucaVax A phase III trial was planned investigating the effect of GV1001 in stage III NSCLC. Patients with unresectable disease who got chemoradiotherapy with curative intent are eligible. Chemoradiotherapy has to consist of platinum-based doublets which must not contain gemcitabine and radiotherapy with a dose of up to 66 Gy. Patients are randomized in a double-blind manner to either GV1001 or placebo. The trial was planned to start in April 2012 but is not recruiting, yet (clinical trials.gov, NCT01579188).

In summary, there is some evidence for immunological responses after vaccination of patients with GV1001. GV1001 seems to be well tolerated and showed some efficacy in early trials. However, ongoing phase III trials in pancreatic cancer are failed, while the phase III trial in NSCLC did not start recruitment. It is open if the development of GV1001 will be continued.

3.4 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: two adenocarcinomas, one squamous cell carcinoma, and one large cell carcinoma. All cell lines were transfected with an antisense gene for TGF-β (Nemunaitis et al. 2006). Using defined human cancer cell lines presenting antigens for vaccination of NSCLC patients is a reasonable approach, because data from gene expression profiling in adenocarcinomas indicate that the majority of tumor-related genes are co-expressed by different lung tumor subtypes (Hayes et al. 2006). In addition, this approach has the advantage that the vaccine can be used "off the shelf."

TGF-β has a broad variety of effects on tumors, which may be even controversial (Katz et al. 2013). TGF-β signaling can result in tumor promotion by several modes of action. Immune evasion is one of them, and blocking it may be a useful tool to increase the effect of a vaccine. There are other direct effects of TGF-β which promote tumors, for example promotion of invasion and angiogenesis and development of metastases.

Belagenpumatucel-L is administered intradermally (2.5×10^7 cells in a volume of 0.4 mL) monthly for 18 months and then once at 21 and 24 months in the absence of disease progression (clinical trial.gov, NCT00676507).

Phase II trials Belagenpumatucel-L was investigated in an open-label, three-arm phase II trial (Nemunaitis et al. 2006). Three doses of the vaccine were investigated: 1.25, 2.5, and 5×10^7 cell/injection (cohorts 1, 2, and 3, respectively). Patients were

eligible if they had a confirmed stage II (n = 2), IIIA (n = 12), IIIB (n = 15) or IV (n = 46) NSCLC. Treatment with belagenpumatucel-L was started after at least 30 days following the initial cytotoxic therapy.

The treatment was safe with no difference in serious adverse events across dose cohorts. All but two-grade 3/4 adverse events were attributed to disease progression.

Dose-related survival results were observed. Cohorts 2 and 3 were significant different compared to cohort 1 (p = 0.0069). Also 1- and 2-year survival probability was higher for cohorts 2 and 3 compared to cohort 1: 68 and 52 % compared to 39 and 20 %, respectively. The median survival time was 581 days in dose cohorts 2 and 3, i.e., significantly higher than in cohort 1 (252 days, p = 0.0186).

In PBMC of all patients, an increased intracellular cytokine production was measured at week 8 compared to base line. Further, patients with stable disease (SD) or better had higher frequencies of IFN-γ, IL-6 and IL4. Seven of nine patients with negative ELISPOT at baseline developed a response at week 12. The majority of stage IIIB/IV NSCLC patients with SD or better produced a markedly elevated ELISPOT response. With regard to the humoral immune response, a correlation of positive response and clinical outcome was described.

The clinical data were confirmed in a smaller phase II study in 21 patients with advanced stage NSCLC (Nemunaitis et al. 2009). In addition, further immunological tests were performed in late-stage NSCLC patients of phase II patients (Fakhari et al. 2009). According to these data, immune responders survived 32.5 months compared to 11.6 months for non-responders.

Phase III trial An international multicenter, randomized, double-blind, 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 front-line, platinum-based combination chemotherapy was initiated in 2008 (STOP; Clinicaltrials.gov, NCT00676507). Primary endpoint was overall survival.

STOP did not meet its predefined primary endpoint in the entire patient population (Giaccone 2013); median OS was 20.3 months with the vaccine compared to 17.8 months with placebo (HR 0.94; p = 0.594).

However, prognostic factors for improved outcome were identified in subgroup analyses. The OS was shown to be significantly impacted by the time interval between randomization and the end of frontline chemotherapy (p = 0.002). The OS was improved by 7.3 months with belagenpumatucel-L in 305 stage IIIB/IV patients who were randomised within 12 weeks of chemotherapy completion. In this cohort, the median OS was 20.7 months with belagenpumatucel-L compared to 13.4 months with placebo (HR 0.75; p = 0.083).

Other factors prognostic of better outcome were disease stage, whether prior radiation was received and histology. The patients with confirmed pretreatment radiation showed optimally improved median OS of 29.8 months difference between the treatments; patients receiving belagenpumatucel-L following radiation showed median OS of 40.1 months compared to 10.3 months with placebo (HR 0.45; p = 0.014).

In summary, despite encouraging data from early clinical trials, the phase III trial failed to demonstrate efficacy of belagenpumatucel-L for the intent to treat population. However, subgroup analyses revealed that defined patient populations may benefit from the vaccination. Further studies are warranted to confirm this data.

3.5 Racotumomab (Anti-idiotypic NeuGc-GM3 mAb, 1E10 Antibody)

NGcGM3 ganglioside is expressed by many tumors and seems to be practically undetectable in healthy human tissue (Fernandez et al. 2010; Gomez and Ardigo 2012). GM3 is expressed in >90 % of NSCLC tumors and is involved in tumor induced dentritic cell suppression (van Cruijsen et al. 2009).

Racotumomab is an IgG1 anti-idiotypic monoclonal antibody developed by the Center of Molecular Immunology, Havana, Cuba (Vázquez et al. 2012). Racotumomab is administered intradermally (1 mg/ml) five times every 2 weeks during the induction period followed by monthly vaccinations (clinicaltrials.gov, NCT01460472).

Phase I trials were performed in different tumor types including melanoma, breast cancer, SCLC, and NSCLC (Vázquez et al. 2012). It was found that the administration is safe and that it resulted in a strong and specific immune response against NeuGc containing gangliosides (Hernández et al. 2008).

Phase II trials A multicenter, randomized, placebo-controlled, double-blind trial in patients with advanced NSCLC (stage IIIB/IV) who had achieved stable disease or better after initial onco-specific treatment was conducted (Macías et al. 2012). 176 patients were randomized to the vaccine or placebo. Moderate-to-mild local injection site reaction has been observed in the vaccination arm; otherwise, no difference was seen between both arms. The median OS was 8.3 months in the racotumomab arm and 6.3 months in the placebo arm. The OS rate at 1 and 2 years was 38 and 17 % in the racotumomab arm and 24 and 7 % in the placebo arm, respectively. For patients who received >5 vaccinations/placebo administrations (n = 135), this effect was more pronounced.

In another open, non-randomized phase II trial, patients with advanced NSCLC (stage IIIB/IV) who did progress following initial onco-specific treatment were treated with racotumomab (Gomez et al. 2013). Most of the patients had received 4–6 cycles of cisplatin plus vinblastine. 180 patients were accrued. Median survival was 8.06 months, and the OS rate at 2 years was 21 %. Compared to the above-mentioned trial, a comparable OS was observed for patients who received the vaccine.

Phase III trial A prospective, randomized multicenter, open-label phase III study of racotumomab plus BSC versus BSC in patients with advanced NSCLC was initiated in 2010 (clinicaltrials.gov, NCT01460472). The primary endpoint is OS, secondary endpoints are safety, PFS and immunology. 1,082 patients will be

enrolled, and data are expected in September 2015. Patients are eligible if they suffer from stage III or IV NSCLC and did not progress after standard one line treatment. The trial is conducted in Argentina, Brazil, Cuba and Singapore.

In summary, racotumomab was well tolerated and showed efficacy immunological response in phase II trials and no details on data have been published so far. Obviously, the ganglioside seems to be an interesting target and the phase III trial may provide more information on this vaccine.

3.6 TG4010 (MVA-MUC1-IL2)

TG4010 is an antigen-specific vaccine targeting MUC1 (for MUC1, see also chapter about tecemotide). TG4010 is based on a viral vector, a modified vaccinia of Ankara (MVA), which, in addition to MUC1, expresses interleukin 2. TG4010 is administered subcutaneously at a dose of 108 pfu weekly for 6 weeks and then every 3 weeks (Limacher and Quoix 2012).

Two small phase I trials in 13 patients with MUC1 positive metastatic solid tumors were conducted (Rochlitz et al. 2003). The trials showed that the administration of TG4010 is well tolerated with injection site reactions and flulike symptoms only. It was further observed, that four patients had disease stabilization. One patient with NSCLC, who initially progressed, developed a long lasting response. In addition, some T-cell proliferative immune responses were observed.

Phase II studies A phase II study was conducted in 65 patients randomized to either chemotherapy (cisplatin plus vinorelbine) plus TG4010 (arm 1) or to TG 4010 alone (arm 2) for first-line therapy of stage IIIB/IV NSCLC (Ramlau et al. 2008a). All patients had MUC1-positive tumors as assessed by IHC. The stronger clinical effect was observed in 44 patients randomized to arm 1. 33 of these 44 patients were evaluable for response, 13 achieved a partial response with a response rate of 35.1 %.12 other patents (27.3 %) achieved stabilization of disease. Immunological data (T-cell proliferation test and ELISPOT) were not conclusive. Immunological responses were seen as well before (baseline) as after vaccination. Actually, positive ELISPOTs at any time during the trial correlated with a better survival.

Based on these data, another randomized, open-label phase IIb trial investigated the effect of TG4010 in first-line therapy for stage IV NSCLC which was tested MUC1 positive (Quoix et al. 2011; Ramlau et al. 2008b). 148 patients were recruited, 74 patients were randomized to the combination therapy group (cisplatin plus gemcitabine plus TG4010), and the remaining patients were randomized to the chemotherapy alone group. Progression-free survival (PFS) at 6 months was the chosen primary endpoint. In the combination group, 43.2 (95 % CI 33.4–53.5) were progression free at 6 months, while only 35.1 % (95 % CI 25.9–45.3) were progression free at 6 months in the chemotherapy group. Objective response rate was 41.9 (95 % CI 30.5–53.9) and 28.4 (95 % CI 18.5–40.1) for combination therapy and chemotherapy, respectively. There was no difference in overall

survival. A pre-specified analyses revealed that the percentage of activated natural killer cells (CD16+, CD56+, and CD69+) had predictive value. Patient with normal percentage of activated natural killer cells (73.2 % of patients) benefited more from TG4010 treatment, and this included also median OS which was 17.1 months for the combination group and 11.3 months for the chemotherapy group. The interaction between pretreatment percentage of activated natural killer cells and treatment group was significant for OS ($p = 0.0023$). Overall TG4010 was well tolerated with fever, abdominal pain, and injection site pain as site effects which occurred more frequently in the combination group. While the incidence of serious adverse events was similar in both treatment groups of the ITT population, there was a significantly higher incidence for these events in the combination group for the subpopulation with higher percentage of activated natural killer cells.

Phase IIb/III trial TIME This is a double-blind trial comparing the combination of first-line chemotherapy with TG4010 or placebo in stage IV NSCLC with MUC1 expressing tumor (Quoix et al. 2012). The phase IIb part ($n = 210$) aimed at prospectively validating the level of activated NK-cells, the so-called triple-positive activated lymphocyte levels (CD16+, CD56+, CD69+; TrPAL), as predictive marker with PFS as primary endpoint. In contract to the originally planned approach, recently communicated data indicated that a PFS analysis using a quartile approach for the TrPAL lead to predictive threshold for the phase III part of the trial (press release 2014). The quartile analysis showed that in the 75 % of patients having the lower baseline level of TrPAL and who received TG4010, there was a clinically meaningful improvement in PFS, as indicated by a >25 % reduction in the risk of progression or death compared to placebo. Conversely, in the 25 % of patients with the higher level of TrPAL (highest quartile) and who received TG4010, there was no improvement in PFS. Additionally, in subgroup analyses using the quartile approach, an even larger improvement in PFS was obtained in patients with non-squamous tumors not treated with bevacizumab (73 % of initial study population). The design of the phase III part is now under discussion with regulatory authorities. The study started in 2012 with an estimated primary completion date end of 2015 (clinicaltrials.gov, NCT01383148).

In summary, TG4010 is well tolerated and showed clinical efficacy in combination with chemotherapy in metastatic NSCLC in a randomized phase II trial. A biomarker was identified (activated NK-cells, TrPAL). Data of the phase IIb part of the registration trial will be discussed with regulatory authorities in order to design the phase III part.

3.7 EGF Vaccine (CimaVax)

The epidermal growth factor (EGF) plays an important role in tumor growth, mitosis, and metastasis. The EGF receptor (EGFR) is expressed in NSCLC (Salomon et al. 1995), and two types of anti-EGF receptor approaches have been

investigated in NSCLC: tyrosine kinase inhibitors and monoclonal antibodies (Ciardello and Tortora 2008; Pirker 2013).

An EGF vaccine was developed in Cuba with recombinant human EGF linked to a carrier protein (P64k Neisseria Meningitidis recombinant protein). A pilot trial in 10 patients with solid tumors indicated that this approach is immunologically effective and tolerable (González et al. 1998).

Two pilot trials were performed in 40 stage IIIB/IV NSCLC patients after onco-specific treatment in order to investigate the effectiveness of two different adjuvants in combination with the vaccine. Based on the antibody responses measured, Montanide ISA 51 was chosen for future clinical trials (Gonzáles et al. 2003). Another analysis using pooled data of the pilot trials showed clinical efficacy, in particular in seroconverted patients (Gonzáles et al. 2007).

Phase II trial 80 patients with stage IIIB/IV NSCLC who received first-line treatment were randomized to the vaccine plus supportive care versus supportive care (Neninger Vinageras et al. 2008; García et al. 2008). About 30 % of these patients had progressive disease at the time of randomization. In the treatment arm, patients received a low-dose cyclophosphamide administration 3 days prior to vaccination, then 4 weekly intra-muscular injections of 50 μg EGF followed by monthly injections.

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 with a median OS of 11.57 versus 5.33 months in the vaccination and control arm, respectively. Antigen responses and EGF concentration were measured in 42 patients (26 vaccinated and 16 controls) only. Data showed that patients with good antibody response had the better survival benefit with a median OS of 11.7 months compared to 3.6 months in patients with poor antibody response. Further, an inversed correlation between EGF concentration and survival was demonstrated. Vaccination was safe with mild-to-moderate-related adverse events only.

Phase III trial In 2006, a phase III trial was initiated at 18 sites in Cuba which is still ongoing. 579 patients are randomized 2:1 to vaccine or control (Rodríguez et al. 2010). The therapeutic schedule for the vaccine is the same as in the phase II study; however, the vaccine is administered in four injection sites resulting in a four times higher dose. Patients are stratified for age: >60 years versus ≤60 years. Preliminary results of 160 patients show a trend toward a delayed separation of the survival curves in favor of the vaccine arm (Rodríguez et al. 2010).

A total of 40 patients of this phase III trial who received the high-dose regimen were compared to 40 patients of the phase II trial who received the lower-dose regimen (Rodríguez et al. 2011). The patient groups from both trials were balanced in terms of baseline and tumor characteristics. Both regimens were well tolerated with only grade 1- and grade 2-related adverse events. The humoral response was more pronounced in the high-dose group with an antibody titer of 1:7328 (geometric mean) compared to 1:3160 in the control group. In the high-dose group, the rate of good antibody responders was 54.8 % and the rate of "super good

responders" was 30.8 %. In the phase II trial, the respective rates are 52.8 and 10 %. The median OS in high-dosed patients was 13.57 months compared to 6.47 months in low-dosed patients.

Another phase III open-label, randomized trial in stage III/IV NSCLC patients is ongoing and finished recruitment. Patients in the age of 20–65 years were eligible and randomized to the vaccine or BSC as first-line therapy. According to changes in clinicaltrials.gov (March 2014), the trial has been terminated in order to initiate a new phase III design including biomarker to enrich the patient population and to further strengthen OS benefit (clinicaltrials.gov, NCT01444118). The level of circulating EGF is used as biomarker and a minimum threshold level has been set as inclusion criterion for the trial.

In summary, phase II data on safety, efficacy, and immunology are encouraging. First data of an ongoing phase III trial suggest that an increased dose of the vaccine may have a stronger effect in terms of immunology and efficacy without an increasing risk for the patients. The phase III trial was terminated, and the outcome has not been made public so far. CimaVax was licensed in Cuba for the treatment of adult patients with stage IIIB/IV NSCLC (Rodríguez et al. 2010). Bioven has licensed in the rights on the EGF vaccine developed in Cuba. Bioven names this product EGF *P*athway-*T*argeted Immunization, PTI, instead of vaccine in order to make clear distinction between classical vaccines targeting tumor antigens such as MUC1, MAGE 3, L-BLP-25 presented here, and the PTI approach.

3.8 Talactoferrin Alfa

Talactoferrin alfa is a recombinant human lactoferrin (Kelly and Giaccone 2010). It is structurally and functionally similar to native human lactoferrin as it can be purified from human milk. Lactoferrin has multiple known biological effects such as anti-inflammatory effects or anti-tumor effects. In addition, there are different immune modulatory effects. After oral administration, talactoferrin recruits dentritic cells in the Peyer's patches of the intestine. These dentritic cells present (tumor associated) antigens and initiate an immune stimulatory cascade in the gut associated lymphoid tissue (GALT).

The anti-tumor effect of talactoferrin was demonstrated in mouse models. It was shown that oral talactoferrin induces mucosal IFN-gamma production as well as expansion of CD8+ T lymphocytes and NK-cells and enhancement of CD8+ cytotoxicity (Spadaro et al. 2007).

A phase I trial was conducted in patients with solid tumors. Ten patients who had failed conventional chemotherapy were recruited in the first part of the trial which was intended to escalate the dose and to identify the maximum-tolerated dose (MTD) (Hayes et al. 2006b). Talactoferrin was well tolerated, an MTD was not defined. Talactoferrin induced an increase in circulating IL18. Five patients had

stable disease after 2 months of treatment, seven patients showed a reduction in tumor growth rate. In the second part, further 26 patients were recruited that received the two highest daily doses tested in part one (4.5 or 9 g/day) (Hayes and Falchook 2010). 12 of the total 36 patients suffered from NSCLC. 11 of them have received prior CTX or radiochemotherapy. Out of the 12 patients, seven had SD. The median PFS was 4.3 months, the median OS was 8.8 months. The substance was well tolerated.

Phase II trials Two randomized, double-blind, placebo-controlled phase II studies have been initiated: one in stage IV NSCLC patients that progressed after CTX and one in previously untreated stage IV NSCLC patients.

In the first trial, 100 patients were included of which 81 patients received at least one dose (evaluable population) (Parikh et al. 2011). The majority of patients received one prior therapy, about 1/4 received two or more prior therapies. Talactoferrin was dosed 1.5 mg twice daily for 12 weeks followed by 2 weeks off for a maximum of three cycles until progression. The median OS was 6.1 months in the talactoferrin arm and 3.1 months in the placebo arm and the primary endpoint was met ($p = 0.05$). The 1-year survival rates were 29 and 16 %, respectively.

The second trial recruited 110 stage IV NSCLC patients who did not receive prior therapy for NSCLC (Digumarti et al. 2011). Patients were randomized to CTX plus talactoferrin or CTX plus placebo. Patients in the talactoferrin arm received 1.5 mg twice daily for three 6-week cycles (5 weeks on, 1 week off the drug) until progression. Primary endpoint was response rate (RR). In the evaluable population, RR was 47 % in the talactoferrin arm and 29 % in the placebo arm ($p = 0.05$). Median PFS was 7.0 and 4.2 months (HR = 0.85; $p = 0.24$), and median OS was 10.4 and 8.5 months (HR = 0.87; $p = 0.26$), respectively.

In both phase II trials, talactoferrin was well tolerated with grade 3 and 4 events occurring slightly more frequent in the placebo arms.

Phase III trials Based on the phase II data, two phase III trials were initiated: FORTIS-M and FORTIS-C.

FORTIS-M was a randomized, double-blind, placebo-controlled trial which compared the clinical effect of talactoferrin-alfa plus BSC with placebo plus BSC in patients with stage IV NSCLC who have failed two or more prior treatment regimens (clinicaltrials.gov, NCT00707304). The study did not meet the primary endpoint, i.e., to demonstrate a significant survival benefit for talactoferrin compared to placebo (aggenix press release). 742 patients were enrolled globally at 160 sites. Patients in the talactoferrin arm had a median OS of 7.5 months compared with 7.7 months for the placebo arm (HR = 1.04, $p = 0.66$).

FORTIS-C was a randomized, placebo-controlled study of talactoferrin-alfa in combination with carboplatin and paclitaxel as first-line therapy in patients with stage IV NSCLC. Co-primary endpoints were OS and PFS (clintrials.gov NCT00706862). This trial was stopped early because of the negative results from FORTIS-M (press release). At this time, 94 of the planned 1,100 patients were

enrolled in the US. For the 94 enrolled patients, median PFS was 5.8 months in the talactoferrin arm and 5.6 months in the placebo arm (HR = 0.97; $p = 0.89$). Median OS was 11.4 versus 12.7 months (HR = 1.25; $p = 0.36$), respectively.

In summary, despite encouraging phase II data, talactoferrin-alfa failed to demonstrate clinical efficacy in late-stage NSCLC. The question was raised if this very late stage of disease, i.e., stage IV NSCLC with several previous lines of chemotherapy, is the right candidate for immunotherapy (Madan et al. 2013).

3.9 Tergenpumatucel-L (HyperAcute)

The α(1,3)galactosyl (αGal) gene is functional in mammalian species but inactive in humans (Joziasse and Orial 1999). Due to continuous presentation of the antigen αGal by intestinal and pulmonal bacteria, the human immune system develops specific antibodies recognizing αGal. These antibodies are responsible for a hyperacute reaction to cells expressing αGal, e.g., after xenotransplantation.

Already more than a decade ago, the approach of eliciting hyperacute xenograft response to treat cancer was described (Link et al. 1998). In animal models, it was shown that immunity against αGal can induce antitumor activity (Rossi et al. 2008).

Tergenpumatucel-L is a cell-based vaccine consisting of genetically modified allogeneic NSCLC cells bearing αGal moieties (Morris et al. 2013). Tergenpumatucel is administered intradermally.

Phase II trial 28 patients with metastatic or recurrent NSCLC were recruited into a single-arm phase II trial prior to systemic therapies (Morris et al. 2012, 2013). Patients received eight doses of 300×10^6 cells every 2 weeks. The median OS was 11.3 months. Eight patients had stable disease over more than 16 weeks. Nine out of 16 patients who received follow-up chemotherapy developed a response according to RECIST criteria. The vaccine was well tolerated. All patients showed increased anti-αGal antibody levels and 61 % of patients showed increased interferon-gamma levels (ELISPOT, no details described). These patients were characterized by a longer median OS of 21.9 months compared to 5.5 months for patients with lower IFN-gamma levels (Morris et al. 2012). The authors concluded that, compared to historical controls, the observed survival duration is encouraging. Further, due to the responses seen in the follow-up chemotherapies, the authors concluded that the vaccination has a chemosensitizing effect.

Phase IIb/III trial Based on the phase II data, an open-label, randomized, multi-institutional adaptive design phase IIb/III trial was initiated in January 2013 (clintrials.gov, NCT01774578). Patients are eligible if they have stage IIIB/IV NCSLC and received prior line(s) of chemotherapy. In the phase IIb part, two dosing schedules will be investigated either 300×10^6 cells weekly for 11 weeks and then every 2 months for five additional doses or 300×10^6 cells every 2 weeks for six doses followed by additional 10 monthly doses. In both groups, a total of 16 immunizations is planned. The phase III part will then further investigate the

clinical effect of Tergenpumatucel-L administering the selected dose from phase IIb part. 240 patients will be enrolled and randomized to either the vaccine or to chemotherapy. Primary endpoint is overall survival; results are expected in July 2015.

In summary, data from the phase II study indicate some clinical efficacy, although this trial was not randomized and no details have been published so far.

3.10 Summary—Vaccines

Several vaccines have been/are in phase III clinical development in NSCLC. To date, there is no evidence from phase III trials that vaccines can cause a clinically meaningful benefit in patients suffering from NSCLC. All finished trials did not meet the primary endpoint: MAGRIT (MAGE-A3), START (tecemotide), STOP (Lucanix), FORTIS M and FORTIS C (talactoferrin alpha) and probably also the CIMAVax trial (EGF vaccine). In addition, the LucaVax trial (GV1001) is not followed up and the START2 (tecemotide) follow-up trial was discontinued due to disappointing phase II data from a Japanese study in the same setting. Overall, the results of vaccinations in NSCLC are disappointing so far.

However, there are trials ongoing which can tell us more about the value of vaccinations in NSCLC. TIME (TG4010) as well as the phase III trials with racotumomab and tergenpumatucel-L will generate further data over the next years. Also, new phase III trials with belagenpumatucel-L (Lucanix) and CIMAVax may be initiated based on further analyses of the data.

Outlook: In addition to the development of vaccines as single immunological treatment approach, the combination of vaccines with non-specific immune stimulators, in particular checkpoint inhibitors, seems to be a promising approach which may strengthen the specific immunological effects of vaccines (Rangachari and Brahmer 2013). We are still in an early phase of investigating vaccines. Further understanding of the mode of action and in particular combination therapies may help to make vaccines more successful in NSCLC.

4 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 Spiegel and Socinski (2013). Two pathways, the CTLA-4:B7-1/-2 and the PD-1:PD-L1/-L2 axis, are clinically investigated, and the current status is described below.

4.1 CTLA-4 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 (phase II study for previously untreated subjects with non-small cell lung cancer (NSCLC) or small cell lung cancer (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 versus 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-group, 20 % in the concurrent-group, 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 versus 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). Up to date, no further trials have been initiated.

4.2 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 and 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 with 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). This cohort was also investigated for the association of tumor PD-L1 expression with clinical activity. The tumors were defined as PD-L1 (PD-L1+) positive when ≥5 % of the tumor cells had membrane staining at any intensity. Using this cutoff, 5 of 31 patients defined as PD-L1+ had an OR, while 4 of 32 patients defined as PD-L1− had an OR as well (Antonia et al. 2013). Hence, further evaluation of PD-L1 as a molecular marker of nivolumab therapy is required.

First preliminary data in NSCLC have also been reported recently for lambrolizumab/MK-3475, which is another anti-PD-1 antibody. Thirty-eight patients (19/38) previously treated with two systemic regimens had been enrolled. The ORR (confirmed and unconfirmed using RECIST 1.1) was 21 % applying an independent central images review and 24 % using investigator-assessed irRC. The median duration of response by irRC had not been reached, with a median follow-up of 9 months (minimum 6 months). Pretreatment tumor PD-L1 expression was a statistically significant predictor of response. In patients with evaluable tumor PD-L1 expression, all confirmed responses by RECIST v 1.1 (and irRC) occurred in patients with tumors strongly positive for PD-L1. Fifty percent of the patients had drug-related adverse events, and there was only one case of a grade 3 (pulmonary edema), but no higher drug-related adverse events (Garon et al. 2013). Follow-up data presented at AACR 2014 reported a median PFS of 9 weeks, and a median OS of 51 weeks. PD-L1 IHC score was above a potential cut point in nine patients and below a potential cut point in 22 patients (seven patients could not be evaluated), and significant associations between tumor PD-L1 expression and ORR (57 vs. 5 %) were observed (Gandhi et al. 2014).

BMS-936559, an anti-PD-L1 antibody was 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 (Spiegel and Socinski 2013). 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 grade 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 versus docetaxel in previously treated advanced or metastatic NSCLC, one trial in squamous and the other trial in non-squamous histology (clinicaltrials.gov NCT01642004, NCT01673867). An open-label phase III safety trial of nivolumab in subjects with advanced or metastatic NSCLC who have progressed during or after receiving at least one prior systemic regimen to estimate the incidence and characterize the outcome of high-grade, select adverse events has also been recently initiated (clinicaltrials.gov NCT02066636). Additional phase II trials are actively recruiting or ongoing (clinicaltrials.gov NCT02041533, NCT01721759).

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 versus 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). Further, phase I and II trials with MK-3475 in NSCLC in monotherapeutic setting and in combination settings with paclitaxel, carboplatin, pemetrexed, bevacizumab, ipilimumab, and erlotinib have been initiated (clinicaltrials.gov NCT02039674). The anti-PD-L1 antibody MPDL3280A is also further explored in one phase III and three phase II trials in advanced NSCLC (clinicaltrials.gov NCT02008227, NCT01846416, NCT02031458,). Moreover, a phase III trial with the anti-PD-L1 antibody MEDI4736 as sequential therapy in patients with locally advanced, unresectable NSCLC (stage III) who have not progressed following definitive, platinum-based, concurrent chemoradiation therapy has been initiated (clinicaltrials.gov NCT02087423).

5 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 (not described here because the development was stopped). Also, for tecemotide and Lucanix, a clinical benefit in terms of OS could not be demonstrated in large phase III trials. The appropriate patient selection, either with gene signature or using the optimal combination treatment, may help yield the survival improvement with vaccines. Over the next few years, we will get a clearer picture about the role of vaccines in the treatment of NSCLC. Currently, the most promising results in NSCLC 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.

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Novel Approaches for Vaccination Against HPV-Induced Cancers

Emma King, Christian Ottensmeier and Kevin G.J. Pollock

Abstract To date, more than 5 % of all cancers are as a result of human papillomavirus (HPV) infection, and this incidence is increasing. Early recognition of disease is associated with good survival, but late presentation results in devastating consequences. Prevention is better than cure, and there are now successful prophylactic vaccination programmes in place. We discuss these and the prospect of therapeutic vaccinations in the near future to address a growing need for improved therapeutic options.

Contents

E. King (✉) · C. Ottensmeier
University of Southampton, Southampton, UK
e-mail: e.king@soton.ac.uk

K.G.J. Pollock (✉)
Health Protection Scotland, Glasgow, Scotland
e-mail: kevin.pollock@nhs.net

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1 HPV Lifecycle—Exposure, Infection and Clearance

Infections cause more than 15 % of human cancers, and approximately one-third of these are as a result of human papillomavirus (HPV). HPV is a ubiquitous double-stranded DNA virus, and it is estimated that there are more than 100 different HPV types that can infect humans. They are subdivided into low and high risk (HR), pertaining to their malignant potential. It is estimated that a third of all primary school children are infected with low-risk HPV, resulting in cutaneous warts (Bruggink et al. 2012). Cervical smear samples in unvaccinated women suggest that approximately half of women aged 20–21 carry an infection with HR HPV types and 58 % are infected with any HPV type (Kavanagh et al. 2014). Prevalence studies using oral swabbing and testing for HPV suggest that 37–60 % of people are infected, although there are a number of caveats to consider with such prevalence studies (Steinau et al. 2014). Given that oropharyngeal and cervical cancers are relatively rare (although the former are increasingly incident), it is evident that most people who are exposed to HPV through sexual contact will clear the virus via cell-mediated immunity (Stern et al. 2000).

The HPV virus contains both 'early' and 'late' genes: the early genes, E1 and E2, are virus-encoded replication factors; E4 and E5 act to regulate late viral functions by mechanisms that are not yet well understood. Interestingly, E3 exists in only a few papillomavirus types, but not HPV16 (the HR subtype that is responsible for more than 97 % of HPV-driven malignancy in head and neck squamous cell carcinoma): the gene is not expressed as a protein and has no known function. Late genes L1 and L2 are synthesised following amplification and produce capsid proteins, which the virus needs for full assembly and release of infectious virions (Shulzhenko et al. 2014).

In the cervix (where most extensively studied), HPV virions infect the basal cell layer, as a result of microwounds that expose the basal layer. Viral capsids bind initially to the basement membrane and then to keratinocytes, as they move into the wound (Kines et al. 2009). Both Annexin A2 and S100 calcium-binding protein A10 are integral but with separate roles during HPV16 binding, entry and trafficking of HPV16 (Dziduszko and Ozbun 2013). Following cellular entry and uncoating, HPV is replicated in the nucleus to about 100 episomal copies per cell. This phase is orchestrated by the early genes, E1, E2, E6 and E7. E1 and E2 initiate viral DNA replication, and E6 and E7 maintain long-term replication competence (Frattini and Laimins 1994; Sedman and Stenlund 1995). In undifferentiated basal cells, the viral proteins are expressed at very low levels, favouring immune evasion. As the basal cells divide, each daughter cell contains HPV within the nucleus: E2 is central to this process as it binds to specific ACCN6GGT motifs in the viral genome and attaches it to mitotic chromosomes, tethering the virus genome to the host chromosomes during meiosis (Mcbride et al. 2006). In addition, E2 maintains a stable viral copy number, essential to allow continued persistence in basal cells; it can both positively and negatively regulate the early viral promoter that regulates E1, E6 and E7 as well as E2 itself (Steger and Corbach 1997).

Although the undifferentiated basal cells contain very low levels of viral proteins, as the infected host cell leaves the basal layer and differentiates, high levels of protein synthesis are induced. This delayed expression of viral proteins undoubtedly delays expression of viral antigens until they are in a less immune-competent compartment. However, sustaining long-term viral infection is a strategy not without problems: the virus does not produce its own replicative enzymes, host cell replication is required, and as the host cell differentiates, replication stops. To overcome this problem, the virus forces the differentiated cell to continue to replicate, largely under the control of E7 and its ability to inactivate retinoblastoma (Rb) protein (Felsani et al. 2006). The late viral promoter increases the copy numbers from hundreds to thousands, and then, the capsid proteins are produced. L1 and L2 are considered highly immunogenic, but are not expressed until the most differentiated strata, minimising immune impact. The virions are shed into the environment without lysis or necrosis, avoiding an inflammatory response. Thus, HPV infection is entirely intraepithelial with only limited transfer of antigen to adjacent lymph nodes, minimising an adaptive immune response.

The entry of HPV into epithelial cells is best documented in cervical cells. Data relating to HPV infection in the oropharynx and other genital areas are poorly documented.

Dependent upon type, HPV infection ranges from benign cutaneous warts (primarily due to HPV 1, HPV 2, HPV 27 and HPV 57) (Bruggink et al. 2012), to genital warts, recurrent respiratory papillomatosis (HPV 6 and HPV 11) and some HR 'oncogenic' HPV types can precipitate cancers; notably the majority of cervical cancers, 75 % of oropharyngeal cancer, 90 % of anal cancers and 40 % of vulval, vaginal and penile cancers are as a result of HR HPV (Anantharaman et al. 2013; Jemal et al. 2013).

Viral infection of the host can have several outcomes. Acute infection may develop, which is followed by recovery from the virus and total elimination of the virus mediated by cellular immunity. Alternatively, a chronic infection may persist, with prolonged carriage of the virus with or without further relapses of acute disease. Instead of immune-mediated clearance following infection, a latent stage may develop, during which clinical signs of disease are absent and new virions are not produced and released. It is possible that such latent infections may undergo subsequent reactivation leading to new virion synthesis, with or without the re-emergence of clinical disease: this late re-emergence may underlie disease recurrence including genital warts and may also contribute to the development of the aforementioned cancers (Maglennon and Doorbar 2012).

2 Malignant Transformation

In most patients, the host immune system prevails and the HPV infection resolves. However, in a significant minority, the infection is present for long periods allowing additional cellular changes and mutations to occur, leading to cancer. The ancient

relationship between HPV and its host relies on productive infection to maintain a continued viral existence: under this perspective, induction of malignant transformation is beneficial to neither host nor virus, but undoubtedly occurs. Although the role of E6 and E7 in the basal layer is uncertain during infection, it is crucial in malignant transformation. During malignant progression, the HPV genome can integrate into a host cell chromosome and as a result, E6 and E7 were thought to remain the only viral proteins that continue to be expressed. HPV genome integration is a terminal event and not a manifestation of the normal viral life cycle. Interestingly, viral integration is not a prerequisite for malignant transformation and episomal persistence can also result in malignant transformation (Olthof et al. 2014): It is likely that both episomal and integrated virus exists in the same cell. In anogenital carcinoma, there is a significant correlation between the frequency of integrated viral DNA and progression of dysplastic lesions to malignancy (Vinokurova et al. 2008). In addition, integration appears to be influenced by the subtype: in cervical malignancy, 55 % of HPV16 and 92 % of HPV18 cases have viral integration. Finally, in 39 % of oropharyngeal malignancies, viral integration was detected. Interestingly, in the latter cohort, there was no significant difference in the viral copy numbers of E2, E6 or E7 in either integrated or episomal cases (Olthof et al. 2014).

HPV-induced malignant transformation has been most extensively studied in the cervix: levels of both E6 and E7 increase in parallel to the increases in degree of dysplaia, i.e. cervical intraepithelial neoplasia 1(CIN) to CIN3. In CIN1 lesions, the virus can typically complete its life cycle and produce viral particles: clinically, patients have flat cervical warts. CIN2 lesions have elevated levels of E6 and E7 compared to CIN1, predisposing them to the accumulation of genetic changes as a result of diminished tumour suppressor gene (TSG) activity. Specifically, E6 downregulates p53, and E7 downregulates Rb.

Cancers arise secondary to the action of E6 and E7 oncoprotiens, and only HR HPVs harbour significant malignant potential. Both E6 and E7 lack intrinsic enzymatic activity and function by associating with, and functionally reprogramming key components of the host cellular signal transduction networks. The E6 protein most frequently interacts with an E3 ubiquitin ligase, E6-associated protein (E6AP) (Huibregtse et al. 1991). The ‘ubiquitin cascade’ adds multiple ubiquitin monomers to the protein, destined to be degraded by proteosomal degradation, and this includes p53 (Scheffner et al. 1993). When p53 is bound by E6 and E6AP, it is unable to induce apoptosis and is degraded. E6 can also inhibit p53 activation by blocking the alternate reading frame p14 pathway and by interacting with histone acetyltransferase, hADA3 (Khoronenkova and Dianov 2011; Kumar et al. 2002). In addition, E6 in combination with E6AP can promote telomerase activity, via E6AP (Klingelhutz et al. 1996). Finally, HR E6 proteins (and not low-risk E6 proteins) can interact with PDZ domain-containing proteins (including hDIg) (Kiyono et al. 1997), MAGI-1 (Glaunsinger et al. 2000), hScrib (Nakagawa and Huibregtse 2000), MUPP1 (Lee et al. 2000) and PTPN3 (Jing et al. 2007) affecting epithelial cell polarity (McLaughlin-Drubin and Munger 2009; Muench et al. 2009) [ref].

Interestingly, E7 is considered the 'main' HPV oncoprotein and at low frequency is able to immortalise human epithelial cells. HPV16 E7, from both HR and LR HPV, binds the cullin 2 ubiquitin ligase complex and silences pRB and associated proteins (p105, p107, p130). The HR HPV E7 binds with much greater affinity than LR E7 (Munger et al. 2001). This binding results in E2F transcription factor repression, allowing entry into S-phase. pRb degradation results in p16 upregulation encoded by the sequence on the CDKN2a gene (Khleif et al. 1996). This results in high p16 expression in HPV-driven malignancy. Interestingly, p16 is a potent TSG, but E7 also directly activates cyclins A and E downstream of p16, negating the TSG effect (Zerfass et al. 1995). However, increased expression of p16 acts as a good biomarker for HPV-driven malignancy.

There are many additional host cell factors that HR E7 proteins bind to including HDACs 1, 2 and 3, p21, p27 (cell cycle inhibitory functions), ATM (DNA damage sensor) and p600 (anoikis). Interestingly, abrogation of pRb function by HR E7 protein leads to increased stabilisation of p53 potentially leading to increased apoptosis. As a result, the HR E6 proteins have evolved to induce degradation of p53 to block apoptosis (Howie et al. 2009). E5, E6 and E7 oncoproteins are all considered anti-apoptotic, and the main contributors to malignant transformation, E2 and E7 are also pro-apoptotic proteins, and a balance therefore exists (Garnett and Duerksen-Hughes 2006).

Interestingly, E6 and E7 also act as potent mitotic mutators, thereby increasing the occurrence of mutations that contribute to carcinogenic progression (McLaughlin-Drubin and Munger 2009). The E6 and E7 genes are located in the same open reading frame and are transcribed as a single transcript. E2 protein differentially regulates E6/E7 expression: the transcription of E6/E7 is controlled by E2. E2 binds to its promoter which upregulates p97, which in turn activates the transcription of E6. Interestingly, E2 protein stabilises p53 and maintains apoptosis in HeLa cells (Webster et al. 2000). HPV 16 and HPV 18 E2 proteins have been shown to activate transcription of HPV 16 E6 and E7 oncogenes (Bouvard et al. 1994); however, there are numerous other factors that affect the repression and activation of E6 and E7 oncoproteins. When HPV integrates into the host genome, E2 is inhibited, resulting in a loss of E2 apoptosis and E2-mediated regulation of E6 and E7 (Arisa-Pulido et al. 2006). Clearly, episomal virus does not lose E2-mediated regulation.

Although E6 and E7 are cited as the main oncogenes, E5 also has a role to play. E5 is the smallest HPV oncoprotein and in HPV16, E5 is primarily localised within the endoplasmic reticulum (ER) (Conrad et al. 1993; Borzacchiello et al. 2010). E5 expression in oropharynx malignancy is associated with high EGFR expression, which is linked with poor outcome (Um et al. 2014). E5 protects against apoptosis through inhibition of death receptor apoptosis and ER stress-induced apoptosis (Jiang and Yue 2014). E5 protein may cooperate with E6 and E7 to immortalise cells, and play an inhibitory role in apoptosis (Jiang and Yue 2014). E5 may contribute to the early stages of cancer initiation, but in integrated viruses, E5 is often lost and is not necessary for the maintenance of the transformed phenotype.

Interestingly, in low-risk subtypes, E5 is missing or lacks an ORF and/or a translation start codon (Schiffman et al. 2005).

We understand now that the host immune system is central to survival in many solid tumours, including HPV-induced head and neck malignancy (Ward et al. 2014a). It is likely that it is also important during the early stages of malignant transformation: in order to restrict an immune response, it is probable that both the adaptive and innate immune systems are affected by HPV-infected cells. Healthy keratinocytes constitutively express low levels of interferon-inducible genes in the absence of added interferon. However, cells infected by high-risk HPV E6 and E7 proteins repress the transcription of many interferon target genes including Stat-1, IRF-1 and IRF-3. In addition, both E6 and E7 can minimise the expression of TLR-9, important to sense double-stranded DNA (Ghittoni et al. 2010). Furthermore, keratinocytes constitutively express low levels of several proteins that are upregulated following viral infection. HPV infection does not result in upregulation of key pro-inflammatory cytokines including IL-1, IL-6, TNFa and TGFb, but does upregulate anti-inflammatory IL-10 (Alcocer-González et al. 2006). Clearance of HPV-infected lesions is via cell-mediated responses and cytolysis, and dendritic cells are central to this process. HPV L2 proteins have been shown to suppress maturation, migration and cytokine secretion by dendritic cells (Fahey et al. 2009). MHC class I is downregulated as a result of E6, E7 or E5. In addition, E7 has been reported to downregulate TAP, interfering with antigen presentation via MHC class I (O'Brien and Saveria Campo 2002). Clinically, immunosuppressed patients are at increased risk of both benign and malignant HPV infections. Rates of anal HPV infection are extremely high in HIV-positive patients, especially in those men that have sex with men, resulting in high rates of anal intraepithelial neoplasia (AIN) and anal cancer (Gami et al. 2014).

3 HPV-Related Cancer

The World Health Organization (WHO) published their 'position paper' on HPV in 2009. It identified that in 2005, there were 500,000 cases of cervical cancer and 260,000 related deaths worldwide. The rates were variable from 1 to 50 per 100,000 females, higher in Latin America and the Caribbean, sub-Saharan Africa and Asia. Most were diagnosed when older than 40 years. Up to 80 % can be prevented by screening programmes, and mortality rates remain significantly higher in the developing world.

Vulvar, vaginal, penile, anal and oropharyngeal cancers, and their precancerous lesions are all relatively rare, and most of these cancers occur in adults aged more than 50 years. HPVs are estimated to cause at least 80 % of anal cancer, 75 % of oropharyngeal cancer and 40–60 % of vulvar, vaginal and penile cancers, though prevalence rates do vary.

The estimated burden of non-cervical HPV-related cancers in Europe is higher in men than in women and is driven primarily by head and neck cancers. It has been

estimated that 17,403 cancer cases attributable to HPV (15,497 attributable to HPV 16/18) occur each year in *men* in Europe. This compares with an estimated 9,308 non-cervical cancer cases attributable to HPV 16/18 each year in *women* in Europe (Hartwig et al. 2012).

3.1 Cervical Cancer

HPV infections in the genital tract are the most common sexually transmitted infections. Although progression to malignancy is rare, the high prevalence of the virus makes HPV-related cancers among the most common malignancies. In the UK, cervical cancer is the second most common cancer in women under 35 years [1]. HPV types 16 and 18 are essential precipitants in at least 70 % of cervical cancers (Smith et al. 2007) but may contribute in excess of 80 % of cervical cancers in particular geographic areas, such as Scotland (Cuschieri et al. 2010). Worldwide, 0.5 million new cases of cervical cancer are reported with 274,000 associated deaths annually, making it the second most prevalent cancer in women (Brotherton et al. 2011). Certain HR HPV types, recognised as class I carcinogens by the WHO, are necessary risk factors for the development of cervical cancer.

Not all cervical precancers progress to an invasive cancer: Approximately 25 % of CIN2 and 3 lesions completely regress within a short time frame (4 months) (Trimble et al. 2005). Detection of antibodies against E6 and E7 in serum does not predict which lesions will regress (Trimble et al. 2009a). However, the presence of CD8+ T cells in cervical dysplastic lesions does predict dysplasia regression (Trimble et al. 2010).

High-risk viruses in the cervix include HPV 16, HPV 18, HPV 31, HPV 33 and HPV 45, and together they cause 97 % of cervical cancers worldwide. By contrast, HPV 6 and HPV 11 frequently infect the genital tract, but are rarely detected in malignancy (Lorincz et al. 1992). In all, there are more than 40 HPV subtypes frequently detected within the female genital tract (Schiffman et al. 2005): Schiffman et al. prospectively followed 10,000 women and identified HPV16 as uniquely likely to both persist and to cause neoplastic progression when it persisted. Remarkably, 20 % of HPV16-infected women were either diagnosed with CIN3 or cancer either at enrolment or within 5 years. Other carcinogenic types were not as persistent, but could induce malignancy at a less frequent rate, and many were persistent, without significant malignant potential. Most women clear the infection within 12–18 months. However, a 10 % minority fail to clear the infection, resulting in a persistent infection: if this is HR HPV, there is a risk of malignant progression.

HPV is not only implicated in squamous cell carcinoma (SCC). In the cervix, HPV DNA is detected in most adenocarcinomas, adenosquamous carcinomas and carcinomas with neuroendocrine differentiation (Howley and Lowy 2007). HPV16 is most commonly associated with squamous cell carcinomas, while HPV18 is the predominant type found in adenocarcinomas and neuroendocrine carcinomas.

The Papanicolaou (Pap) smear allows the recognition of cellular abnormalities in HPV-infected cells, and screening has reduced the number of cervical cancers by 80 % in the USA, over the past 50 years. Early stages (I–IIa) of cervical cancer can be treated successfully, but locally advanced cancers are characterised by high recurrence rates and a poor prognosis. The standard therapy of locally advanced cervical cancer is a combination of radiotherapy and cisplatin-based chemotherapy with an overall 5-year survival of less than 50 %. Patients with stage IV or recurrent cervical cancer treated with cisplatin alone or in combination with topotecan only have a median survival of less than 1 year [57].

3.2 Anogenital Cancer

Anogenital malignancies include those arising from the vulva, vagina, penis, scrotum and anus. The majority (90 %) of vulval carcinomas are SCC, representing 4–5 % of malignancies in women (Dittmer et al. 2012). The majority of these are in older women and not related to HPV. However, there is an increasing incidence in younger (<50) women, and 43 % are related to HPV 16 and HPV 18. Early lymph node spread is frequent due to the prominent lymphatic supply, and surgery is the primary mode of treatment. Survival varies from early- to late-stage disease from 90 to 18 %.

Vaginal cancer is rare with under 260 new cases diagnosed in the UK each year (CRUK), less than 1 out of every 600 cancers diagnosed in women. Seventy percentage of vaginal cancers are caused by HPV, the majority HPV16. Treatment is radiotherapy (with or without chemotherapy) and salvage surgery if required.

Penile cancer is rare in men in developed countries, but common in underdeveloped countries. Risk factors include non-circumcision and HPV infection. HPV DNA has been identified in both benign and malignant penile lesions including condylomata acuminate, Bowens disease and SCC (Schoeneich et al. 1999). Lymphadenopathy is present between 28 and 64 % of cases at presentation. Surgery is the main form of treatment.

Scrotal carcinoma is also associated with HPV, predominately subtype 18. In a recent study, 40 % of patients had HPV-driven scrotal SCC (Matoso et al. 2014). It is again treated surgically with wide local excision, and survival is related to stage at presentation.

Anal carcinoma is an uncommon malignancy. In the general population, the incidence is between 0.8 and 1.4 cases per 100,000 people-years. This rises to 35 and 128 cases per 100,000 in men practicing anal intercourse and those HIV-positive patients practicing anal intercourse, respectively. Risk groups include HPV16 infection and high-grade AIN. Most AIN is associated with HPV (6, 11, 16 and 18). The risk for progression to malignancy from AIN is 10 % at 5 years (Scholefield et al. 2011). HPV is responsible for approximately 3,000 anal cancer cases in the USA per year (Markowitz et al. 2007).

3.3 Oropharynx Cancer

Head and neck SCC is the sixth leading incident cancer worldwide. In the USA, there were 11,500 deaths in 2012 from this malignancy (Siegel et al. 2012). Of these, HPV is responsible for approximately 3,500 of the cases (Markowitz et al. 2007). There has been a significant increase (225 %) in HPV-driven head and neck malignancy over the past 15–20 years, and this is predicted to continue to rise (Chaturvedi et al. 2011), especially in the Western world, where smoking is in decline. By 2020, the number of HPV head and neck cancers will exceed cervical cancers if this trend continues. Compared to HPV-independent head and neck cancer, HPV-driven patients are younger, frequently white males, non- or light-smokers and with only moderate or light alcohol consumption. It is likely that HPV head and neck cancer is a sexually transmitted disease (Gillison et al. 2008). HPV-driven cancer in the head and neck is predominantly related to the oropharynx. The oropharynx is an anatomical subsite that includes both palatine and lingual tonsils, soft palate and lateral pharyngeal wall. Both distinct sets of tonsillar tissue consist of organised lymphoid tissue, surrounded by stratified squamous epithelium. The surface area is significantly increased due to multiple crypts, facilitating antigen capture and immunosurveillance. This epithelium has an incomplete basal cell layer and basement membrane (to facilitate antigen trafficking), dispensing with the need for microtrauma for viral access (Pai and Westra 2009).

Traditional risk factors for head and neck cancer were smoking and alcohol. This cohort of patients have a significantly worse prognosis than those with HPV-driven malignancy (Ward et al. 2014b). Treatment includes surgery, chemotherapy or radiotherapy, either alone or in combination. In the HPV-driven group, survival does not depend on treatment type, instead on the infiltration of lymphocytes into the tumour (Ward et al. 2014a, b).

4 Prophylactic Vaccination—Cohorts Vaccinated, Uptake, Serological Evaluation

Both the prophylactic bivalent (Cervarix [HPV16 and 18]) and quadrivalent (Gardasil [HPV 6, HPV 11, HPV 16 and HPV 18]) vaccines prevent infection using L1 virus-like particles (VLP). Both prevent cervical HPV 16 and HPV 18 infection and confer protection against subsequent virally induced CIN (Paavonen et al. 2009; Brotherton et al. 2011; Pollock et al. 2014). Low levels of neutralising antibodies against L1 are detectable in 50–70 % of patients, 6–18 months following HPV infection (Viscidi et al. 1997; Carter et al. 2000; Safaeian et al. 2010). E1, E2, E6 and L2 do not evoke any measurable antibody response following natural infection (Mariani and Venuti 2010).

Although it is assumed that that the HPV vaccine protects via neutralising antibody, this mechanism has only ever been demonstrated in a preclinical model

using passive transfer of serum immunoglobulins (Suzich et al. 1995). More recent evidence suggests that AS04-adjuvanted vaccines (such as Cervarix) stimulate NF-κB with increased cytokine production as a result of increased numbers of activated, antigen-loaded dendritic cells and monocytes in the lymph nodes draining the injection site, further increasing the activation of antigen-specific T cells (Didierlaurent et al. 2009). Systemic immunisations with L1 VLP generates antibody concentrations fourfold higher than following a natural infection, as a result of both route of administration and concentration of antigen (Harro et al. 2001). Although the commercial HPV vaccines have proven efficacy, the correlation between either antibody levels or B-cell memory has not been established (Stanley et al. 2012). Animal models suggest that only very low levels of antibody are required to be protective (Day et al. 2010).

Although both Gardasil and Cervarix use L1 VLP, quadrivalent vaccine produces neutralising antibodies to HPV L1, which are type restricted and possess limited cross-reactivity. However, bivalent vaccine confers a degree of cross-protection against some phylogenetically related types including HPV 31, HPV 33 and HPV 45 (Kavanagh et al. 2014). While L2 does not produce a neutralising antibody response in natural infections (it is not highly immunogenic, unlike L1), following deliberate immunisation with L2 protein, neutralising antibodies were protective against viral challenge in cows and rabbits. Strikingly, these antibodies could cross-neutralise a broad range of HPV subtypes (Karanam et al. 2009). However, this vaccine remains poorly immunogenic, consistent with other protein vaccines, compared to the L1 VLP. Attempts to improve its immunogenicity are currently being tested in animal models (Tyler et al. 2014).

Population-based surveillance data from countries such as Australia, Denmark and the United Kingdom provides early encouragement that prophylactic HPV vaccination is significantly associated with a reduction in both low- and high-grade cervical abnormalities (CIN1-3) in young women (Brotherton et al. 2011; Crowe et al. 2014; Baldur-Felskov et al. 2014; Pollock et al. 2014). Furthermore, the quadrivalent vaccine, which additionally includes HPV types 6 and 11 that are associated with 85–95 % of genital warts, has also been shown to be strongly associated with a reduction in genital warts in both females and heterosexual males (Ali et al. 2013). In Australia, the decrease in genital warts in heterosexual men was observed prior to the implementation of vaccination of boys and is likely due to herd immunity.

In the Western world, cancers of the anus, penis, scrotum, vagina and vulva (henceforth described as non-cervical genital cancers) are increasing in incidence (Parkin and Bray 2006). The increase in non-cervical genital cancers may be associated with a concomitant rise in HR oncogenic HPV infections, with HPV 16 and HPV 18 estimated to contribute between 74–93 % of these cancers (Olsen et al. 2012). Autoinoculation of HPV occurs both from cervix to anus and from anus to cervix in the same woman, and it appears to be relatively common (Moscicki et al. 2012). Although no natural history studies of anal intra-epithelial neoplasia (AIN) are available in women, women with other HPV-associated lesions, including high-grade CIN and vulvar cancer, have higher rates of anal cancer. Therefore, it seems

biologically plausible that girls vaccinated with the HPV vaccine will have a significantly reduced propensity in developing non-cervical genital cancers and oropharyngeal malignancy (Garland et al. 2009; Giuliano et al. 2011; Kreimer et al. 2011; Olsen et al. 2012; Herrero et al. 2013) with a successful prophylactic vaccination programme.

While the benefits of the HPV vaccine are now being realised (Crowe et al. 2014; Ali et al. 2013; Pollock et al. 2014), efficacy is dependent on a number of critical factors. National vaccine programmes, which target preadolescent girls through school-based delivery, are likely to be more successful in preventing HPV infection and disease (Sinka et al. 2014; Pollock et al. 2014). However, such coordinated programmes require a robust and well-governed infrastructure and tend only to be a feature of affluent countries. In spite of this, a high HPV vaccine uptake has been achieved in Rwanda, showing what can be achieved (Hopkins and Wood 2013). Nevertheless, the burden of disease attributable to HPV infection is significantly greater in deprived countries, where recognised barriers such as high vaccine cost must be overcome if global burden of disease is to be reduced (Campo and Roden 2010).

Gender-neutral vaccination has been recommended in the USA, Canada, Austria and Australia. Considered cost-effective modelling has preceded such decisions suggesting that when the burden of disease in men is included in the models, depending upon vaccine price and vaccine uptake as well as other factors, male vaccination can become cost-effective (Stanley 2014). Although the HPV vaccine is not currently offered to boys within the United Kingdom, the UK Joint Committee on Vaccination and Immunisation is appraising the evidence as to whether vaccination of boys would be cost-effective. In a recent Norwegian analysis, public health priority and cost-effectiveness appears to be directed towards increasing vaccine uptake in girls rather than expanding vaccination coverage to boys, but this is crucially dependent upon vaccine tender price (Burger et al. 2014).

There are indirect benefits of the HPV vaccine. Achieving high HPV vaccine uptake may reduce inequalities in cervical cancer prevention by mitigating inequalities observed in the cervical screening programme. Knowledge and awareness of HPV infection, cervical cancer and screening in young girls who have been vaccinated against the virus is surprisingly low (Bowyer et al. 2013). Thus, it may be assumed that in older women knowledge and awareness of the virus and its association with cervical cancer will be even lower. By having a high-profile HPV vaccine campaign with prompt dissemination of the realised benefits, it provides an opportunity to emphasise the importance of attendance at cervical screening for both vaccinated women and older, unvaccinated women. Given that there is a small minority of mothers and daughters from disadvantaged backgrounds who do not participate in either cervical screening or HPV vaccination, it is imperative that awareness of HPV is raised through targeted efforts to reach these deprived groups (Spencer et al. 2014).

4.1 Prophylactic Vaccines for Established Lesions

Although both licensed HPV vaccines are most effective in individuals with no prior exposure to HPV, there are reports of vaccination after HPV DNA was demonstrated in cervical specimens (Hildesheim et al. 2007). They demonstrated that the bivalent vaccine did not improve the clearance rate of the virus. In addition, there are reports of resolution of HPV-induced warts after quadrivalent HPV vaccination (Silling et al. 2014; Kreuter et al. 2010). These studies enrolled patients who were immunocompromised and suggest that there may be clinical benefit in post-exposure treatment of persistent warts. However, in a recent case series, there was no clinical improvement in immunocompetent patients with HPV 6-positive condylomatas who received quadrivalent vaccine (Kreuter and Wieland 2013). It is likely that the decreased expression of L1 in chronic premalignant lesions and loss of L1 expression in SCC (and the infected basal cells) is responsible for the unsuccessful outcome of prophylactic vaccine for established malignant disease (Yoshida et al. 2008). The majority of healthy controls and cervical cancer patients are able to mount a systemic Th1 response against L1 (van Poelgeest et al. 2006). Well-designed clinical trials are required to elucidate the immunological mechanisms required for wart clearance.

5 Therapeutic Vaccines

HPV-driven malignancy unlocks a unique opportunity for cancer immunotherapy. For this patient cohort, the viral oncoproteins (E5, E6 and E7) responsible for malignant transformation and progression are known, permitting targeted treatment. In addition, there is a known immunosuppressive environment that cancer 'creates' and this must also be overcome to permit an effective immune response following vaccination. Recently, regulatory T cells (Tregs) within head and neck cancer were shown to express more immunosuppressive molecules compared to circulating Tregs (Jie et al. 2013): These molecules included cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), T-cell immunoglobulin and mucin protein 3 (TIM-3) and programmed cell death ligand-1 (PD-1). In addition to the checkpoint receptors, cancer visibility to the immune system can also be lessened due to reduced expression of HLA I molecules and also transporters associated with antigen processing (TAP) (Li et al. 2010).

5.1 Proteins and Peptides

There are data to show the likely benefit of immunotherapy in HPV-driven lesions. In a small study, a HPV16 E6/E7-based peptide vaccine caused regression of

HPV-associated premalignant vulvar lesions (Kenter 2009). In addition, a second study in patients with vulval intraepithelial neoplasia (VIN) showed that using an E6E7L2 fusion protein, efficacy was associated with the induction of HPV-specific CD4 and CD8 T cells (Daayana et al. 2010; Welters et al. 2010). A number of other trials with a range of vaccines have shown variable clinical results, but none have shown a convincing correlation between clinical outcome and immunogenicity (Ressing et al. 2000; Baldwin et al. 2003; Smyth et al. 2004; Trimble et al. 2009b; Santin et al. 2008; Frazer et al. 2004; Matijevic et al. 2011; Brun et al. 2011; Maciag et al. 2009).

Current trials evaluating alternative peptide vaccines include a phase I/II study to determine the safety and immune modulating effects of the therapeutic HPV16 E6/E7 long-peptide vaccine (ISA101). This vaccine will be used at different doses with or without IFNα as combination therapy with carboplatin and paclitaxel in women with HPV16-driven advanced or recurrent cervical cancer (NCT02128126). In addition, another phase 1 trial is evaluating the treatment of high-grade squamous intraepithelial neoplasia of the cervix using a vaccine consisting of four HPV16 E6 peptides in combination with Candin® (NCT01653249). Candin is a yeast extract and has anti-HPV effects; it has been used to treat common warts caused by LR HPV. Furthermore, synthetic peptides (SLP-HPV-01® with or without interferon-α injections) are being evaluated in men with AIN (NCT01923116).

In addition, protein vaccines using HPV16-derived peptides presented as a Trojan-type construct to prevent proteolysis and facilitate HLAI processing have been trialled in head and neck patients (Voskens et al. 2012). They were combined with MAGE-A3 proteins (HLA restricted), and although a peripheral blood response could be detected (PBMCs from 4 of the 5 patients were able to recognise both the full Trojan constructs and constituent HLA-II peptides), there was no clinical response in any of the 5 patients with advanced cancer. In a study with vulval lesions, vaccination of HPV16 E6 and E7 long peptides with incomplete Freund's adjuvant in 20 women with VIN reported 50 % complete response and 75 % having a durable clinical response (Kenter et al. 2009). Unfortunately, when these long peptides were trialled in patients with high-grade CIN, the trial was terminated early due to unacceptable side effects including flu-like symptoms and injection site morbidity (de Vos van Steenwijk et al. 2012).

5.2 Viral Vectors

A modified vaccinia virus (TG4001), designed to express HPV16 E6 and E7 and IL2, was shown to induce a clinical response in 10 patients (48 %) with CIN 2 and 3 lesions following 3 weekly subcutaneous injections (Brun et al. 2011). A collaboration between transgene and EORTC was announced with a view to trial this vaccine in head and neck patients (EORTC: transgene collaborates with EORTC on phase 2b trial with TG4001 in head and neck cancer).

5.3 DNA Vaccines

DNA vaccines remain attractive due to their stability, ease of production and high expression of antigen in transfected cells, but their limited immunogenicity remains problematic (Huang et al. 2010). However, advances in delivery, including electroporation, are likely to significantly impact on immunogenicity (Sardesai and Weiner 2011). In a completed phase I trial, a microencapsulated DNA vaccine (ZYC-101) consisting of multiple HLA-A2-restricted E7 epitopes was evaluated in women with high-grade CIN. Thirty-three percentage of the patients had a complete response (Sheets et al. 2003). There is also a phase I trial currently recruiting head and neck patients testing pNGVL4a-CRT/E7 (Detox) DNA vaccine in combination with cyclophosphamide (NCT01493154). This same vaccine is being used in combination with topical imiquimod in a phase I trial for CIN 3 patients (NCT00788164). Another study (NCT00988559) is evaluating the efficacy and safety of different routes of administration of the same DNA vaccine [pNGVL4a-CRT/E7(detox)] in patients with HPV16+ CIN2/3. Patients will be enrolled in one of six treatment groups including intradermal vaccination (with a needle-free delivery device, a gene gun), intramuscular and intralesional vaccination.

NCT02172911 is an open-label study to evaluate the safety, tolerability and immunogenicity of VGX-3100 (2 separate DNA plasmids encoding E6 and E7 proteins of HPV 16 and HPV 18) and INO-9012 (DNA plasmid encoding human interleukin 12) delivered by electroporation (EP) in patients with biopsy-proven HPV 16 or HPV 18 cervical SCC.

5.4 Bacterial Vector Vaccines

Bacterial vector vaccines have been investigated, with *Listeria monocytogenes* generating the most interest due to its ability to infect APCs in the cytosolic compartment, permitting both MHC class I and II presentation (Wood and Paterson 2014). ADXS11-001 (ADXS-HPV) is a live-attenuated *L. monocytogenes* (Lm)-LLO immunotherapy in trial for the treatment of head and neck cancer HPV-associated dysplasia and malignancy as a window study prior to surgical resection (NCT02002182). This American study is due to complete recruitment ($n = 30$) in January 2015. A second study evaluating the same vaccine in CIN has just been terminated due to lack of enrolment (NCT01116245). Unfortunately, the UK head and neck trial REALISTIC has recently been terminated due to patient infection with Listeria resulting in a serious adverse reaction and withdrawal of the study by the sponsor (NCT01698792). In addition, there are 2 studies looking at the dose range (NCT01356823), efficacy and immunogenicity (NCT01735006) of recombinant HPV 16/18 bivalent vaccine expressed in *Escherichia coli* in vaginal–intraepithelial, vulval–intraepithelial, cervical–intraepithelial neoplasia and cervical cancer. Both trials are reported as ongoing but not recruiting.

For completeness, tumour cell and dendritic cell vaccines have been explored for HPV-driven disease, but production problems (including purity) and administration have remained problematic and will not be discussed further.

6 Summary

It is clear that HPV has relied on its host for both replicative machinery and productive infection for many generations. In the majority of cases, the host's immune system can clear the infection with limited morbidity. In a significant minority, a HR infection remains, providing the initial platform for malignant transformation. We do not yet understand what predisposes individual patients to harbour rather than clear the infection, but it is intriguing to understand which part of the virus/host interaction influences this outcome. It is apparent that this group of malignancies will provide insight into both prophylactic and therapeutic vaccination strategies and hopefully provide understanding that can be transferred to other solid malignancies where the obvious treatment target is not as clear-cut. It will also be interesting to observe what 'help' the immune system requires in addition to the vaccination model: whether in the form of an adjunct or discrete immunostimulatory molecules (i.e. anti-CD40 monoclonal antibodies). It is clear that this is a rapidly emerging field and data generated will facilitate our understanding of the host–tumour interaction.

Conflicts of Interest None declared.

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Tapping the Potential of DNA Delivery with Electroporation for Cancer Immunotherapy

Kimberly A. Kraynyak, Angela Bodles-Brakhop and Mark Bagarazzi

Abstract Cancer is a worldwide leading cause of death, and current conventional therapies are limited. The search for alternative preventive or therapeutic solutions is critical if we are going to improve outcomes for patients. The potential for DNA vaccines in the treatment and prevention of cancer has gained great momentum since initial findings almost 2 decades ago that revealed that genetically engineered DNA can elicit an immune response. The combination of adjuvants and an effective delivery method such as electroporation is overcoming past setbacks for naked plasmid DNA (pDNA) as a potential preventive or therapeutic approach to cancer in large animals and humans. In this chapter, we aim to focus on the novel advances in recent years for DNA cancer vaccines, current preclinical data, and the importance of adjuvants and electroporation with emphasis on prostate, melanoma, and cervical cancer.

Contents

K.A. Kraynyak · A. Bodles-Brakhop · M. Bagarazzi (✉)
Inovio Pharmaceuticals, 660 W. Germantown Pike, Suite 110,
Plymouth Meeting, PA 19462, USA
e-mail: mbagarazzi@inovio.com

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1 Introduction

Cancer, a growing global problem, is a group of diseases described by the uncontrolled growth and spread of abnormal cells which can result in death. Cancer accounts for approximately one in eight deaths worldwide and is a growing problem for low- and middle-income countries that lack the funds and/or medical capability to prevent or treat the disease. Both external factors, such as tobacco, infectious organisms, chemicals, and radiation, and internal factors, such as inherited mutations, hormones, immune conditions, and mutations, that occur from metabolism can cause cancer. According to the American Cancer Society in 2014, about 585,720 Americans are expected to die of cancer, almost 1,600 people per day. Cancer is the second most common cause of death in the USA, exceeded only by heart disease, accounting for nearly 1 of every 4 deaths. However, it is estimated that nearly 14.5 million Americans with a history of cancer were alive on January 1, 2014 and by January 1, 2024, that number will increase to nearly 19 million (DeSantis et al. 2014) demonstrating the need for improved treatment options that are financially feasible.

The initial work of Wolff et al. showed that DNA plasmids injected intramuscularly could generate long-term gene expression in vivo and set the stage for the role of plasmid DNA (pDNA) in the scientific and medical fields (Wolff et al. 1990). DNA vaccines are plasmids that encode for parts of a pathogen (antigens) to induce a pathogen-specific immune response in the immunized host. This prophylactic vaccination serves to prevent infection altogether or lessen the disease burden caused by that pathogen. DNA immunotherapies consist of plasmid-encoding antigens to induce an antigen-specific immune response to help fight an existing condition. Enhancing the immune response is one strategy currently being investigated as a means to effectively recognize and kill transformed cells in order to overcome cancer. In this chapter, we aim to discuss the potential of DNA vaccines and delivery with electroporation (EP) in the prevention and treatment of cancer-related disease, concentrating on melanoma, prostate, telomerase reverse transcriptase (TERT), and human papillomavirus (HPV)-related cancers. First generation DNA vaccines have been notoriously ineffective at generating potent immune responses in large animals and humans. However, the efficacy of DNA vaccines was dramatically improved when combined with EP delivery. Accordingly, we will focus on EP delivery as well as molecular adjuvants to boost the immune response. Moreover, the utilization of effective pDNA vaccines delivered by EP could prevent further tumor recurrences, due to the establishment of persistent immune memory. The overwhelming impact of cancer necessitates the need for a novel solution to this serious and fatal multifaceted disease. The combination of naked pDNA with an adjuvant and the delivery method of EP has produced encouraging positive results (Flingai et al. 2013) and may someday provide possibility and hope for many suffering from cancer.

1.1 Current Cancer Treatments

The treatment for cancer is as diverse as the disease itself. Each type of cancer has its own set of treatments with an assorted arsenal that has been approved and can be utilized. There are approximately six broad treatment options including watchful waiting or active surveillance, surgery, radiation therapy, hormone therapy, chemotherapy, and biologic therapy. While chemotherapy remains the mainstay of current cancer treatment, it has a limited therapeutic benefit, is significantly toxic, and can lead to severe side effects and often resistance. Overall survival is not always dramatically improved. Novel therapeutic alternatives and techniques are therefore required.

Prostate cancer is the most common type of cancer in men, and it is estimated that there will be 233,000 new cases in 2014 with approximately 30,000 deaths in the USA alone. There are over 25 approved drugs for the treatment of prostate cancer according to the National Cancer Institute. The last several years have seen the arrival of novel therapeutic agents for prostate cancer (Abdulla and Kapoor 2011; Adamo et al. 2012). Numerous recently approved immunotherapeutics such as sipuleucel-T, androgen axis inhibitors including abiraterone acetate and enzalutamide, a chemotherapeutic agent, cabazitaxel, and a radiopharmaceutical, radium-223, have resulted in positive results with increased survival for patients with castration-resistant prostate cancer (Agarwal et al. 2014). However, there is controversy over the clinically relevant therapeutic improvement (Jacobs et al. 2014) leaving an opening for the development of novel therapeutics such as DNA-based vaccines.

Skin cancer is in the top five of common types of cancers diagnosed in the USA, with an estimated 76,000 new cases annually. For melanoma patients, there are roughly 20 drug treatments available that have been approved by the FDA; however, these therapies, overall, have been demonstrated to be only marginally successful as metastatic melanoma is resistant to native apoptotic and anti-growth signals, is highly metastatic and deploys several local immune evasion mechanisms. Since 2011, 4 new agents have been approved for the treatment of patients with metastatic melanoma (ipilimumab, vemurafenib, dabrafenib, and trametinib), and several new agents are currently in phase 3 trials with hopes of even more agents, including DNA-based vaccines, being approved for this fatal disease (Saranga-Perry et al. 2014).

Breast cancer is a heterogeneous complex of diseases, a spectrum of many subtypes with distinct biological features that lead to differences in response patterns to various treatment modalities and clinical outcomes, making it difficult to develop a successful therapeutic strategy (Yersal and Barutca 2014). According to the National Cancer Institute, 235,000 new breast cancer cases are projected in the USA in 2014 with approximately 40,000 deaths. Currently, there are 5 approved drugs for the prevention of breast cancer with over 60 drugs approved for the treatment with many of them used in combination. Again, the need for improved therapeutics is critical for the treatment of breast cancer, and DNA vaccines are potential targets for the development of such novel therapeutics.

Cervical cancer is almost always caused by HPV infection and accounts for approximately 12,000 new cases in 2014 and results in about 4,000 deaths. There are currently 4 treatments approved for the prevention of cervical cancer including Cervarix® (GlaxoSmithKline), a bivalent vaccine containing VLPs of types 16 and 18, and Gardasil® (Merck), a quadrivalent vaccine containing VLPs of types 6, 11, 16, and 18 (Aggarwal 2014). Potentially precancerous dysplastic tissue, also known as cervical intraepithelial neoplasia (CIN), is sometimes managed with a watch and wait approach and depending on the severity can result in surgical intervention. There is currently no non-surgical alternative treatment option. Cervical cancer is commonly treated with a combination of chemotherapy and radiation, including cisplatin, brachytherapy or internal radiation therapy, and external radiation therapy. A targeted therapy, such as bevacizumab, may also be utilized. This area of cancer is greatly lacking in noninvasive therapeutic strategies, and DNA vaccines may provide a much needed solution.

1.2 DNA Vaccines

pDNA is employed in DNA vaccines and gene therapies against many infectious, acquired, and genetic diseases, including HIV-AIDS (Muthumani et al. 2013; Kalams et al. 2013), Hepatitis C (Latimer et al. 2014; Lang et al. 2008), Ebola (Shedlock et al. 2013), Malaria (Ferraro et al. 2013), different types of cancer, enteric pathogens, and influenza (Yan et al. 2014b; Laddy et al. 2007, 2008, 2009). Compared to conventional vaccines, DNA vaccines have many advantages such as high stability, non-infectious, focusing the immune response to only those antigens desired for immunization, the ability to give repeat doses (boost the immune response), and long-term persistence of vaccine-induced protection. It has been shown that a multivalent vaccine can be employed to target multiple antigens or even multiple pathogens simultaneously (Hirao et al. 2011). Furthermore, the simplicity, low cost to produce, and rapid production of DNA vaccines give them an advantage over many conventional vaccines or treatments and should make the technology accessible to developing countries. DNA vaccines have been shown to elicit tumor-protective cytotoxic T lymphocyte (CTL) immunity but to date have not reached their full potential in larger animals or humans.

Cancer is a worldwide leading cause of death, and several malignancies are incurable or not as successfully treated by current therapies as previously discussed. Therefore, new anti-tumor therapies are necessary to improve the outcome for patients with cancer. The induction of cellular immunity directed against tumor-specific antigens is emerging as a potential pathway for novel and effective drug development. DNA vaccines may prove to be a reliable form of cancer immunotherapy based on safety profile, stability, and can be ease of production. Moreover, tumor-specific antigens are expressed for a longer period of time compared to RNA- or protein-based vaccines. The selection of antigens, vector, delivery route, dose, timing, adjuvants, and boosting agents all affect the outcome of vaccination,

in particular, the magnitude and quality of immunity elicited (Kutzler and Weiner 2008). Here, we will discuss the development of DNA vaccines and their potential application in treating cancer.

DNA vaccines are also being investigated as cancer treatments in companion animals. Testing anticancer medicine in companion animals such as dogs as an intermediary step of translational research program also provides clinical data for FDA/USDA approval as a veterinary anticancer drug or vaccine. The veterinary community is in need of novel medicine for the prevention and treatment of canine and feline cancers. More importantly, the benefit of testing anticancer vaccines in companion animals is that spontaneous tumors in dogs may provide invaluable information for human trials due to their heterogeneous nature. p62 (SQSTM1) is a key component of autophagic machinery which is also involved in signal transduction and is over-expressed in a variety of human tumors. It was noted that the administration of p62-encoding plasmid acted as a novel, effective broad-spectrum anti-tumor, and anti-metastatic vaccine (Venanzi et al. 2013). The potential effect of a p62 DNA vaccine on mammary tumors of dogs was also examined (Gabai et al. 2014). It was noted that the p62 DNA vaccine administered intramuscularly decreased or stabilized growth of locally advanced lesions and was associated with lymphocyte infiltration and tumor encapsulation via fibrotic reaction.

All these studies show the importance of tapping the potential of DNA vaccines for the development of novel therapeutics that will benefit cancer patients and concurrently provide much needed anticancer medicines for our four-legged friends. In order to produce the greatest benefit that DNA vaccines can deliver, it will be important to also evaluate effective adjuvants that may boost the immune response as well as utilize EP as a delivery method.

1.3 Adjuvants

Adjuvants are becoming critical components of most clinical vaccines and are used to enhance adaptive immune responses to antigen effecting both the quantity and quality of the immune response. Numerous studies are examining the potential of such adjuvants to increase the efficiency of DNA vaccines.

The potency of DNA-based vaccines co-administered with molecular cytokine adjuvants as part of a vaccine cocktail has been demonstrated to boost the adaptive immune response (Villarreal et al. 2013). Currently, there are numerous cytokine adjuvants that are showing potential as DNA vaccine assistants for cancer therapies. Cytokine adjuvants are of particular interest as they have the ability to induce $CD4^+$ and $CD8^+$ T cell responses that are critical for an effective therapeutic immune response. Cytokines such as the interleukin family members IL-12, IL-15, and IL-33 are all under investigation as potential adjuvants for use with DNA vaccines.

Previously, IL-12 DNA was shown to act as a molecular adjuvant when optimized for high level of expression and delivered using in vivo EP. Co-administration with IL-12 DNA resulted in the detection of systemic IL-12 cytokine in the

plasma and was paralleled by a rapid increase of IFN-γ (Jalah et al. 2012). Hirao et al. (2008) have shown in non-human primates that the inclusion of IL-12 into a DNA vaccine delivered with EP resulted in the induction of higher, more rapid responses with increases in IL-2, TNF-α, and IFN-γ compared to the group not receiving IL-12 demonstrating that the combination elicits the most favorable outcome. Furthermore, it was found that when IL-12 was combined with a SIV-mac239 DNA vaccine, significantly higher SIV-specific cellular immune responses developed and persisted up to 6 months after the last vaccination (Jalah et al. 2012). A single therapeutic injection of an optimized murine IL-12 DNA plasmid showed significantly more potent control of tumor development in the B16 melanoma cancer model in mice (Jalah et al. 2013) indicating that optimization of the adjuvant can contribute to the success of therapeutic vaccines. Recently, IL-12 DNA was shown to augment the T cell immunity induced by a DNA vaccine in humans (Kalams et al. 2013).

In another example of the beneficial addition of cytokine adjuvants, the therapeutic response of an HPV DNA vaccine was improved with the inclusion of IL-33 (Villarreal et al. 2014). It was noted that the IL-33 immunoadjuvant induced potent anti-tumor immunity and resulted in regression of established tumors in mice. IL-28B was also examined by Morrow et al. for its potential to booster the immune response as an adjuvant in a non-human primate study for DNA vaccination (Morrow et al. 2010). The IL-28B adjuvant continued to boost the immune response three months after the final immunization.

Other adjuvants such as the novel chemokine adjuvant CCL27/CTACK have been examined for the ability to enhance immune responses to an HIV-1 or SIV antigen in mice and rhesus macaques (Kraynyak et al. 2010). Significant IFN-γ secretion and $CD8^+$ T cell proliferation was observed. Furthermore, plasmids encoding the mucosal chemokines CCL27 and CCL28 have been shown to be effective adjuvants in eliciting antigen-specific immunity in vivo and protected from morbidity and mortality associated with a lethal intranasal influenza challenge (Kutzler et al. 2010). The inclusion of molecular adjuvants into a DNA vaccine or immunotherapy can help drive an immune response tailored to the particular pathogen being targeted.

Other adjuvants have been also examined for their potential to aid the immune response of DNA vaccines in cancer. The Toll-like receptors (TLR) which play a crucial role in innate immune responses to infection are under consideration. TLR3 and TLR9 have been shown to be capable of eliciting strong T cell responses and have been shown to help control the growth of established B16 melanoma tumors after immunization (Zaks et al. 2006). A DNA vaccine incorporating TLR adaptor molecule such as the Toll-interleukin-1 receptor domain-containing adaptor-inducing beta interferon resulted in enhanced cellular immune responses and significantly reduced tumor growth compared to DNA vaccine alone (Takeshita et al. 2006). More recently, it has been shown that TLR3 and TLR7 agonists can be used to enhance the immune response to DNA vaccine encoding HPV-16 E7 (Sajadian et al. 2014).

1.4 Electroporation

To date, numerous delivery methods have been investigated to boost efficacy of non-viral gene therapies such as lipid-mediated entry into cells, jet injection, gene gun delivery, and sonoporation. In this review, we will focus on EP whereby a DNA-based vaccine is delivered intradermally or intramuscularly followed by electric pulses (millisecond electrical fields), resulting in temporary pores in the cellular membrane facilitating significant cellular uptake of the vaccine. Although the exact mechanism of cellular macromolecule entry is still under discussion, entry of small molecules such as anticancer therapeutics seems to occur by simple diffusion after the pulse, and larger molecules such as pDNA are thought to enter through a multistep mechanism involving the interaction of the DNA molecule with the destabilized membrane during the pulse and then its passage across the membrane. The cell membrane then reseals and the cellular machinery uses the DNA code to produce the desired antigen. Antigen-presenting cells then engulf the produced antigen and transport them to the lymph nodes where the antibodies or T cells are produced to eliminate the cancerous cells. The delivery method of EP for DNA-based vaccines in the treatment of cancer and its complications has been previously reviewed (Bodles-Brakhop and Draghia-Akli 2008).

Numerous preclinical studies have examined the effectiveness of EP as a delivery method for DNA vaccines. EP appears to have a great impact on immunogenicity and efficacy by increasing antigen delivery up to a 1000-fold over naked DNA delivery alone (reviewed by (Sardesai and Weiner 2011)). Furthermore, there are currently several ongoing clinical studies examining the administration of DNA vaccines with EP (Khan et al. 2014). DNA vaccines administered with EP in a comparison study with an adenovirus-based vaccine revealed significant differences with the DNA vaccine plus EP being able to booster the immune response with each subsequent vaccination, whereas the adenovirus vaccination failed to boost the immune response after the first immunization (Hirao et al. 2010).

The skin, compared to muscle, is an attractive tissue for DNA vaccination in a clinical setting due to the accessibility of the target, the ease of monitoring, tolerability, and most importantly the immunocompetent nature of the dermis (Broderick et al. 2014). Furthermore, it has been shown that EP of the skin with the use of a topical anesthetic cream prior to vaccination does not affect the number or function of the antigen-specific T cells induced (Roos et al. 2009). Recently, a novel dual depth device that has the ability to deliver both intradermal and intramuscular DNA vaccines simultaneously has been proven to be superior to delivery to either tissue alone for induction of antigen-specific antibody and cellular immunity (Lin et al. 2011). The strategy of intratumoral EP of DNA vaccines has also been explored with successful results. Intratumoral EP with tetanus toxin fragment C and IL-12 cDNA induced an IFN-γ-producing T cell response to tumor-associated antigen, heavy inflammatory infiltration, regression of established tumors, and immune memory to protect mice from repeated tumor challenge (Radkevich-Brown et al. 2010).

The effectiveness of the delivery method of EP has been shown to improve anti-tumor immune responses when used in a prime/boost strategy (Buchan et al. 2005). Furthermore, in a head to head comparison of EP with gene gun and conventional intramuscular injection in the ability to generate antigen-specific cytotoxic CD8+ T cell responses as well as anti-tumor immune responses against an HPV-16 E7 expressing tumor cell line using the pNGVL4a-CRT/E7(detox) DNA vaccine, EP was the most effective delivery method (Best et al. 2009). In addition, different procedures of DNA vaccine delivery, namely intradermal injection, gene gun delivery, and intramuscular injection alone or with EP, were compared in a murine transgenic model of mammary carcinoma overexpressing HER2/neu. In this study, intramuscular delivery followed by EP elicited better protection against HER2/neu spontaneous tumor development and induced an immune response (Smorlesi et al. 2006).

Overall, EP has been shown to be an efficient method for enhancing the uptake and expression of DNA vaccine candidates, enabling the use of a lower dose of vaccine with similar or higher efficacy. Optimization of EP will also increase its acceptability as a delivery method by potentially reducing the associated pain with the procedure. The current of intradermal vaccination by EP impacts antigen expression, inflammation, and the induction of both humoral and cellular immunity. It was observed that a lower (0.1 A) current reduced inflammation and improved antigen expression compared with a 0.2 A current and highlights the need for optimization of EP conditions in vivo (Hutnick et al. 2012). Minimally invasive low-voltage EP delivery has been accomplished with no evidence of injection site inflammation or local tissue damage (Broderick et al. 2012; Lin et al. 2012). In general, the delivery of DNA vaccines via either intramuscular, intratumoral, or intradermal EP has been proven to enhance antigen-specific antibody and cellular immunity compared to delivery to tissue alone (Lin et al. 2011).

2 Potential of DNA Vaccines in Cancer—Preclinical Studies

2.1 Melanoma

The delivery of pDNA by in vivo EP has been widely tested in preclinical melanoma models. Intratumoral delivery has been shown to generate a direct anti-tumor effect, whereas delivery to other sites can result in additional therapeutic effects such as anti-angiogenesis, reviewed by Heller and Heller (2010).

It has previously been shown that a combination of therapies can provide significant results. In a mouse model, in vivo EP of plasmids encoding granulocyte–monocyte colony-stimulating factor (GM-CSF) and interleukin-2 (IL2) into B16 mouse melanomas combined with electrochemotherapy resulted in the induction of long-term immunity to recurrence and resistance to challenge (Heller et al. 2000). Furthermore, it has been shown that optimization of a plasmid vaccine delivered by

EP can dramatically improve outcome compared to EP with plasmids encoding the full-length autologous melanocyte antigen tyrosinase-related protein-2 in mice when challenged with B16F10M melanoma cells (Kalat et al. 2002).

The combination of cancer vaccination with angiogenesis inhibition is appealing, due to favorable safety profile of both approaches, as well as possible biological synergies. The administration of pDNA vaccination (encoding gp100, TRP2, and Ii-PADRE) and anti-angiogenesis (pDNAs encoding angiostatin and/or endostatin) in B16F10 murine model was investigated. In an optimized administration protocol, the melanoma vaccination together with intratumoral delivery of pDNAs encoding angiostatin and endostatin resulted in 57 % tumor-free survival over 90 days after challenge (Chan et al. 2009). Furthermore, the intradermal EP with a survivin DNA vaccine has been shown to suppress angiogenesis in vivo and elicit protection against highly aggressive syngeneic B16 melanoma tumor challenge (Lladser et al. 2010).

The HIV-1 accessory protein Vpr (viral protein R) has previously been demonstrated to induce G2 cell cycle arrest as well as in vitro growth inhibition/killing of a number of tumor cells by apoptosis. When established, subcutaneous B16.F10 melanoma tumors were injected intratumorally with plasmid Vpr followed by EP complete tumor regression with long-term survival was noted (McCray et al. 2006). In a follow-up study, refinement of the pVPr plus EP regimen resulted in a higher percentage of complete tumor regression with long-term survival (McCray et al. 2007). It has also been shown that the HIV accessory protein Vpr has anti-proliferation/anticancer properties (Muthumani et al. 2009) and when administered as a plasmid by in vivo EP can result in regression of established B16.F10 melanoma tumors in mice (Muthumani et al. 2010).

The p42.3 gene is involved in cell cycle regulation, tumorigenesis and has significantly high expression levels in cancer cells including melanoma (Mao et al. 2014). When tested as a DNA vaccine in the B16F10 melanoma mouse model by EP, prophylactic and therapeutic effects were observed such as tumor growth inhibition and decrease in tumor weight (Liu et al. 2013).

The ability to treat melanoma with a DNA-based vaccine is gaining ground although there are still obstacles to be overcome. The utilization of canines which offer many similarities to human counterparts has led to a study where the therapeutic efficacy of a human chondroitin sulfate proteoglycan-4 (hCSPG4) DNA-based vaccine has been investigated. Homology between the human and canine forms is 80 % warranting its use. Intramuscular administration followed by EP resulted in significantly longer overall and disease-free survival times in 14 vaccinated dogs as compared with 13 non-vaccinated controls with the development of antibodies against both hCSPG4 and cCSPG4 (Riccardo et al. 2014). These results demonstrate the important potential advances for the veterinary field as well as providing an opportunity for translation into the human clinical setting.

Most recently, the role and significance of myeloid-derived suppressor cells (MDSCs) in the maintenance of the tumor microenvironment has been examined as they have the ability to suppress immune responses and, as such, facilitate tumor

growth. A novel synthetic tyrosinase (Tyr) DNA vaccine therapy delivered by EP in both prophylactic and therapeutic models was investigated in conjunction with the role of MDSCs in immune suppression of T cells in an antigen-specific B16 melanoma murine system. A robust and broad immune response was induced as well as a reduction in the number of MDSCs in the tumor microenvironment. Overall, DNA vaccination by EP resulted in a significantly reduced melanoma tumor burden and increased survival in vivo (Yan et al. 2014a).

2.2 Prostate Cancer

The identification and measurement of prostate-specific antigen (PSA) has led to earlier diagnosis of prostate cancer. The knowledge that PSA expression is limited to prostate cells and has been shown to be found at higher expression levels in prostate-specific cancer cells makes it a good candidate for the development of immunotherapies and DNA-based vaccines (Kim et al. 1998). The safety and immunogenicity of a PSA DNA vaccine was evaluated in rhesus macaques. Overall, it was found to result in induction of PSA-specific humoral responses with no adverse effects (Kim et al. 2001b) establishing it as a potential cancer vaccine target. Furthermore, it has been shown that the co-immunization of PSA vaccine with cytokine adjuvants can result in significant enhancement of the PSA-specific antibody responses (Kim et al. 2001a). The intradermal EP of the prostate cancer vaccine encoding PSA has been shown to result in significantly higher gene expression levels and increased levels of PSA-specific T cells compared to DNA vaccine alone, without EP (Roos et al. 2006). Furthermore, the optimization of the intradermal EP delivery of a PSA DNA vaccine has been shown to enhance efficiency (Roos et al. 2008). The intramuscular EP of plasmid PSA has also been shown to be an effective strategy for inducing effective anti-tumor immune responses, tumor reduction, and increased survival (Ahmad et al. 2010).

Prostate stem cell antigen (PSCA) is a cell surface antigen expressed in normal human prostate and it is also over-expressed in prostate cancer correlating with increased tumor stage and increased bone metastases (Reiter et al. 1998). Intramuscular EP of a PSCA plasmid vaccine results in effective anti-tumor immune responses, inhibition of tumor growth, and increased survival while also inhibiting metastases (Ahmad et al. 2009).

Other novel DNA vaccines delivered by EP have also been evaluated. A DNA vaccine encoding the prostate-specific antigen prostatic acid phosphatase (PAP) delivered with EP in mice resulted in strong PAP-specific cellular immune responses and inhibited tumor growth in the transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse model that closely resembles human prostate cancer (Spies et al. 2012). A dual antigen approach has also been investigated as a potential therapeutic strategy toward prostate cancer with the premise that a more comprehensive collection of antigens could improve the effectiveness of the vaccine. Highly optimized

DNA vaccines encoding both PSA and PSMA were evaluated in a mouse model inducing robust antigen-specific immune responses (Ferraro et al. 2011) and warranting further investigation of this novel dual approach.

2.3 Human Papillomavirus Infections and Associated Cancer

DNA vaccination has become an effective strategy for the development of therapeutics against many types of cancer, including cervical carcinoma. Human papillomavirus (HPV) is the leading cause of a broad spectrum of disease, including genital warts, precancerous lesions, cervical, and anal cancer, and is a worldwide public health problem of epidemic proportions. Persistent infection with HPV is the main etiological factor in cervical cancer, the second most common cancer in women worldwide. HPV, particularly HPV type 16, is the primary causative agent of cervical cancer; thus, HPV-associated cervical malignancies might be prevented or treated by the induction of the appropriate specific immune responses in patients (Han and Sin 2013). Moreover, HPV16 accounts for more than 90 % of HPV-related head and neck squamous cell carcinomas (Marur et al. 2010). HPV18 is the most prevalent high-risk HPV after type 16. Therefore, HPV18 antigen development, with the goal of increasing anti-HPV18 cellular immunity, has been shown to result in a strong cellular immune response against HPV18 E6 and E7 antigens in a murine model. Moreover, when applied to rhesus monkeys, this construct is also able to elicit cellular immunity, providing evidence as a candidate for further study in the eventual context of immunotherapy for HPV-associated cancers (Yan et al. 2008).

Viral E6 and E7 oncoproteins are suitable targets for therapeutic vaccination (Gan et al. 2014), and it has been shown that HPV E6 and E7 are promising tumor antigens as they are regulatory proteins that are constitutively expressed in HPV-associated cancer cells (Morrow et al. 2013) and, therefore, were investigated as potential HPV DNA vaccine candidates (Seo et al. 2009). In one approach, E6 and E7 antigens were engineered to generate an optimal HPV DNA vaccine by codon optimization, fusion of E6 and E7, the addition of a tissue plasminogen activator signal sequence, and the addition of CD40 ligand (CD40L) or Fms-like tyrosine kinase-3 ligand (Flt3L). The results indicated that the inclusion of a tpa signal sequence, Flt3L, fusion of E6 and E7, and codon optimization induced strong E6- and E7-specific $CD8^+$ T cell responses that were further enhanced by intramuscular EP leading to complete tumor regression (Seo et al. 2009). The addition of adjuvants to a HPV-16 E7-based DNA vaccine was investigated as a means to improve immunogenicity (Ohlschlager et al. 2009). DNA encoded cytokines (IL-2, IL-12, GM-CSF, and IFN-γ) and the chemokine MIP1-alpha were co-applied either simultaneously or at different time points pre- or post-E7SH vaccination. The timing of adjuvant administration was found to be critical for success in mice, with MIP-1α pretreatment (day 5) in combination with sequence-optimized IFN-γ (day 3) resulting in the more significant tumor regression. In a further study, it was shown that the immunogenicity of the HPV-16 E7SH DNA vaccine was greatly enhanced

by the introduction of a highly optimized CpG cassette into the plasmid backbone, as well as by improving DNA delivery using EP technology (Ohlschlager et al. 2011).

Although most effects have been directed against the high-risk subtypes 16 and 18 of HPV, investigation into the so-called lower-risk subtypes 6 and 11 that are also associated with otolaryngologic malignancies, carcinoma of the lung, tonsil, larynx, and low-grade cervical lesions is needed. Optimized DNA vaccines that encode HPV 6 and 11 consensus E6/E7 fusion proteins (p6E6E7 and p11E6E7) were delivered to mice using in vivo EP. p6E6E7 and p11E6E7 proved to be highly immunogenic vaccines that mounted very robust HPV 6 or HPV 11 E6- and E7-specific T cell immune responses (Shin et al. 2012).

2.4 Other Potential Cancer Targets

To date, there is no effective vaccine available for breast cancer patients. Although major advancements have been achieved especially with the advent of HER-2 transgenic mice (Lollini et al. 2013), there is still a lot of work to be done. However, it has been shown that intramuscular EP with DNA plasmids coding for the extracellular and transmembrane domains of the protein product of the Her-2/neu oncogene can result in tumor clearance (Quaglino et al. 2004). Furthermore, it has been shown that a single administration with EP was enough to markedly delay carcinogenesis progression in mice with multiple microscopic invasive carcinomas and keep about 50 % of mice tumor free at one year of age (Curcio et al. 2008).

Moreover, the first administration of a Her2-pDNA vaccine in humans has been reported (Norell et al. 2010). The vaccine, encoding a full-length signaling-deficient version of the oncogene Her2, was administered together with low doses of GM-CSF and IL-2 to patients with metastatic Her2-expressing breast carcinoma who were also treated with trastuzumab in a pilot clinical trial. This pilot clinical trial demonstrates that Her2-pDNA vaccination in conjunction with GM-CSF and IL-2 administration is safe, well-tolerated and can induce long-lasting cellular and humoral immune responses against Her2 in patients with advanced breast cancer.

High levels of human telomerase reverse transcriptase (hTERT) are detected in more than 85 % of solid tumors, while normal cells showed undetectable levels of telomerase expression making it an attractive immunotherapeutic target as a universal cancer vaccine. A DNA-based hTERT vaccine delivered by EP was examined in mice and non-human primates. Robust and broad immune responses were generated in vaccinated mice compared to the control group, and in an HPV16-associated tumor model, vaccination of phTERT with EP slowed tumor growth and improved survival rate in both prophylactic and therapeutic studies. The success of these results suggests that phTERT may have a role as a broad therapeutic cancer vaccine candidate (Yan et al. 2013). Similar immune responses were achieved in non-human primates (Yan et al. 2013). More recently, it has been shown that an intradermal administration of a hTERT vaccine with EP induces an intense specific $CD8^+$ T cell response (Calvet et al. 2014) and has warranted further investigation in a phase 1 clinical trial.

2.5 Clinical Trials

According to the Journal of Gene Medicine, there have been over 2000 clinical trials worldwide investigating gene therapy with 379 trials investigating pDNA (http://www.wiley.com//legacy/wileychi/genmed/clinical/). This is a staggering increase over the last five years (Bodles-Brakhop et al. 2009). The majority of the gene therapy clinical trials address cancers (64.1 %, n = 1,331). Of these, there are 216 studies that are examining the potential of naked pDNA in the treatment of various cancers. Cancer DNA vaccines administered by EP are shown in Fig. 1.

A vaccine that encodes a domain of fragment C of tetanus toxin to induce $CD4^+$ T cell help, fused to a tumor-derived epitope from prostate-specific membrane antigen (PSMA) for use in HLA-A2(+) patients with recurrent prostate cancer was investigated for safety and tolerability. The immune response in an open label phase I/II two arm dose escalation trial with and without intramuscular EP was also investigated (Low et al. 2009). The results indicated that delivery of the vaccine with EP yielded the highest humoral response that persisted up to 18 months. Reports of an encouraging phase 1 safety, tolerability, and immunogenicity trial for

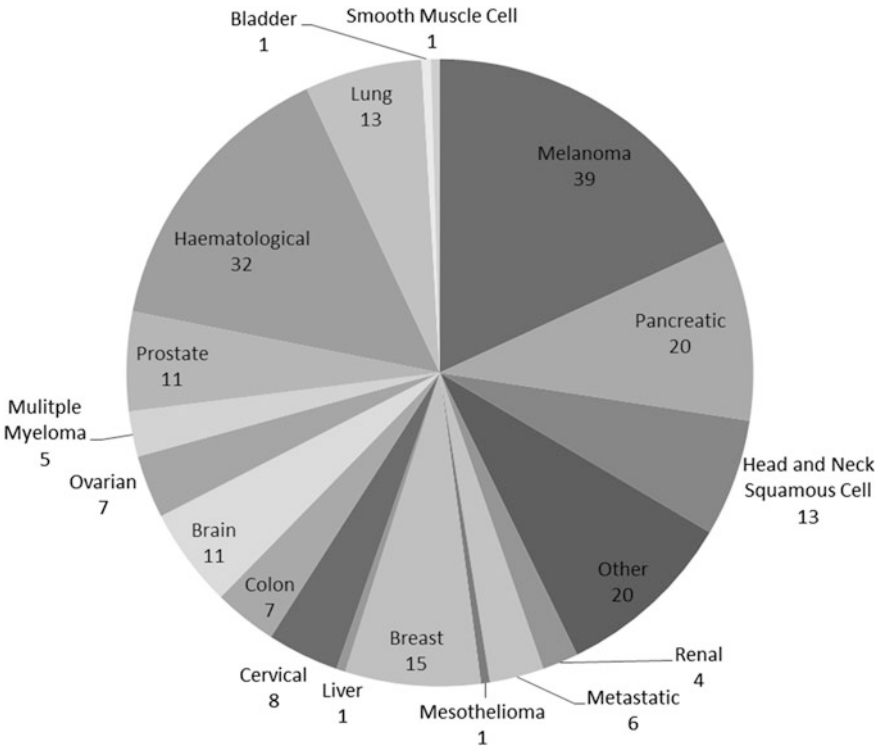

Fig. 1 Clinical trials of naked plasmid DNA for the treatment of cancer by type. The number of clinical trials using naked plasmid DNA for individual cancers is indicated

Table 1 Clinical trials involving DNA vaccines administered with EP for the treatment of cancer

NCT ID	Phase	Status	Sponsor	Condition	Intervention	Objective
NCT00859729	1 & 2	Completed	Uppsala University	Prostate cancer	pVAXrcPSAv53, DERMA VAX™ ID DNA delivery system	Feasibility and safety, immune response, anti-tumor effect
NCT01440816	2	Recruiting	OncoSec Medical Incorporated	Merkel cell carcinoma	IL-12 gene, IT EP-mediated plasmid DNA vaccine therapy	Twofold increase in IL-12 successful outcome, safety, survival, immunological effects
NCT01064375	1 & 2	Unknown	Maria Liljefors	Colorectal cancer	tetwtCEA DNA, DERMA VAX™ (EP device)	Safety and immunogenicity
NCT00685412	1	Completed	Inovio Pharmaceuticals	Human papilloma virus	VGX-3100, DNA plasmid delivered via IM injection + EP using CELLECTRA® device	Safety and tolerability, humoral and cellular immune responses
NCT01634503	1	Completed	Genexine, Inc.	Cervical cancer	GX-188E administered by EP	Safety and immunogenicity
NCT01304524	2	Ongoing	Inovio Pharmaceuticals	Cervical intraepithelial neoplasia 2/3 or 3	VGX-3100, DNA plasmid delivered via IM injection + EP using CELLECTRA® device	Histopathological regression, Virologically-proven clearance
NCT02139267	2	Not yet open	Genexine, Inc.	Cervical cancer	GX-188E, administered IM by EP	Histopathological regression of cervical lesions
NCT00471133	1	Completed	Ichor Medical Systems Incorporated	Melanoma	Xenogeneic tyrosinase DNA vaccine, TriGrid™ delivery system for IM by EP	Safety and feasibility, immunological and anti-tumor response
NCT01334060	2	Suspended	University Hospital Southampton NHS Foundation Trust	Haematological malignancies	p.DOM-WT1-37 DNA vaccine and p. DOM-WT1-126 DNA vaccine, IM by EP	Molecular response, time to disease progression, overall survival

(continued)

Table 1 (continued)

NCT ID	Phase	Status	Sponsor	Condition	Intervention	Objective
NCT02172911	**1 & 2**	**Recruiting**	**Inovio Pharmaceuticals**	**Cervical cancer**	**VGX-3100 and INO-9012 delivered via IM EP with CELLECTRA®-5P**	**Safety and tolerability, immunogenicity**
NCT01493154	1	Terminated	Sidney Kimmel Comprehensive Cancer Center	Head and neck cancer	pNGVL4a-CRT/E7(Detox) DNA vaccine using the IM TriGrid™ delivery system	Safety and immunogenicity
NCT02100085	1	Ongoing	Genexine, Inc.	Cervical intraepithelial neoplasia 3	GX-188E administered by EP, follow up study from NCT01634503	Immune response, lesion and infection status, compared to that of the final visit in phase I study
NCT01188850	1	Completed	Inovio Pharmaceuticals	Human papilloma virus	VGX-3100, delivered via IM injection + EP using CELLECTRA® device	Safety and tolerability, humoral and cellular immune responses
NCT01138410	1 & 2	Recruiting	Scancell Ltd	Malignant melanoma	SCIB1 administered by IM injection using the TDS-IM EP device (Ichor Medical Systems, Inc.)	Safety, tolerability, biological, and clinical effects
NCT01579318	2	Recruiting	OncoSec Medical Incorporated	Cutaneous T cell lymphomas	IT injection of pIL-12 with EP	Response rate, safety, and immunogenicity
NCT02204098	1	Not yet open	Washington University School of Medicine	Breast cancer	Mammaglobin-A DNA vaccine given IM using an integrated EP administration system	Safety and immunogenicity
NCT01502293	2	Recruiting	OncoSec Medical Incorporated	Melanoma	Plasmid interleukin-12, IT with EP	Safety, response rate, overall survival
NCT01664273	1	Terminated	Copenhagen University Hospital at Herlev	Metastatic malignant neoplasm	Plasmid AMEP injected IM and immediately followed by application of electric pulses via a needle electrode inserted into the muscle	Safety, efficacy, pharmokinetics

(continued)

Table 1 (continued)

NCT ID	Phase	Status	Sponsor	Condition	Intervention	Objective
NCT02241369	**1**	**Recruiting**	**Inovio Pharmaceuticals**	**Aerodigestive malignancies (e.g., squamous cell carcinoma)**	**INO-3106 alone or in combination with INO-9012 (DNA plasmid encoding human IL-12) delivered by EP**	**Safety, tolerability, and immunogenicity**
NCT02163057	**1 & 2**	**Recruiting**	**Inovio Pharmaceuticals**	**Head and neck squamous cell cancer**	**VGX-3100 and INO-9012 delivered via IM EP**	**Safety, tolerability, and immunogenicity, anti-tumor effects**
NCT01764009	1 & 2	Ongoing	BioAlliance Pharma SA	Melanoma	Naked DNA coding for protein AMEP delivered by IM EP	Safety and tolerability
NCT01045915	1	Terminated	BioAlliance Pharma SA	Melanoma	Naked DNA coding for protein AMEP delivered by IM EP	Determination of dose-limiting toxicity
NCT00685412	1	Completed	Inovio Pharmaceuticals	Cervical cancer	VGX-3100, DNA plasmid delivered via IM injection + EP using CELLECTRA® device	Safety and tolerability, humoral and cellular immune responses
NCT02301754	1	Recruiting	Invectys	Solid tumors	INVAC-1, a DNA vaccine encoding human telomerase reverse transcriptase (hTERT), delivered ID with EP	Safety, immunogenicity and pharmacodynamics

There are a total of 22 clinical trials worldwide, with 9 of these studies currently open of which 7 are occurring in North America (USA), 1 in Europe, and 1 in East Asia. Three studies, highlighted in bold, are examining the safety, tolerability, and immunogenicity of a DNA vaccine administered with a plasmid adjuvant by EP (search conducted November 2014)

EP electroporation; *ID* intradermal; *IM* intramuscular; *IT* intratumoral

a therapeutic HPV16/18 candidate vaccine, VGX-3100, delivered by in vivo EP indicate it is capable of driving a robust immune response to antigens from high-risk HPV serotypes and could contribute to elimination of HPV-infected cells and subsequent regression of the dysplastic process (Bagarazzi et al. 2012).

3 Summary

Overall, the preclinical and clinical studies discussed demonstrate that DNA vaccines administered with an adjuvant by EP could provide a potential alternative to standard therapies. To date, four DNA plasmid products are currently licensed for veterinary applications. This includes two infectious disease vaccines, one for West Nile virus in horses (Ft Dodge Animal Health) and one for infectious haematopoietic necrosis virus in salmon (Novartis). A melanoma cancer vaccine for dogs (Manufacturer: Merial) and a growth hormone releasing factor therapy for pigs (Manufacturer: VGX Animal Health) are also approved. The canine melanoma vaccine (Trade name: Oncept) consists of highly purified pDNA capable of expressing the human tyrosinase protein and is a therapeutic vaccine administered to dogs with stage II or stage III oral melanoma to aid in extending overall survival (Peruzzi et al. 2010). The vaccine was evaluated by the Canadian Centre for Veterinary Biologics of the Canadian Food Inspection Agency for licensing in Canada. The vaccine is given by transdermal administration, targeting intramuscular deposition using the VET JET needle-free transdermal vaccination system (a high-pressure device placed on the skin which deposits injectate transdermally into the muscular tissue) with four doses of vaccine given at two-week intervals, with a booster dose administered every six months for the life of the animal. The results of a double-arm clinical trial revealed immune responses, no adverse effects, and an extended overall survival time compared to historical controls that were treated with a chemotherapy regimen (Gavazza et al. 2013). These results in pet dogs represent exceptional translational models for advancement of cancer research because they reflect the complex heterogeneity also observed in human cancer. Currently, there are three clinical trials that are examining the potential of DNA vaccines administered by EP with an adjuvant as highlighted in Table 1 in bold. These studies and others are paving the way for future clinical studies and will play an important role in advancing DNA vaccines as a significant therapeutic option.

Most recently, it has been shown that a combination therapy of old and new treatment concepts may prove effective and should be considered when the health and well-being of a patient is under consideration. For example, a combination therapy consisting of intratumoral IL-12 gene therapy, human tyrosinase (hTyr) DNA vaccination, and metronomic cyclophosphamide (CPX) was evaluated in a B16-F10 mouse model revealing that each component of this combination treatment contributed a unique immunologic trait with associated clinical benefits

(Denies et al. 2014). As we progress toward developing DNA vaccines for cancer, the inclusion of adjuvants and delivery by EP should be investigated fully in combination approaches with and without existing therapies in an effort to make every effort to provide the best outcome for patients.

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Targeted Immunotherapy Designed to Treat MUC1-Expressing Solid Tumour

Bruce Acres, Gisele Lacoste and Jean-Marc Limacher

Abstract Several approaches to antigen-specific immunotherapy of cancer antigen-specific immunotherapy of cancer have been tested clinically. In this chapter, we will describe studies done with the antigen MUC1. Tested MUC1 therapeutic vaccines include the following: monoclonal antibodies (MAbs) specific for MUC1; synthetic and recombinant polypeptides from the protein sequence of MUC1; dendritic cells carrying MUC1; RNA and DNA vaccinations; and recombinant viruses carrying the MUC1 DNA sequence. Chemotherapy of cancer aims to be toxic to the cancer cells with manageable side effects to the patient. In contrast, antigen-specific immunotherapy of cancer aims to treat the patient, such that the patient is then able to control and eventually eliminate their cancer cells. It is therefore important to know the immune status of each cancer patient prior to therapy.

Contents

G. Lacoste (✉) · J.-M. Limacher
Department of Medical Affairs, Transgene SA, 400 Blvd Gonthier d'Andernach, Parc d'Innovation CS80166, 67405 Illkirch-Graffenstaden Cedex, France
e-mail: lacoste@transgene.fr

B. Acres
Bruce Acres Consulting, Strasbourg, France

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1 Introduction

1.1 Historical Background

The ferocity of a full-blown immune reaction to some pathogens and, most dramatically, in organ graft rejection has tempted immunologists for generations, to hope that the destructive power of this force could be focused onto cancer cells. The realization of that dream had a slow start, due to the sequential revelations of the many complexities of the immune system. Now, armed with much better knowledge of the vast array of accelerators and brakes used by the immune system, some progress is being made in the treatment of cancer with immunotherapy (Weber 2014).

The application of immune stimulation to cancer treatment was first attempted over a century ago. Most often with sarcomas, some early success was observed when William Coley injected patients with a concoction of bacterial extracts, Coley's toxins (Coley 1893, 1906). The reaction to the toxins and their eventual success in the elimination of some tumours has been attributed to a strong cytokine reaction to bacterial products.

A more antigen-specific approach was also tried in the early 1900s. Patients were injected with their own tumour cells or extracts, or tumour cells of the same type from other patients (Coca and Lebredo 1912; Coca 1909). Again, some successes were noted, but growth of new tumours was also observed.

During the following decades, the miracle of penicillin led many to believe that any disease could be treated with small chemical molecules. This was in addition to the magic of radiotherapy and advances in surgery. Therefore, these new treatments soon overshadowed any attempts to coax the immune system to eliminate cancer cells.

Fifty years later, interest in immunology was reawakened by the seminal work of Medawar (Billingham et al. 1953) and Burnet (1960), who showed some of the complexities of the immune response in the demonstration that the immune system could be rendered tolerant to some antigens and that the specific immune response was the result of cellular clonal expansion. Therefore, evidence was starting to accumulate to show that the immune response could be manipulated. Over the same period of time, the 1950s, it was demonstrated that the rodent immune system had ability to react and destroy autologous tumour cells (Foley 1953; Prehn and Main 1957; Baldwin 1955). Inbred stains of mice were available, along with continuous tumour cell lines from various mouse strains. The antigens responsible for this recognition were initially called TSTA for tumour-specific transplantation antigens, but are now called tumour-associated antigens or TAAs. Work soon followed to identify some of these antigens from rodent (Harris et al. 1973; De Plaen et al. 1988) and human (Eichmuller et al. 2001) tumour cells.

During the next decade, immunology began to elucidate various immune control mechanisms such as IgG feedback, high-dose and low-dose tolerance, and T suppressor (and contra-suppressor) cells.

In 1975, a major leap forward occurred in the laboratory of Kohler and Milstein: the discovery that antibody-producing murine plasma cells could be fused to murine plasmacytoma cells to create a 'hybridoma'. These hybrid cells could be cloned, grown to large quantities (Kohler and Milstein 1975) and produce almost limitless quantities of antibodies with a single specificity. This was also a turning point with the discovery and purification of many antigenic molecules including TAA. One such antigen, MUC1, was initially thought to be a collection of distinct molecules associated with several different tumour types. Several tumour-specific antibodies were derived in several different laboratories, using different tumour cells as the immunizing antigen. Each laboratory had a different name for their antibody and a different name for their TAA (Keydar et al. 1989; Sekine et al. 1985; Kim et al. 1988; Burchell et al. 1984; Stacker et al. 1989; Lan et al. 1987).

1.2 MUC1

Once the genes for the corresponding proteins were cloned, several of these were shown to be the same MUC1 tumour-associated antigen. This TAA was subsequently shown to be abnormally expressed on a variety of cancer cells, predominantly epithelial tumours (Jonckheere and Van Seuningen 2010). MUC1 is so commonly over-expressed in a tumour-associated form (see below) that it has been estimated to be a potential target of immune therapy for about 80 % of all cancer cases (Kimura and Finn 2013). It has also been shown to be expressed on several normal tissues (Peat et al. 1992) albeit in a different glycoform.

MUC1-specific monoclonal antibodies (MAbs) are able to distinguish healthy MUC1 from cancer-associated MUC1 (Burchell et al. 1984; Sekine et al. 1985; Keydar et al. 1989; Lan et al. 1987; Kim et al. 1988; Stacker et al. 1989). The reason for this was demonstrated in the laboratory of Joyce Taylor-Papadimitriou, who, in an elegant series of experiments, showed that antibodies generated to the human milk fat protein, mucin-1, chemically stripped of its carbohydrate side chains, could react specifically to cancer-associated MUC1 glycoprotein (Burchell et al. 1987; Burchell and Taylor-Papadimitriou 1993). This suggested that cancer-associated MUC1 is non- or at least under-glycosylated with respect to the normal MUC1 glycoprotein.

It was eventually shown that cancer-associated MUC1 has about as many glycosylated sites on the protein core as normal MUC1, but that the carbohydrate side chains are much shorter and terminated with a sialyl group (see Fig. 1). The reason for this was shown to be a cancer-associated perturbation in the relative expression of the numerous glycosyl transferase enzymes responsible for the assembly of the carbohydrate side chains on the MUC1 protein (Dalziel et al. 2001; Picco et al. 2010).

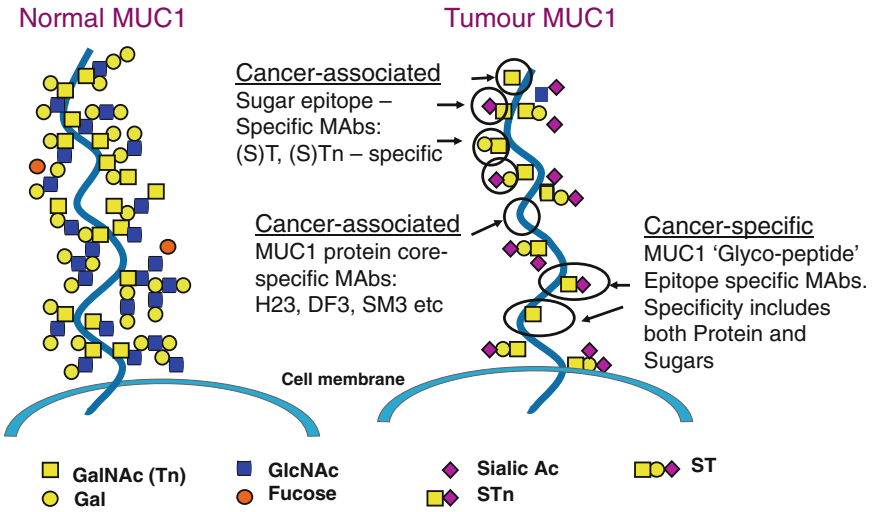

Fig. 1 The MUC1 protein on cancer cells is modified and expresses several types of novel, cancer-associated antigenic epitopes (Re-published, with permission, from Acres and Limacher (2005))

Many pre-clinical and laboratory experiments in mice and rats have provided a wealth information on the potential for MUC1-based vaccines combined with sophisticated vaccinations strategies for the treatment of cancer. These have been reviewed by Tang and Apostolopoulos (2008) and Roulois et al. (2013). This review will focus on a description of the biology of MUC1 and MUC1-based vaccines that have been tested in clinical studies.

2 Biology of MUC1

The molecular biology and biochemistry of MUC1 have been nicely reviewed recently by Nath and Mukherjee (2014). Briefly, MUC1 is a long, rigid protein which towers above the glycocalyx at the cell surface. It is comprised of an intracellular, C-terminal segment, rich in conserved tyrosines; a single-pass trans-membrane region and a large extracellular region comprised mainly of a tandemly repeated 20 amino acid segment, the variable number tandem repeats (VNTR), which can be repeated from 20 to 120 times. In healthy epithelial tissues, MUC1 expression is restricted to the luminal side of epithelial cells lining secretory ducts. It acts as a lubricant for material passing though the duct, but also protects the epithelial cells from the same contents and from bacteria. In contrast, on tumour cells, MUC1 expression is over-expressed, is hypo-glycosylated and is not restricted to any particular cell surface.

Studies have shown that MUC1 contributes to the transcriptional regulation of genes associated with not only immune regulation and inflammation, but also drug resistance, apoptosis, proliferation, angiogenesis, metastases and tumour cell invasion (Nath and Mukherjee 2014). Thus, MUC1 is not only an important target antigen for cancer immunotherapy due to its over-expression and cancer-associated modifications, but MUC1 is a very important target antigen since the reduction of MUC1 expression by immune selection of MUC1-negative variants would have a serious negative impact of the ability of the tumour cells to replicate, invade and metastasize.

3 MUC1 Immunology

3.1 Antibody Responses

Some patients with MUC1-expressing tumours do spontaneously show signs of an antibody response to MUC1, which seems associated with better prognosis (von Mensdorff-Pouilly et al. 2000) without overt signs of autoimmunity. These antibodies were identified using an ELISA with synthetic, non-glycosylated MUC1 VNTR peptides. Early vaccine studies with MUC1 showed that blood samples taken from patients prior to MUC1 immunotherapy did sometimes have a demonstrable level of antibodies to MUC1. Patients who had no anti-MUC1 prior to immunotherapy did not develop antibodies to MUC1 following vaccination, as determined using the same ELISA as described above (Scholl et al. 2003). It has been shown, using human MUC1 transgenic mice, that antibodies to human MUC1 (hMUC1) do not cause any signs of autoimmunity, even when the mice are infused with MUC1-specific antiserum (Tempero et al. 1999).

Immune tolerance to MUC1, at the level of antibodies, appears to be well established as demonstrated in transgenic mice (Rowse et al. 1998; Acres et al. 2000) when using an ELISA in which synthetic non-glycosylated VNTR peptide was used. Interestingly, evidence of a T cell response was observed in MUC1-vaccinated hMUC1 transgenic mice. In another hMUC1 transgenic mouse system, an anti-MUC1 antibody and T cell response could be generated using a complex and very powerful immune stimulation: mouse tumour cells fused with autologous dendritic cells (Gong et al. 1998) or specific glycopeptides (Sorensen et al. 2006).

There is now an accumulation of data showing the complexity of the antibody response to MUC1 by cancer patients and the role of MUC1 glycosylation. Sorensen et al. (2006) showed that some cancer patients do have a detectable antibody response to glycoforms of MUC1. What is even more interesting is that when they used MUC1 VNTR glyco–peptide arrays to assess serum for patients in a vaccine study, they were able to show that patients had no antibody response to MUC1 glycopeptides prior to vaccination. However, once vaccinated with a VNTR peptide glycosylated with the Tn antigen (see Fig. 1), patients generated IgG antibody response to that peptide and peptides with similar glycosylation, but not to unglycosylated MUC1 (Wandall et al. 2010) or unrelated glycoforms.

3.2 MUC1 Glycosylation

Figure 1 demonstrates the glycosylation of MUC1. 'Normal' or 'healthy' MUC1 is decorated with long, complex and branched glycosyl side chains, which are often terminated with a fucose residue. In this conformation, the protein core is covered and not accessible to antibodies specific for protein core epitopes (Burchell and Taylor-Papadimitriou 1993). In contrast, the cancer-associated MUC1 presents several novel epitopes to the immune system:

1. Cancer-associated sugar epitopes, the so-called T and T antigens along with their sialylated counterparts: Sialyl-T and Sialyl Tn;
2. Cancer-associated protein core epitopes, which are exposed by the shorter, truncated carbohydrate side chains and are recognized by MAbs such as SM3 (Burchell et al. 1984) and H23 (Keydar et al. 1989); and
3. Cancer-associated epitopes which are the combination of the protein core and short sugar side chains, as recognized by the antibodies DF3 (Sekine et al. 1985) 2D9 and 5E5 (Tarp et al. 2007).

Some MAbs specific for tumour antigens have now found their way into the clinic and, since the turn of the millennium, have achieved notable successes, such as Herceptin for breast cancer (Brenner and Adams 1999) and rituximab for human B cell malignancies (Maloney 2012). These are now commercially available treatments. The application of anti-MUC1 MAbs to cancer patient treatment has also been tested clinically (Table 1). The lack of dramatic success may well be due to the release of a soluble form of MUC1 from the cancer cell, the CA15.3 tumour marker, such that it acts as a decoy for antibody molecules. Even if there was no soluble MUC1 released, the cell bound form is such a long molecule that antibodies attached to it could well be too far from the cell surface to have any significant impact.

3.3 T Cell Responses to MUC1

T cell responses to MUC1 have been reviewed in detail by Roulois et al. (2013). It is noteworthy that the first-reported human T cell response to MUC1, described in detail, was very unusual in that the T cells were not specific to the combination of MUC1 peptide sequences within the target cell surface major histocompatibility complex (MHC) I (Barnd et al. 1989). Rather, they are MHC unrestricted and were shown to be specifically blocked by the antibody to MUC1 protein core, SM3. This special type of T cell generated some scepticism since it could not be found in any murine responses to MUC1 or other tumour antigens. Nevertheless, independent laboratories have also described this cell population in humans (Noto et al. 1997; Takahashi et al. 1994). There has been some speculation that this could be a sub-population of NKT cells (Wajchman et al. 2004). Whatever their exact lineage,

Table 1 A summary of MUC1-based immunotherapeutics (Re-published, with permission, from Limacher and Acres (2014))

Type of product	Agent	Stage of development	Disease setting	Main results	References
Monoclonal MUC1-specific antibodies	MAb-AR20.5 (Brevarex®)	Phase I	Adenocarcinomas	Antibody response	De Bono et al. (2004, 354)
	AS1402	Phase I	Breast cancer, metastatic	Correct tolerance	Pegram et al. (2009, 356)
		Randomized Phase II	Breast cancer, metastatic, with letrozole	No improvement	Ibrahim et al. (2011, 357)
Radiolabeled MUC1-specific antibodies	C595-^{67}Cu	Phase I	Bladder cancer	Tumour uptake	Hughes et al. (2001, 359)
	C595-^{111}In	Phase I	Bladder cancer	Tumour uptake	Hughes et al. (2001, 359)
	NCRC48-^{111}In	Phase I	Bladder cancer	tumoral uptake	Kunkler et al. (1995, 362)
MUC1 VNTR peptides vaccines	MUC1-MFP (mannan fusion protein)	Phase I	Adenocarcinomas	Antibody and T cell immune response	Karanikas et al. (2001, 366)
		Randomized Phase III	Breast cancer	Decreased recurrence rate	Apostolopoulos et al. (2006, 371)
	L-BLP25 (Stimuvax®)	Randomized Phase II	Non-small-cell lung cancer, advanced	Improved overall survival in stage III patients	Butts et al. (2005, 372, 2011, 374)
		Phase III	Non-small-cell lung cancer, non-resectable stage III	Improved survival in patients who received concurrent chemoradiothaerpy	Butts et al. (2014, 375)
	MUC1-KLH	Phase I	Breast cancer, high risk	Antibody response	Gilewski et al. (2000, 376)

(continued)

Table 1 (continued)

Type of product	Agent	Stage of development	Disease setting	Main results	References
Vaccines based on poxviruses encoding MUC1 DNA sequence	PANVAC (CV 301)	Randomized Phase II	Colorectal, cancer after surgical resection of metastases (treated with vaccine + DC)	Improved overall survival	Morse et al. (2013, 377)
		Phase III	Pancreatic cancer, advanced, after failure of gemcitabine	No improvement of survival	Therion Biologics press release (2006)
		Randomized Phase II	Breast cancer, metastatic, combination with chemotherapy	Improved progression-free survival	Heery et al. (35th CTRC-AACR San Antonio Breast Cancer Symposium)
	TG4010	Phase II	Kidney cancer, metastatic	Favourable overall survival	Oudard et al. 2011, 88)
		Randomized Phase II	Prostate cancer, PSA failure	Improvement in PSA-doubling time	Dreicer et al. (2009, 378)
		Randomized Phase II	Lung cancer, advanced, combination with chemotherapy	Improved response rate	Ramlau et al. (2008, 90)
		Randomized Phase II	Lung cancer, advanced, combination with chemotherapy	Improved 6-month PFS and response rate, TrPAL biomarker	Quoix et al. (2011, 379)
MUC1 mRNA vaccines	In vitro-transcribed naked RNA	Phase I/II	Kidney cancer, metastatic	Stabilizations/response	Rittig et al. (2011, 380)
Adoptive cell therapy	MUC1-stimulated PBMCs	Phase I/II	Breast cancer, advanced	Change tumour burden	Wright et al. (2009, 381)
		Phase I	Ovarian cancer	One complete clinical response	Dobrzanski et al. (2009, 382), Corbiere et al. (2011)
		Phase I/II	Pancreatic cancer	Long-term survival	Lepisto et al. (2008, 383)
	MUC1-MFP-pulsed DCs	Phase I	Adenocarcinomas	Cellular immune response, two long stabilizations	Loveland et al. (2006, 385)
	Dendritic cells and CTL transfer	Phase I	Pancreatic cancer	Clinical response	Kondo et al. (2008, 408)

these cells could be very important in the patient's immune response to MUC1 expressing tumour cells, since so many tumour cells express low or no MHC I molecules. Whether these cells are activated and/or amplified by MUC1 vaccines is not yet known.

MHC I-restricted MUC1 epitopes for more 'traditional' cytotoxic T lymphocytes (CTL) have also been identified (Apostolopoulos et al. 1997; Barnea et al. 2002; Brossart et al. 1999; Dreicer et al. 2009; Heukamp et al. 2001; Roulois et al. 2011). The majority of these epitopes lie outside of the VNTR section of the MUC1 sequence. Therefore, the MUC1-based therapeutic vaccines, which include only the VNTR segment or peptides from that segment, are unlikely to stimulate such a CTL response. In addition, there are some cancer-associated forms of MUC1 devoid of the VNTR (Baruch et al. 1997; Roulois et al. 2013).

Like most non-melanoma TAA, immune monitoring of patient CTL responses to MUC1 has been a challenge. Responses, measured using lymphocytes from peripheral blood of patients, appear to be weak and transient. Measurable CTL specific for MUC1 are not rare in these patients, but do not seem to be systematically activated or amplified by vaccination. Measurable CTL are detectable in circulation of about 20–30 % of patients prior to MUC1 vaccination. Existing T cell immunity to MUC1 has been detected in blood samples from both healthy individuals and breast cancer patients, again with no overt signs of autoimmunity. Pre-existing T cell immunity to MUC1 has been associated with better response to MUC1-based immunotherapy in metastatic renal cell carcinoma and late-stage lung cancer patients (Oudard et al. 2011; Ramlau et al. 2008). The appearance of MUC1-specific CTL, nor its disappearance, was associated with better clinical outcome (Quoix et al. 2011). Nevertheless, there was an association between having a MUC1 CTL response anytime on the study, whether before or during therapy, and clinical improvement (Oudard et al. 2011; Ramlau et al. 2008). In a study with minimal remaining disease following therapy (prostate cancer therapy patients following radiation or surgery for their cancer, who have rising PSA with no apparent disease), a de novo CTL response to MUC1 during MUC1 vaccine immunotherapy was loosely associated with clinical improvement (Dreicer et al. 2009). These observations question the approach of assessing the MUC1-specific CTL response in the periphery, while the key events of this immune process happen in tissues, either lymphatic or tumour.

Monitoring patient CTL responses has become more complex with recent evidence to indicate that CTL may react to glycosylated peptides as well as to naked peptides as previously thought. It has been immunological dogma to believe that polypeptides are associated with MHC I complex without glycosylation. However, it now appears that glycopeptides can be presented to CTL in the cleft of MHC I (Ninkovic et al. 2009; Haurum et al. 1999)

In normal cells, emerging data suggest that the extensive glycosylation could prevent the MUC1 from being processed by the proteasome and thus, MUC1 peptides would not be presented with MHC class I molecules on the cell surface (Hiltbold et al. 2000; Hanisch et al. 2003; Ninkovic et al. 2009). MUC1 expressed by tumour cells is hypoglycosylated so would be more easily processed by antigen-

presenting cells. This could be one potential reason why normal cells are not vulnerable to T cells targeting MUC1 epitopes.

Nevertheless, this does complicate monitoring of patient CTL responses during a clinical study due to the numbers of possible glycosylations of each MUC1 peptide epitope. This will require more patient blood per sample and more resources since glycopeptides are very expensive to produce, or new assays to detect T cell responses to a wider variety of MUC1 antigenic epitopes will be required.

CD4+ T cells, while not direct effector T cells, are nonetheless important for the development of both CTL and antibody responses. The state of glycosylation may also affect presentation to CD4+ T 'helper' cells in the context of MHC II (Vlad et al. 2002). CD4+ T cells produce a variety of cytokines which can indirectly affect the growth of tumours by, for example, restricting blood flow to the tumour mass. The traditional method for measuring CD4+ T cell activity is by lymphocyte proliferation, although ELISpot or intracellular cytokine measurement after stimulation with long peptides can also indicate CD4+ reactivity. In clinical studies, MUC1-specific CD4+ activity has been detected in lymphocytes from patient peripheral blood samples; however, no correlation with clinical activity has been described (Oudard et al. 2011; Ramlau et al. 2008).

4 MUC1 Therapeutic Vaccines in the Clinic

MUC1-based immunotherapies in clinical studies are summarized in Table 1. The application of MUC1-specific MAbs has been described above. There are two MUC-based therapeutic vaccines in clinical development, which comprise long, non-glycosylated polypeptides from the VNTR sequence from MUC1 and are most likely to elicit antibody responses. These are mannan fusion protein, MFP (Apostolopoulos et al. 2006), and Stimuvax® (Powell and Chow 2008; Wu et al. 2011).

McKenzie et al. made the important observation (Apostolopoulos et al. 1995) that coupling the carbohydrate mannan to their MUC1 VNTR-GST (glutathione-S-transferase) fusion protein affected the MUC1 VNTR-specific immune response. There are mannose receptors on dendritic (DC) cells. When mannan was coupled to the fusion protein under oxidized conditions, it induced a cytotoxic T lymphocyte (CTL) response in mice, while coupling under reducing conditions, a less effective antibody response was generated. In contrast, MFP produced under oxidizing conditions produced antibody rather than CTL in humans (Apostolopoulos et al. 1998). A small Phase III study testing MFP in breast cancer was undertaken. Over 8 years later, it was observed that rate of recurrence of breast cancer in the MFP-treated patients was significantly lower than in the non-vaccinated patient group (Apostolopoulos et al. 2006). This was an interesting observation, even though initial, protocol defined, end points were not achieved. Stimuvax® (L-BLP25 or Tecemotide) is a therapeutic vaccine based on 25-mer peptides including the pattern repeated in MUC1 VNTR which are incorporated in a liposome formulation. In a Phase II trial, 171 non-small-cell lung cancer (NSCLS) patients received

subcutaneous vaccinations of L-BLP25 weekly for 8 weeks, followed by maintenance vaccinations at 6-week intervals plus best supportive care (BSC) or BSC alone. Median survival in patients receiving L-BLP25 plus BSC compared to those receiving BSC alone was 17.2 months versus 13.0 months, respectively. In the subset of patients with stage IIIb loco-regional disease, median survival time was 30.6 versus 13.3 month (Butts et al. 2011). A Phase III trial in unresectable stage III NSCLC patients was conducted (START). It did not reach its primary end point of overall survival, but a large pre-specified subgroup of 806 patients who received concurrent chemoradiotherapy demonstrated a significantly longer survival for the patients who received Stimuvax®: 30.8 versus 20.6 months (Butts et al. 2014). Based on these results, a second Phase III was launched (START2) but unfortunately prematurely terminated for strategic reasons.

As described above, most potential CTL epitopes on the MUC1 molecule lie outside of the VNTR. It is therefore of interest to incorporate the entire MUC1 molecule into therapeutic vaccines. Two such vaccines are described in Table 1, both of which utilize poxviruses as vectors for the MUC1 gene sequence. Poxviruses are useful because large DNA sequences can be inserted, and they are safe and reasonably easy to produce and very immunogenic. In both the PANVAC and TG4010 therapeutic vaccines, additional sequences for immune-stimulating molecules are co-expressed.

PANVAC is based on two poxviruses, vaccinia and fowlpox, engineered to express two tumour antigens, CEA and MUC1, plus a complex series of co-stimulatory molecules, all of which have been administered either together with the cytokine GM-CSF or as infected dendritic cells, in a Phase II study at the NIH. Some clinical benefit was observed.

TG4010, which by comparison, is simpler, since it uses one poxvirus: the non-propagative modified virus of Ankara (MVA), which incorporates MUC1 and the immune stimulatory cytokine IL2. TG4010 has been tested in Phase II studies in breast, kidney, prostate and lung cancers. Now, two controlled Phase IIb studies in late-stage NSCLS have been completed.

MUC1 epitope peptides have been characterized by cell binding assay and iTopia® and have been used to test T cell response by patients treated with TG4010. ELISpot was used to test T cells from lung, prostate and kidney cancer patients who have been treated with TG4010. Many of these epitope peptides have now been incorporated into tetramers (Quoix et al. 2011). At least, some MUC1-specific T cells using most peptides were observed. The strongest and most frequently detected CTL were identified using the HLA-B7 epitopes, with both ELISpot and tetramers. Neither of the HLA-B7 epitopes tested are from the tandem repeat of MUC1. HLA-A2, the most frequent HLA type in Caucasians, has received a lot of attention when looking for TAA epitopes. MUC1 peptides that bind to HLA-A2 have been identified (Heukamp et al. 2001; Barnea et al. 2002; Brossart et al. 1999; Apostolopoulos et al. 1997; Ramlau et al. 2008) and were included in the analyses of immune responses by lymphocytes from T4010-treated patients. CTL detected with these A2 peptides, either in ELISpot or using tetramers, were less frequent as with the B7 peptides.

In both PANVAC and TG4010, the MUC1 antigen is co-expressed with additional signals to provide a local stimulation to a cellular immune response. In PANVAC, these signals are in the form of the co-stimulatory molecules B7.1 (CD80), ICAM-1 (CD54) and LFA-2 (CD58). The vaccine is often given together with another positive immune signal in the form of GM-CSF. TG4010 also provides the positive immune signal IL-2 in addition to the MUC1 antigen. Including these positive immune stimulatory signals is like pressing a bit harder on the accelerator of the immune system. It is felt that this is required since the immune system of late-stage cancer patients is often described as 'tired' or 'anergic' such that additional immune systems can provide a secondary boost to the local immune response. From this perspective, the viral vectors themselves are seen by immune system as a danger system, further contributing to co-signalling messages during antigen presentation by dendritic cells to lymphocytes.

Another concept, which holds great potential, is to inhibit the immune inhibitors. Using the same analogy as above, it is like removing, or at least weakening, the brakes on the immune system. This is done by applying antibodies which inhibit negative immune signals such as PD-1 (Herbst et al. 2014; Hamid et al. 2013; Tumeh et al. 2014) or CTLA-4 (Robert et al. 2014; Ribas et al. 2012). This may require more caution since it removes some elements of control, which may be required to guide the immune response through its many dips and turns and avoid collateral damage such as autoimmunity.

A similar approach, removing growth controls altogether from the amplification of immune effector cells, is the in vitro amplification and activation of a patient's own tumour infiltrating or other tumour-specific T cells. These cells develop without any of the in vivo checks and balances exerted on immune development in vivo. They are grown to large numbers in vitro and then injected back into patients. A recent editorial in Nature Biotechnology (2013) advises caution for the treatment of cancer with in vitro-activated, tumour antigen-specific T cells. In theory, the T cells are exquisitely specific for tumour antigens. Nevertheless, severe toxicity can be associated with these treatments.

It has been estimated that decades can elapse between, for instance, the first lung cancer cell division and eventual diagnosis of lung cancer. During that time, the cancer and the immune system develop a relationship which is both dynamic and patient specific. At the time of diagnosis, some patients have evidence of a dominant inflammatory response with abnormally high blood levels of inflammation-related proteins and cytokines such as CRP, sCD54, IL-6, IL-10 and CSF-1. Inflammation is usually associated with poor clinical response to treatments such as chemotherapy or immunotherapy. Other patients do not show any particular signs of inflammation, but have a measurable immune response to tumour antigens, cellular and/or antibody, both of which have been associated with better response to therapy (Ramlau et al. 2008; von Mensdorff-Pouilly et al. 2000; Oudard et al. 2011). Some patients have abnormally high levels of cells with activated natural killer and/or natural killer T cell phenotype (CD16+ CD56+ CD69+) at the time of diagnosis (Quoix et al. 2011). These cells have come to be called TrPAL for triple-positive activated lymphocytes. Abnormally, high levels of these cells in lung

cancer patients, prior to therapy, are associated with poor response to TG4010 in combination with chemotherapy. No such association is seen in response to chemotherapy alone.

The correlation between lower levels of TrPAL cells and better response to TG4010 therapy has now been further supported in a second Phase IIb study in stage IV lung cancer patients (Quoix et al. 2014).

Therefore, the status of the patient's immune system should be considered when deciding whether to treat with some immunotherapeutics. In addition to the TrPAL biomarker, other soluble signs of immune imbalance were also associated with poorer or better response to TG 4010, namely sCD-54, IL-10 or CSF-1 (Quoix et al. 2011).

Chemotherapy is designed to be toxic for the disease with hopes for usually manageable side effects to the patient. In contrast, vaccine immunotherapy treats the patient with the intention that the patient's own immune system will then treat the cancer. Thus, it is imperative that the patient's immune system is still adequately and correctly reactive.

The fine specificity of the 'therapeutic' T cells following an immunotherapeutic vaccination is an interesting question. One could question whether the immune response to the vaccine is responsible for observed clinical responses. Alternatively, the response to the vaccine may sufficiently disturb the established anergy, which allows the immune system to react and to find the most effective therapeutic antigen specificity. Such antigen spreading has been observed in melanoma (Corbiere et al. 2011).

5 Conclusion

Lung cancer is the leading cause of cancer-related mortality in both men and women. Most new cases of lung cancer are NSCLC, and about 60 % of cases are diagnosed as unresectable or advanced. In patients with unresectable, localized, NSCLC chemotherapy combined with radiation is a viable option. For patients with advanced or metastatic NSCLC, there are fewer treatment options. Chemotherapy has demonstrated some improvement in survival, but the treatment of these patients remains unsatisfactory. More effective therapies need to be found. Most targeted therapies for NSCLC are based on either small inhibitory molecules or MAbs which interfere with tumour growth, angiogenesis or both (Auberger et al. 2006). Vaccine-based therapeutic immunotherapy is designed to induce or amplify an immune response directed against the population of tumour cells (O'Mahony et al. 2005). Due to its over-expression on many cancer cells and in cancer-specific forms, MUC1 is a potential target for immunotherapy of many cancers (Cheever et al. 2009). Recent data indicate that there is a true potential for the treatment of late-stage lung cancer patients, as well as many other MUC1+ cancers, with therapeutic vaccines such as TG4010.

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Chimeric DNA Vaccines: An Effective Way to Overcome Immune Tolerance

Federica Riccardo, Elisabetta Bolli, Marco Macagno, Maddalena Arigoni, Federica Cavallo and Elena Quaglino

Abstract The fact that cancer immunotherapy is considered to be a safe and successful weapon for use in combination with surgery, radiation, and chemotherapy treatments means that it has recently been chosen as Breakthrough of the Year 2013 by Science editors. Anticancer vaccines have been extensively tested, in this field, both in preclinical cancer models and in the clinic. However, tumor-associated antigens (TAAs) are often self-tolerated molecules and cancer patients suffer from strong immunosuppressive effects, meaning that the triggering of an effective anti-tumor immune response is difficult. One possible means to overcome immunological tolerance to self-TAAs is of course the use of vaccines that code for xenogeneic proteins. However, a low-affinity antibody response against the self-homologous protein expressed by cancer cells is generally induced by xenovaccination. This issue becomes extremely limiting when working with tumors in which the contribution of the humoral rather than the cellular immune response is required if tumor growth is to be hampered. A possible way to avoid this problem is to use hybrid vaccines which code for chimeric proteins that include both homologous and xenogeneic moieties. In fact, a superior protective anti-tumor immune response against ErbB2$^+$ transplantable and autochthonous mammary tumors was observed over plasmids that coded for the fully rat or fully human proteins when hybrid plasmids that coded for chimeric rat/human ErbB2 protein were tested in ErbB2 transgenic mice. In principle, these findings may become the basis for a new rational means of designing effective vaccines against TAAs.

F. Riccardo · E. Bolli · M. Macagno · M. Arigoni · F. Cavallo · E. Quaglino (✉)
Department of Molecular Biotechnology and Health Sciences,
Molecular Biotechnology Center, University of Torino, via Nizza 52,
10126 Torino, Italy
e-mail: elena.quaglino@unito.it

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Contents

1 Introduction

Its specificity and long-term memory furnishes the immune system with great potential for specifically eliminating tumor cells without harming normal cells and for preventing cancer recurrence. Considerable effort has therefore been invested in discovering molecular biomarkers associated with each specific cancer and in designing effective vaccines for their targeting, all with the final aim of boosting or restoring the ability of patients' immune system to fight cancer. Thus, cancer vaccines have the potential to become a therapeutic treatment which is used synergistically in combination with traditional therapies. Recent decades have seen several types of anticancer vaccines successfully designed, manufactured, and preclinically tested, with this final aim in mind. However, only few have been proven to be effective in inducing a measurable immune response when used in clinical trials and overall clinical benefit has been so far limited. Indeed, only a single anticancer vaccine (Provenge®, or APC8015) has so far been approved by the Food and Drug Administration (FDA) (Kantoff et al. 2010).

One problem, in terms of clinical setting, is that cancer vaccine studies are generally carried out in end-stage cancer patients with high tumor burden and impaired immune capacity. Studies are, therefore, now moving toward patients with minimal disease, thereby preventing recurrence and metastasis (Frelin et al. 2010). Other factors that may account for this failure concern the choice of target antigens, vaccine formulation, and overcoming tolerance. We herein discuss these limitations and propose the use of chimeric DNA vaccines against oncoantigens as a possible solution.

2 Choice of Successful Vaccination Targets

2.1 Oncoantigens

The starting point in the development of an effective anti-tumor vaccine is identifying an appropriate target antigen. Several reports have demonstrated that cancer control through active immunotherapy is an attainable goal only when the vaccine targets a tumor-associated antigen (TAA) that displays a high level of expression in cancer cells but low levels in healthy cells, holds a potent oncogenic role, and is not prone to escape from immune recognition. TAAs with these features are defined as oncoantigens (Lollini et al. 2006).

Oncoantigens can be secreted or expressed by both cancer and non-neoplastic cells that form the tumor microenvironment (Iezzi et al. 2011). They can be classified into three classes according to their function and cellular localization. Class I oncoantigens (receptors, adhesion molecules, etc.) are expressed on the cell surface, class II (growth factors, angiogenic factors, etc.) are present in the tumor microenvironment, while class III (non-receptor tyrosine kinases, transcription factors, cell cycle molecules) are intracellular proteins.

The benefits of using oncoantigens as targets for effective immunotherapy rest on several pillars. Firstly, they are not susceptible to cancer immunoediting (Dunn et al. 2004) as they play a key role in tumor growth and survival and, furthermore, their loss impairs or blocks tumor progression. Inevitably, tumor cells are less likely to down-regulate this protein and, even if oncoantigen-loss variants occur, they will cause impaired tumorigenic potential and negative selection (Friedman et al. 2005). Secondly, their low expression level on normal cells makes the risk of inducing an autoimmunity reaction following oncoantigen vaccination almost negligible. Thirdly, they can be targeted by different immune mechanisms according to their diverse functions and localizations. Class I oncoantigens are considered to be the best targets for effective anticancer immunotherapeutic strategies (Iezzi et al. 2012) as they are susceptible to the attack of both T cell and antibodies at the same time.

2.2 ErbB2 as a Prototype Oncoantigen

Of all the TAAs identified as possible targets for cancer immunotherapy so far, ErbB2 has the makings of an “ideal” oncoantigen as it is expressed at low levels by few healthy cells in adult life, and its overexpression and dysregulation occur in several types of cancer, including invasive breast cancers, colorectal cancers, ovarian cancers, pancreatic cancers, and prostate cancers. These aberrations are associated with poor prognosis because of greater tumor aggressiveness and an increased risk of recurrence (Spears et al. 2012). Moreover, its localization on the tumor cell surface makes ErbB2 a target for both cell-mediated and antibody-mediated immunity. As a consequence, the development of innovative therapeutic

options that specifically target ErbB2 has become one of the most outstanding achievements in clinical oncology.

Since the intrinsic kinase activity of the ErbB2 receptor is essential for many of its oncogenic functions, several small-molecule tyrosine kinase inhibitors (TKI) have been tested (Albanell and Gascon 2005) and approved by the FDA. These include Gefitinib (Iressa®) and Erlotinib (Tarceva®) (Rukazenkov et al. 2009), which have been approved for the treatment of patients with ErbB2$^+$ advanced or metastatic non small cell lung cancer (NSCLC) and Lapatinib (Tyverb®) for the treatment of ErbB2$^+$ breast cancer patients. Unfortunately, ErbB2 inhibitors often fail to elicit a clinical response, even when tumors express high levels of the activated receptor. Several explanations are possible for this and include insufficient target inhibition due to poor drug penetration of the tumor, or alternatively, it is possible that some tumors are intrinsically resistant to TKI or can acquire different mechanisms of resistance to therapeutic inhibitors (Wykosky et al. 2011).

The field of passive immunotherapy has seen the successful clinical testing of monoclonal antibodies (mAbs) that target ErbB2 and, thus, the FDA approval of Trastuzumab and Pertuzumab. The first has become standard of care treatment for women with ErbB2-overexpressing metastatic breast cancer. Its clinical efficacy is clear, both when used as a single agent (Vogel et al. 2002) and when used in combination with a variety of chemotherapy agents (Cortes and Roche 2012). Pertuzumab has recently shown promising results when used against metastatic breast cancer, leading to the exploration of a combination of targeted therapies in the adjuvant setting (Baselga et al. 2012). Although the clinical results observed in Trastuzumab and Pertuzumab therapy have been encouraging, the high rate of cardiac side effects (Shak 1999) and the evidence of the short-lived therapeutic action of mAbs, associated with the relapse of most treated patients, suggests that tumors intrinsically possess or acquire mechanisms for escape (Anido et al. 2006; Castiglioni et al. 2006). All these limitations highlight the need to develop other strategies to provide a wider immunotherapeutic armamentarium against ErbB2$^+$ tumors.

Active therapies, such as cancer vaccines, offer a number of advantages over passive therapies, including the induction of a specific long-lasting immunological memory against the antigen and of a wider vaccine-elicited immune response that may be able to overcome tumor resistance mechanisms (Eschenburg et al. 2010). In reality, while the ability of several anti-ErbB2 vaccine formulations to induce a specific humoral and cellular response has been demonstrated in cancer patients (Kutzler and Weiner 2008; Norell et al. 2010), along with their safety, their therapeutic clinical benefits remain questionable. Several hypotheses have been put forward to explain these results, and besides the deleterious impact on the immune system caused by treatments such as chemotherapy and radiotherapy prior to vaccination, the difficulty in breaking the immune tolerance against the self-ErbB2 antigen would appear to play a key role in hampering an effective anti-tumor immune response.

2.3 Oncoantigens are Self-tolerated Molecules

The priceless opportunity provided by anti-tumor vaccination relies on its ability to specifically amplify a patient's own immune response against definite oncoantigens in order to induce long-lasting memory against cancer cells (Eschenburg et al. 2010). However, one of the most important challenges is found in the intrinsic nature of oncoantigens: most of them are self-tolerated proteins, overexpressed by cancer cells but non-mutated and therefore showing various degrees of tolerogenicity (Even-Desrumeaux et al. 2011). Even if the overexpression of self-oncoantigens exceeded the threshold for T cell activation and break immune tolerance, they may show low immunogenicity potential (Zinkernagel and Hengartner 2001). Tolerogenicity causes reactive high-avidity T cells to be deleted or transformed into natural $Foxp3^+$ regulatory T cells (Treg), a T cell subset with immunosuppressive properties that limits the magnitude of effector T cell responses (Josefowicz et al. 2012; Stritesky et al. 2012). As consequence of central tolerance, low-frequency tumor-specific T cell precursors are present and display a suboptimal TCR affinity or an immunosuppressive phenotype. Potentially self-reactive, low-avidity T cells that evade from central tolerance mechanisms are controlled in the periphery by steady-state antigen-presenting cells, which present tumor antigens to T cells in the presence of coinhibitory receptors (e.g., programmed cell death ligands, PD-L1 and PD-L2) or in the absence of costimulatory signals (e.g., CD40 and CD86), rendering T cells almost unresponsive (anergic or exhausted) or $Foxp3^+$ (inducible Treg). Both natural Treg and inducible Treg further suppress self-reactive T cells by antigen-specific and non-specific mechanisms that involve the expression of inhibitory molecules, such as cytotoxic T lymphocyte antigen (CTLA)-4, and the secretion of anti-inflammatory cytokines, such as TGF-β and IL-10, which suppress dendritic cell (DC)-mediated induction and effector T cell function (Sakaguchi et al. 2001; Yagi et al. 2004). These dysfunctions in T cell response against tumors may all contribute to the failure of the anticancer vaccines that have been evaluated in human clinical trials (Kutzler and Weiner 2008).

The real challenge for anti-tumor vaccines is, therefore, to overcome all these types of T cell dysfunctions, inducing the activation of non-tolerized, non-exhausted, and self-cross-reactive low-affinity T cell clones (Jacob et al. 2006).

2.4 Tumors Limit Vaccine-Induced Immune Response

Besides the difficulty in inducing an effective anti-tumor immune response against self-tolerated molecules, recent data demonstrate that tumors can also escape from immune destruction via dominant negative regulatory pathways that impart a state of peripheral T cell tolerance against TAAs. These inhibitory pathways can take the form of cytokines, suppressive cell populations (e.g., Treg, myeloid-derived suppressor cells, (MDSC) and tumor-associated macrophages) (Takenaka et al. 2013;

Pollard 2004; Kim et al. 2006), amino acid-catabolizing enzymes (e.g., indoleamine-2, 3-dioxygenase, and arginase), and the ligation of inhibitory receptors on activated T cells (e.g., CTLA-4 and PD-1) (Pentcheva-Hoang et al. 2009). Indeed, increased Treg frequencies in the blood, draining lymph nodes, and tumor tissues of cancer patients have been described and associated with impaired immune response against cancer (Liyanage et al. 2002). For this reason, Treg depletion in cancer immunotherapy may be a useful adjuvant strategy. The targeting of the CD25 Treg surface marker with specific mAb has been shown to be effective in enhancing vaccine-induced anti-tumor activity in various preclinical models (Ludwig-Portugall et al. 2009; LaCelle and Jensen 2009; Goforth et al. 2009; Viehl et al. 2006; Casares et al. 2003; Quezada et al. 2008). Moreover, the use of US FDA-approved humanized mAb against CD25 (Daclizumab) in a peptide vaccination trial of breast cancer patients was shown to be effective in inducing an anti-peptide immune response (Rech et al. 2012). However, the autoimmune reactions potentially associated with the administration of anti-CD25 mAb and the risk of inhibiting the tumor-specific cytotoxic T cells that transiently express this molecule after activation are critical issues for its application in human patients. Therefore, alternative strategies with which to potentiate vaccine-induced immune responses and improve tumor control have to be developed (Stewart and Smyth 2011). One possible method is combining anti-tumor vaccines with the use of antibodies that block or suppress inhibitory receptors on activated T cells (Sarnaik et al. 2011; Yuan et al. 2011). Improved advanced metastatic melanoma patient survival has recently been described in a phase III clinical trial which combined a gp-100 peptide vaccine with CTLA-4 blockade using the fully human antagonistic anti-CTLA-4 mAb, Ipilimumab (Gajewski 2010; Hodi et al. 2010). However, it is worth noting that severe adverse events occurred in around 15 % of patients treated with Ipilimumab, most of which were immune-related, and included seven patient deaths.

In conclusion, even if the combined use of mAbs blocking checkpoint inhibitors together with anticancer vaccines is a potentially powerful strategy, safer drugs have to be developed.

3 Chimeric DNA Vaccines

3.1 Xenogeneic Vaccines

One way to overcome the major limitations of a successful anticancer vaccine, namely the host immune tolerance to self-oncoantigens and the tumor-induced immunesuppression, is to use xenogeneic proteins or peptides from a different species, which is significantly homologous with the self-ortholog, as immunogens. This strategy has been widely studied, and several papers have demonstrated its potential (Jacob et al. 2006; Kianizad et al. 2007; Soong et al. 2013; Yuan et al. 2009).

Many oncoantigens are phylogenetically conserved to various degrees of similarity; however, they are recognized by the immune systems as "non-self-antigens" as they are not identical in their sequences. Therefore, a vaccine coding for a xenogeneic oncoantigen can circumvent immune tolerance and may acquire the proper immunogenic and anti-tumor potential.

Although the xenogeneic vaccination mechanism of action has not been completely elucidated, it is believed that subtle differences between epitopes of the orthologue and the native protein are responsible for eliciting T and B cell responses against the xenoantigen that may cross-react with the native target. From the mechanistic point of view, the foreign epitopes of an orthologue protein which is used as vaccine may lead to the formation of heteroclitic epitopes (Dyall et al. 1998; Kianizad et al. 2007) that possess an enhanced ability to bind to MHC glycoproteins and effectively prime both $CD8^+$ and $CD4^+$ T cells which are able to cross-react against the original non-mutated peptide. The release of helper cytokines by activated $CD4^+$ T cells may rescue bystander anergic T and B lymphocytes (Jensen and Kapp 1986) and lead to the activation of DCs (Smith et al. 2004). Moreover, the interaction between B cells, reacting with self-epitopes, and their cognate-activated $CD4^+$ T cells provides proper signals to B cells and triggers the production of high-affinity antibodies which may potentially cross-react with the self-protein (Zhang et al. 2001; Sobel et al. 1994).

The efficacy of this strategy has been extensively demonstrated in several murine models of cancer both in prophylactic and therapeutic settings (Cavallo et al. 2014). Moreover, xenovaccination has recently been shown to hamper cancer growth and to improve survival in veterinary cancer patients, mainly in dogs affected by spontaneous disease (Riccardo et al. 2014; Alexander et al. 2006; Kamstock et al. 2007; Yu et al. 2011). These positive results obtained in veterinary trials led to the approval by the United States Department of Agriculture (USDA) of the first xenogeneic DNA vaccine against tyrosinase, ONCEPT (Merial), for the treatment of oral malignant melanomas (Grosenbaugh et al. 2011) in 2010. Although the therapeutic efficacy of ONCEPT has been recently questioned (Ottnod et al. 2013), its licensing has renewed enthusiasm for heterologous DNA vaccination as an alternative and potentially effective immunotherapeutic strategy. Finally, the safety and immunogenicity of xenovaccination against human tumor antigens have also been demonstrated in several phase I/II human clinical trials (Fong et al. 2001; Wolchok et al. 2007; Yuan et al. 2009, 2013; Ginsberg et al. 2010; Eriksson et al. 2013), thus facilitating the opening up of new perspectives in the management of cancer.

3.2 An Easy and Versatile Means of Oncoantigen Delivery: DNA

Many different oncoantigen sources have been tested for use as vaccines in preclinical models and in clinical studies (Berzofsky et al. 2004). These include whole tumor cells, tumor lysates, purified tumor antigens (protein- or peptide-containing

vaccines), recombinant viruses, and nucleic acids (DNA and mRNA) that encode for the oncoantigen of choice. Even if proof of principle of the potential efficacy of all these strategies has been demonstrated, specific pitfalls have to be taken into consideration when developing a successful anti-tumor vaccination strategy.

The use of autologous whole tumor cells or tumor cell lysates as the source of tumor antigens for vaccination (Kircheis et al. 2000; Kayaga et al. 1999) faces a number of logistical challenges which come in the form of the preparation of patient-specific therapies and high production costs. Thus, researcher interest has shifted toward molecularly defined synthetic vaccines which are more suitable for large-scale pharmaceutical manufacturing processes (Mocellin et al. 2009). However one of the major issues with peptide-based vaccination is the fact that individual peptides will only be useful in patients who possess appropriate HLA molecules capable of presenting that peptide. However, encouraging data from two trials in patients with advanced renal carcinoma suggest that targeting multiple tumor-associated HLA-restricted peptides elicits specific vaccine-induced immune responses associated with clinical benefit (Walter et al. 2012).

A more hopeful scenario seemed to be presented by DC-based anticancer vaccines. The best example can be traced to 2010, when sipuleucel-T (also known as Provenge), a DC-enriched autologous cell preparation which is expanded ex vivo in the presence of a prostate acid phosphatase/granulocyte macrophage colony-stimulating factor (PAP/GM-CSF) fusion protein, was approved by the US FDA for use in patients with asymptomatic or minimally symptomatic metastatic castration-refractory prostate cancer (Cheever and Higano 2011). Overall, experience with DC has shown that these cells can be safely administrated with no, or only mild, side effects. The induction of an immune response against the target antigens has been demonstrated, but the actual clinical benefits for patients with cancer have so far been marginal (Sabado and Bhardwaj 2013).

Advances in recombinant techniques have provided the opportunity to introduce oncoantigens, or simply epitopes (if needed, along with costimulatory molecules and cytokines), into viral vectors. Indeed, a number of trials for the treatment of cancer which use recombinant viruses, i.e., adenovirus, vaccinia, and the avipox virus, that express oncoantigens have been reported or are in progress (Odunsi et al. 2012; Madan et al. 2012; Marshall et al. 2000). However, the use of these vectors in clinical cancer immunotherapy brings with it some important limitations which can be traced to the potential dangers associated with live virus use and the presence of preexisting anti-vector immune responses and a high prevalence of anti-viral neutralizing antibodies in patients.

The high versatility, ease of production, and low cost of manufacturing combined with the high stability and easy scalability of DNA plasmids may provide an important base from which to further develop this approach into the effective cancer therapies of the twenty-first century (Fioretti et al. 2010). A DNA vaccine is composed of a plasmid DNA, which includes the entire or partial coding region of the TAA of interest under the control of a mammalian promoter, a transcription termination signal, and a prokaryotic antibiotic resistance gene (Glenting and Wessels 2005). Sometimes, instead of the partial or entire TAA sequence, a string

of epitopes which is specifically selected as a highly immunogenic sequence of the TAA can be used as immunogens (Aurisicchio et al. 2014). The plasmid DNA vaccine mode of action appears to be twofold. On the one hand, DNA plasmids appear to act as a pathogen-associated molecular pattern which is able to stimulate the innate immune system (Klinman et al. 1997). On the other hand, DNA plasmids directly transfect resident cells that start producing the vaccine-encoded antigen.

Several DNA vaccines against TAAs have been successfully tested in preclinical models of different cancers. In parallel, human trials performed with DNA vaccines, which target some of the best-known tumor antigens, have shown clinical evidence of strong immunogenicity and preliminary evidence of clinical activity (Chudley et al. 2012; Thomas and Kwak 2012; Eriksson et al. 2013; Diaz et al. 2013; Ginsberg et al. 2010; Norell et al. 2010).

Of the several means of delivering a naked plasmid in vivo, sophisticated electroporators have recently rendered intramuscular plasmid electroporation, one of the most effective methods for securing safe, efficient DNA immunization both in animals and humans (Aurisicchio et al. 2013; Low et al. 2009). The induction of local inflammation at the injection site and the production of a pro-immunogenic microenvironment results in effective antigen cross-presentation which has been indicated as the principal route by which DNA vaccines elicit a potent antibody and cytotoxic response (Cavallo et al. 2006; Heath et al. 2004; Shedlock and Weiner 2000; Rice et al. 2008; Liu et al. 2008).

However, rather limited overall efficacy in inducing effective anti-tumor protection has so far been demonstrated (Stevenson et al. 2010; Kutzler and Weiner 2008) and no DNA vaccines have been licensed for human use to date. Preclinical studies have revealed that one reason for failure is the insufficient induction of the $CD4^+$ T cell help engagement provided by DNA vaccine coding for the oncoantigen alone (Janssen et al. 2003). Besides the use of xenogeneic sequence of the oncoantigen, several other strategies have been developed and tested. Among them, the coinjection of the vaccine with a $CD4^+$ T cell inducing peptide such as the Pan HLA-DR epitope (PADRE) peptide or the use of long peptides, which have the advantage of both potentially activating T cell help and also being preferentially processed by DCs (Melief and van der Burg 2008). A more recent approach is represented by the use of DNA vaccines coding for fusion proteins between the oncoantigen and tetanus toxin or plant viral coat proteins (Stevenson et al. 2011). This strategy has been demonstrated to be highly effective when tested in mice, and although not yet in clinical trials, it has considerable potential.

Nevertheless, it is important to mention that DNA vaccination is the youngest of the vaccination strategies for cancer immunotherapy and, thus, has probably not yet lived up to its potential. Moreover, if one takes into account the fact that a timeline of more than a century was needed for the development of antibodies into effective cancer drugs, DNA vaccines may yet be considered to be on fast track development.

3.3 Hybrid Plasmids Coding for Chimeric Antigens

Thanks to the capability of xenoantigens to induce a potent cross-reactive T cell immune response against self-tolerated TAAs, xenovaccination using DNA has been demonstrated to effectively halt tumor growth in several preclinical mouse model of cancer (Cavallo et al. 2014). However, it must be noted that an antibody-mediated contribution may be fundamental in hampering tumor growth rather than just T cell immunity, depending upon the oncoantigen used and the disease model. Indeed, as discussed above, the role of $CD8^+$ T cells is prominent in the case of oncoantigens with intracellular localization (class III oncoantigens), while the contribution of $CD4^+$ T cells and antibodies might be determinant in the case of oncoantigens expressed on the cell membrane (class I oncoantigens). Several studies have demonstrated that self-reactive antibodies are better induced by autologous rather than xenogeneic vaccination, reflecting the exquisite specificity for the cognate antigen of antibodies induced by DNA vaccines (Jacob et al. 2006, 2010; Quaglino et al. 2010). Therefore, one of the major issues concerning xenogeneic vaccination is the low-affinity antibody response generally induced against the self-homolog protein, thus limiting the anticancer efficacy of the vaccine.

One possible means by which to induce a combined potent cross-reactive T cell and antibody response is to use hybrid plasmids which code for chimeric proteins that include both xenogeneic and homologous oncoantigen domains. The potential efficacy of these plasmids relies on the presence of the homologous sequence which ensures the specificity of the immune response and the presence of the xenogeneic determinants which are instrumental in circumventing immune tolerance. In particular, the chimeric protein produced by transfected cells can be uptaken by DCs and also be recognized and internalized by B cells. In this way, peptides from both the xenogeneic and homologous domains of the internalized chimeric protein are presented by DCs and B cells through MHC class I (MHC I) and class II (MHC II) glycoproteins. The presentation of xenogeneic peptides by DCs is instrumental for the effective priming and the expansion of both $CD8^+$ and $CD4^+$ T cells which are specific for xenogeneic moieties and potentially cross-reactive against the homologous one. The interaction between the expanded $CD4^+$ T cells with B cells, which recognize the xenogeneic domain of the chimeric protein, leads to the differentiation of plasma cells which produce antibodies which are specifically against the xenogeneic domain of the vaccine. By contrast, the interaction of expanded $CD4^+$ T cells with B cells which recognize the homologous domain leads to the production of antibodies which are specific for the self-tolerated homologous domain of the protein, by differentiated plasma cells (Fig. 1). The slight differences in the amino acid sequence and in the tertiary structure of the chimeric protein, encoded by the hybrid plasmids, may result in the exposition of subdominant and/or new conformational epitopes, triggering an even more efficient humoral immune response than that induced by the xenogeneic and homologous vaccines.

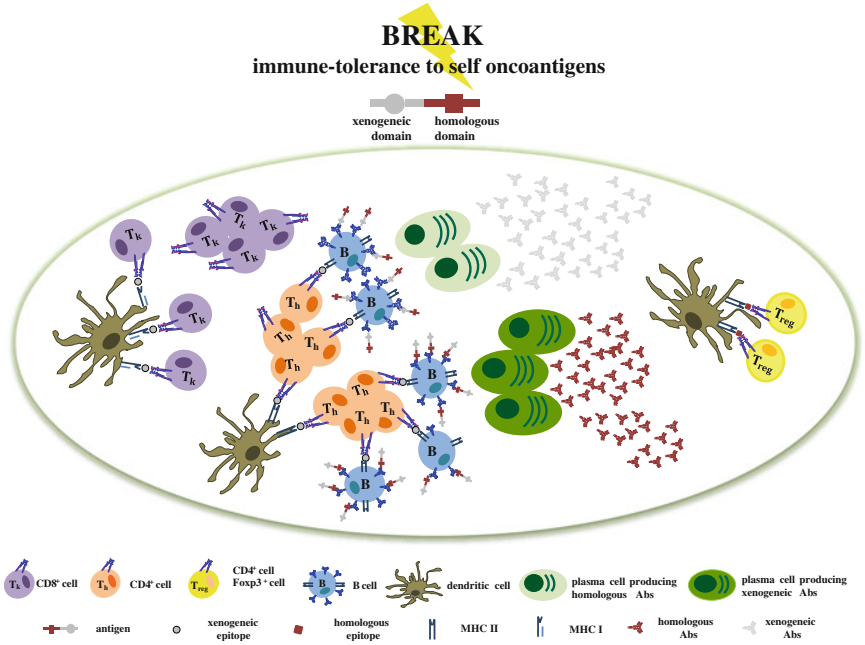

Fig. 1 Schematic representation of the mechanisms leading to the induction of potent cross-reactive T cell and antibody responses using chimeric proteins which combine xenogeneic and homologous domains of the self-oncoantigen. The chimeric protein used as vaccine is taken up by DCs and recognized by B cells that internalize it and present peptides through MHC II. The presentation of xenogeneic peptides (*white circles*) by DCs is instrumental for the effective priming and the expansion of both $CD8^+$ and $CD4^+$ T cells. The interaction between the expanded $CD4^+$ T cells with B cells, which recognize the xenogeneic domain of the chimeric protein, leads to the differentiation of plasma cells which produce antibodies which are specifically against the xenogeneic domain of the vaccine (xenogeneic-Abs). By contrast, the interaction of expanded $CD4^+$ T cells with B cells which recognize the homologous domain (*red squares*) leads to the production of antibodies which are specific for the self-tolerated homologous domain of the protein (homologous-Abs), by differentiated plasma cells. The presentation of peptides which derive from the homologous domain of the vaccine by DCs, through MHC II, leads to the expansion of Tregs

In principle, the strategy of combining heterologous with self-antigens can be applied to any TAA and could provide an initial starting point for the development of an effective anti-tumor vaccine to be used in clinic.

3.4 Significant Mouse Models for the Study of ErbB2$^+$ Breast Cancer

The evaluation of the power of hybrid DNA vaccines, which code for chimeric proteins, unquestionably requires the availability of appropriate animal models. Since immune tolerance to TAAs in cancer patients poses a major obstacle to the

mounting of an effective anti-tumor immune response, the development of genetically engineered mice which carry an oncoantigen as a transgene offers an unprecedented opportunity to evaluate vaccine efficacy. The early expression of the oncoantigen in the thymus of transgenic mice causes immune tolerance that may be analogous to that observed in cancer patients.

In view of the causal correlation between ErbB2 receptor overexpression and mammary carcinogenesis, several laboratories have assessed the tumorigenic potential of ErbB2 in the mammary epithelium of transgenic mice, over the last 20 years (Quaglino et al. 2008). Indeed, several strains of ErbB2-transgenic mice which overexpress the rat or human ErbB2 ortholog and which spontaneously develop mammary tumors have been generated. They mimic crucial features of the human nature of breast cancer, such as slow progression, the natural occurrence of invasion and metastasis, and the presence of a long-lasting interaction between the evolving tumor and the host immune system (Cavallo et al. 2006).

One of the first and more commonly used genetically engineered mouse models in immunological studies is transgenic for an activated form of the rat ErbB2 protein under the transcriptional control of the mouse mammary tumor virus promoter long terminal repeats (MMTV-LTR promoter). The single-point mutation at position 664 in the transmembrane domain of the rat ErbB2 receptor, involving the replacement of valine with glutamic acid, results in the formation of homo- and heterodimers that spontaneously transduce the proliferative signals that are responsible for the transformation of mammary epithelial cells (Boggio et al. 1998). Rat ErbB2 transgenic mice acquire a central tolerance to rat ErbB2 as its early expression in the thymus causes a central deletion of T cell clones that react with high affinity to dominant rat ErbB2 peptides (Rolla et al. 2006; Ambrosino et al. 2006). All rat ErbB2 transgenic females are genetically predestinated to develop mammary tumors at 100 % penetrance. Carcinogenesis is completed over the course of 5 months, and spontaneous lung metastases become evident during the late stages of tumor progression. Notably, mammary carcinogenesis in ErbB2 transgenic mice displays consistent, stepwise, and directly age-related progression that mimics several features of human breast carcinoma (Quaglino et al. 2008). Moreover, the progressive expansion of both Treg cells (Ambrosino et al. 2006) and MDSC (Melani et al. 2003) can be measured in function of tumor progression and both further hamper the induction of an effective immune response. Similar observations have been made in human cancer patients.

Although rat ErbB2 transgenic mice provide an interesting and useful model, the 10% difference in amino acid sequence between rat and human ErbB2 receptors may be of critical importance for the accurate evaluation and translation of immunotherapeutic strategies against ErbB2 in patients. This concept led to the generation of human ErbB2 transgenic mouse models. Indeed, several strains have been generated, including those in which the human ErbB2 transgene is under the transcriptional control of the whey acidic protein (Piechocki et al. 2003), or of the MMTV-LTR promoter (Finkle et al. 2004). Both these mouse models are fully tolerant to human ErbB2 (Piechocki et al. 2003); however, only MMTV-LTR-

ErbB2 transgenic mice develop spontaneous asynchronous mammary tumors, although with long latency and incomplete penetrance.

The availability of these transgenic mice is of crucial importance to the preclinical evaluation of the efficacy of ErbB2 DNA vaccines and their potential benefits. However, they are all subject to major limitations as conventional transgenic mice do not model the sporadic nature of human cancer. Indeed, while the initiating mutation that drives cancer development in humans occurs in a single cell surrounded by a genetically wild-type microenvironment, early and widespread oncogene overexpression in the mammary epithelium does not follow the same expression pattern in ErbB2 transgenic mice giving rise to multifocal and scattered neoplastic lesions all over the mammary glands. Therefore, the efficacy of the anti-ErbB2 vaccines tested so far may be somehow underestimated and the generation of an ErbB2 conditional mouse model in which the oncogene is expressed in a tissue-specific and time-controlled manner could be instrumental to more faithfully gaining insight into the treatment of breast cancer in the future.

3.5 ErbB2 Chimeric DNA Vaccines

As previously described, the ErbB2 oncoantigen can be considered a peculiar archetype of self-tolerated TAA and one of the "ideal" targets to be used as a proof of concept for the effectiveness of hybrid plasmids. Existing immunological tolerance against ErbB2 may, indeed, represent a barrier to the effective vaccination of $ErbB2^+$ cancer patients; however, extreme sensitivity to the antibody-mediated immunity of most $ErbB2^+$ tumors (Wong and Lee 2012) points to DNA vaccination with ErbB2 hybrid plasmids as an appealing strategy for anti-tumor immunotherapy.

Actually, a DNA vaccination strategy using a plasmid which codes for the full-length human ErbB2 protein in conjunction with GM-CSF and IL-2 in patients with advanced breast cancer has been proven to be safe, well tolerated, and effective in inducing long-lasting cellular and humoral immune responses against the oncoantigen. However, measurable therapeutic clinical benefits have not been observed, most probably because of the chemo- and radiotherapy-induced immunosuppression of the patients and of the use of a homologous antigen, which is powerless to effectively circumvent immune tolerance, as a vaccine (Norell et al. 2010).

Considering the potential and the limits of xenogeneic vaccines, a significant amount of effort has been invested in producing hybrid plasmids that code for chimeric ErbB2 proteins which are partly derived from xenogeneic and partly from homologous sequences, with the final aim of inducing both a specific cellular and humoral response. The high amino acid homology (84.5 %) between rat and human extracellular (EC) and transmembrane (TM) domains of the ErbB2 protein has spurred us to generate two different plasmids which code for chimeric rat/human and human/rat EC-TM domains of the ErbB2 protein (named RHuT and HuRT plasmids, respectively) (Quaglino et al. 2010). While the chimeric plasmid RHuT

encodes for a protein in which the 410 NH_2-terminal residues are from the rat ErbB2 domain and the remaining residues are from the human ErbB2 domain, almost symmetrically, HuRT encodes a protein in which 390 NH_2-terminal residues are from the human domain and the remainder from the rat domain. The availability of both rat and human ErbB2 transgenic mice has proven to be of crucial importance to the preclinical evaluation of the potential benefit of using hybrid RHuT and HuRT plasmids as compared to those which code for fully rat (RRT) or fully human (HuHuT) EC-TM ErbB2 domains.

When RRT and the HuHuT plasmids were used as autologous vaccines in rat and human ErbB2 transgenic mice, respectively, they induced high levels of antibodies with consummate specificity to the ErbB2 ortholog, used as an immunogen, but the T cell response was almost nil, as expected. However, a delay in cancer progression was observed, thanks to the pivotal role that antibodies play in the inhibition of $ErbB2^+$ tumors (Quaglino et al. 2010; Jacob et al. 2010). By contrast, when the RRT and the HuHuT plasmids were used as xenovaccines in human and rat ErbB2 transgenic mice, respectively, the humoral and cellular immune response induced were poorly cross-reactive against the self-oncoantigen expressed by the neoplastic cells (Iezzi et al. 2012) and thus completely ineffective in hampering the growth of transplantable or autochthonous ErbB2 tumors (Quaglino et al. 2010; Jacob et al. 2010), suggesting that xenogeneic vaccination may not be the strategy of choice in this model of cancer.

The use of hybrid RHuT and HuRT plasmids was found to be superior both to the fully autologous and to the fully xenogeneic vaccines in inducing a protective anti-tumor immune response against $ErbB2^+$ transplantable and autochthonous tumors, highlighting the importance of hybrid plasmids as effective anti-tumor vaccines.

The generation of a robust B and T cell response to the ErbB2 self-antigen together with a measurable anti-tumor effect in ErbB2 transgenic, tolerant mice (Quaglino et al. 2010, 2011), provided proof of concept of the power of combining target antigen heterologous and self-sequences for effective anti-tumor DNA vaccination. However, the rational design of chimeric DNA vaccines has to be carried out in order to potentially broaden this strategy to any TAA.

3.6 How to Design an Effective Hybrid Plasmid

Although the basic concepts behind the potential advantages of using hybrid instead of xenogeneic or homologous plasmids are clear, setting up the strategy for the design of an effective vaccine which codes for the oncoantigen of interest is less obvious and more complicated.

Seeing as the final goal of the hybrid plasmid strategy is the translation of chimeric vaccine into clinical practice, part of the antigen sequence must be derived from the human sequence. As far as the choice of species from which to derive the xenogeneic sequence used as the vaccine determinant is concerned, no defined

indications are readily available. In principle, this heterologous sequence has to be different enough from that of the self-tolerated antigen in order to provide the heteroclitic peptides which are necessary to overcome T cell tolerance (Kianizad et al. 2007). However, it must, at the same time, be sufficiently similar to guarantee an effective cross-reactive immune response (Jacob et al. 2010). In general, a good degree of homology with the self-homologous oncoantigen can range from 85 to 95% (Cavallo et al. 2014). Practically, the choice of the xenogeneic part, from the several options available, can be dictated by the preclinical model that will be used to evaluate the efficacy of the chimeric vaccine. An example of this is provided by our chimeric human/rat ErbB2 vaccines described above. Moreover, other important issues to take into consideration in the generation of effective vaccines are both the location of the autologous and the xenogeneic domains in the chimeric protein and the ability of the hybrid plasmid to give rise to a protein which presents a structural conformation which is similar to the homologous one. Finally, it has been suggested that vaccine-encoded specific homologous sequences may actually activate the Treg population, thus limiting vaccine-induced anti-tumor immune response (Jacob et al. 2010). Therefore, when designing a hybrid plasmid for a given oncoantigen, self-regions involved in Treg expansion should be defined and replaced with the corresponding xenogeneic sequences. Based on all previous considerations, the design of hybrid vaccines which target oncoantigens should be theoretically performed once the critical regions for immune activation in the self and in the heterologous antigens have been clearly defined.

As far as our oncoantigen of choice, the ErbB2 receptor, and the two different hybrid plasmids generated which combine rat and human ErbB2 sequences are concerned, the rationale behind our design was formed around the availability of a consistent model of breast cancer in which the development of autochthonous mammary tumors is dictated by the expression of a rat ErbB2 transgene. In these mice, the rat ErbB2 is a fully tolerated self-protein. Moreover, rat ErbB2 has a high degree of homology with the human counterpart. To assess the weight of the position of the xenogeneic determinants, we compared the immunogenicity of two hybrid plasmids which differ from one another simply in the location of the rat and human domains in the coded chimeric protein.

Superior immunogenicity and anti-tumor efficacy over fully homologous or fully xenogeneic vaccines was demonstrated by both hybrid plasmids. This suggests, first of all, that the slight differences in the amino acid sequence between the human and rat EC-TM domains of the ErbB2 receptor are enough to circumvent self-tolerance (Quaglino et al. 2010, 2011). Moreover, the anti-tumor efficacy of the RHuT plasmid was found to be superior to HuRT when tested in ErbB2-transgenic and tolerant mice, suggesting that, in this preclinical model, the optimal immune response is elicited when the NH_2-terminal portion of the chimeric protein and the corresponding portion on the targeted ErbB2 orthologue are identical. The availability of mice which are transgenic and tolerant for the human ErbB2 protein allowed us to further confirm this result; HuRT was more immunogenic than RHuT, in this context (Quaglino et al. 2010, 2011). Moreover, it has been demonstrated that there is a Treg activation epitope in the human ErbB2 protein, embedded in the

amino acid sequence ranging from amino acid 501 to 687 (Jacob et al. 2010). The replacement of this Treg-activating sequence with the corresponding rat sequence in the HuRT plasmid may explain its superior efficacy over the fully human ErbB2 vaccine.

This finding was also evident when the ability of ErbB2 hybrid plasmids to activate the suppressive effects of the tumor-induced Tregs, derived from ErbB2$^+$ cancer patients, was compared with that of fully human ErbB2 plasmid (Occhipinti et al. 2014). Indeed, while cancer patient DCs which had been transfected with the fully human HuHuT plasmid promoted the suppressive activity of autologous Tregs, those transfected with chimeric RHuT and HuRT plasmids were found to be ineffective at Treg activation. Therefore, these human surrogate experiments have provided proof of concept that chimeric rat/human ErbB2 DNA plasmids are useful in overcoming tumor-induced T cell dysfunction in ErbB2$^+$ cancer patients.

4 Conclusions

It is when oncology meets immunology that cancer immunotherapy becomes a reality. Strengthening a patient's own immune response against cancer cells is one of the most challenging and exciting concepts in active cancer immunotherapy. Thanks to striking progress in both the understanding of anti-tumor immune response and the characterization of several TAAs, a more rational design and more sophisticated strategies for anti-tumor vaccination have been possible. In this evolving scenario, selecting the best tumor target and the most appropriate vaccine administration route are of paramount importance. Only when vaccines are directed against oncoantigens can effective tumor control be achieved. DNA vaccines are an attractive and potentially effective tool for oncoantigen-specific cancer immunotherapy, and electroporation is one of the most sophisticated and promising strategies of the different delivery approaches tested so far.

However, despite successful studies in murine models, the anti-tumor efficacy of DNA vaccination in human clinical trials has been modest, reflecting the difficulty found in translating the plethora of convincing preclinical and early clinical results to an effective treatment for patients. Existing immunological tolerance against self-oncoantigens and tumor-suppressive mechanisms and the consequent difficulty in inducing an effective anti-tumor immune response may dramatically impact the success of cancer vaccination. Moreover, several studies have reported that despite the robust induction of specific antibodies, a paradoxical expansion of Tregs, of immunosuppressive phenotypes, is associated with vaccines that code for self-sequences.

Vaccination with a xenogeneic protein or peptide has been labeled as an effective means by which to overcome both central and peripheral immunological tolerance to self-proteins. The effective triggering of T cells can be induced, thanks to the generation of the so-called heteroclitic epitope. However, only low-affinity and poor

cross-reactive humoral immune responses have been measured, because of the exquisite specificity of antibodies.

The ability of hybrid plasmids, which combine xenogeneic and homologous domains of the oncoantigen, to harness the power of xenogeneic vaccination to circumvent immune tolerance without losing the specificity of the immune response elicited by autologous vaccines may be a useful addition to the immunotherapeutic armamentarium for the fight against cancer. A demonstration of the power of this strategy is offered by the superior immunogenicity and anti-tumor efficacy elicited by the two rat/human (RHuT and HuRT) hybrid plasmids as compared to that of their fully rat (RRT) or fully human (HuHuT) counterparts in an ErbB2-tolerant context. Taken together, our preclinical data offer a proof of concept for the power of ErbB2 chimeric vaccines in cancer immunotherapy and have laid the foundation for the approval of a phase I clinical trial in patients with ErbB2^{+} carcinomas (EudraCT Number: 2011-001104-34).

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Linked CD4 T Cell Help: Broadening Immune Attack Against Cancer by Vaccination

Natalia Savelyeva, Alex Allen, Warayut Chotprakaikiat, Elena Harden, Jantipa Jobsri, Rosemary Godeseth, Yidao Wang, Freda Stevenson and Christian Ottensmeier

Abstract In the last decade, immunotherapy with monoclonal antibodies targeting immunological check points has become a breakthrough therapeutic modality for solid cancers. However, only up to 50 % of patients benefit from this powerful approach. For others vaccination might provide a plausible addition or alternative. For induction of effective anticancer immunity CD4+ T cell help is required, which is often difficult to induce to self cancer targets because of tolerogenic mechanisms. Our approach for cancer vaccines has been to incorporate into the vaccine design sequences able to activate foreign T cell help, through genetically linking cancer targets to microbial sequences (King et al. in Nat Med 4(11):1281–1286, 1998; Savelyeva et al. in Nat Biotechnol 19(8):760–764, 2001). This harnesses the non-tolerized CD4 T cell repertoire available in patients to help induction of effective immunity against fused cancer antigens. Multiple immune effector mechanisms including antibody, CD8+ T cells as well as CD4 effector T cells can be activated using this strategy. Delivery via DNA vaccines has already indicated clinical efficacy. The same principle of linked T cell help has now been transferred to other novel vaccine modalities to further potentiate immunity against cancer targets.

Contents

N. Savelyeva (✉) · A. Allen · W. Chotprakaikiat · E. Harden · R. Godeseth · Y. Wang · F. Stevenson · C. Ottensmeier
Cancer Sciences Unit, Faculty of Medicine, University of Southampton, Tremona Road, Southampton SO16 6YD, UK
e-mail: N.Savelyeva@soton.ac.uk

W. Chotprakaikiat · J. Jobsri
Oral Biology Department, Naresuan University, Phitsanulok, Thailand

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1 Introduction

Cancer vaccination aims to stimulate the patient's own immune system to fight tumours. The induced immunological response should be specific to the cancer cells, with minimal effect on normal tissues. Vaccines that have been approved by the FDA include prophylactic vaccines against viruses that cause malignancies, for example, the hepatitis B virus which drives hepatocellular carcinoma (Arbuthnot and Kew 2001) and the human papilloma virus (HPV) which causes cervical cancer (Mariani and Venuti 2010). Both vaccines are aimed at inducing antiviral antibody to prevent the infection that causes malignant transformation. The first therapeutic vaccine for patients with established cancer is Provenge® (Sipuleucel T) which has also been recently approved. This dendritic cell (DC) vaccine targets a protein expressed not by an infectious agent but by the cancer, prostate acid phosphatase (PAP). It is used for metastatic castration resistant prostate cancer patients (Kantoff et al. 2010). Several cancer vaccines are currently being developed or are in late stage clinical testing (Quoix et al. 2015) (NCT02187367).

For clinical success there are several major hurdles including tolerance and immunoregulation which must be overcome by vaccination.

1.1 Tolerogenic Pressure

1.1.1 T Cell Immunity

A number of mechanisms exist that prevent the immune system from attacking the host's own tissues, a concept defined as self-tolerance. Failure or deficiency of these mechanisms results in autoimmune responses. Central tolerance in the thymus and bone marrow ensures that newly formed T lymphocytes are not capable of recognizing self-peptides presented by MHC through positive and negative selection (reviewed in Xing and Hogquist 2012).

Although central tolerance is effective, it does not eliminate all potentially self-reactive T cells and hence a T cell repertoire against self-cancer targets is retained. To compensate, peripheral tolerogenic mechanisms act on mature lymphocytes that have escaped central tolerance. Regulatory T cells (Tregs) are crucial for maintaining peripheral tolerance; several subsets of Tregs exist. $Foxp3^+$ regulatory T cells can be induced in the periphery to control the response to tissue-restricted antigens not expressed by the thymus (Gregerson et al. 2009); these are known as iTregs (induced Tregs). A second type of Tregs derives from high-affinity self-reactive CD4 T cells, which are converted to $Foxp3^+$ Tregs in the thymus before exiting to the periphery (Martin et al. 2013). These cells are known as nTregs (natural Tregs) (Hsieh et al. 2012).

Tregs inhibit anti-self-immune responses in the secondary lymphoid organs, as well as within tumours, by a number of mechanisms involving secretion of suppressive cytokines IL-10 (Rubtsov et al. 2008) and tumour growth factor-β (TGF-β) (Wan and Flavell 2007), granzyme-dependent killing of responder cells (Cao et al. 2007), and others mechanisms (Bopp et al. 2007). Contact-dependent inhibition of DC activation through CTLA4 by binding to CD80 and CD86, and LAG-3 (lymphocyte activation gene-3) binding to MHC II have been described (Sansom 2000). Tregs can also operate directly in the tumour by delivering inhibitory signals to T cells (Facciabene et al. 2012; Weinberg et al. 2011).

Other components of the tumour microenvironment, as well as the tumour itself can exert tolerising pressure on T cells (Quail and Joyce 2013). Recently attention has focused on identification of mechanisms that contribute to a dysfunctional or exhausted phenotype of tumour-localized effector T cells (Wherry and Kurachi 2015). Targeting these inhibitory pathways, or so-called checkpoints molecules, is proving to be highly efficient for therapy of cancer (reviewed in Perez-Gracia et al. 2014).

1.1.2 B Cell Immunity

For induction of antibody, tolerogenic mechanisms will affect both the CD4 T cell compartment, essential for induction of antibody, and also B cells directly. Central tolerance will eliminate most self-reactive B cells through receptor editing, deletion and anergy (Nemazee 1996) but low avidity self-reactive B cells may survive. In the periphery self-reactive B cells are subjected to further regulation directly by antigen through the B cell receptor (BCR) and this can result in anergy (Goodnow et al. 1988; Merrell et al. 2006). In contrast to T cells, B cells can undergo affinity maturation in the germinal centre through the process of somatic hypermutation (MacLennan 1994). Therefore, there is a chance for low affinity B cells to increase their affinity in the germinal centre. However this requires functional non-tolerised CD4 T cells and a particular T cell subset, follicular T cells, are crucial (Vinuesa et al. 2005). Again there is an inhibitory counterpart for this process: follicular Tregs have been found to inhibit the germinal centre reaction and hence induction of high-affinity antibody (Chung et al. 2011).

1.2 Choice of Target Antigen: Specific, Neoantigens and Shared Antigens

The tumour antigen is the key component of a cancer vaccine. Tumour antigens (TAs) arise from viruses, mutated proteins or from overexpressed normal proteins (Stevenson et al. 2004b). The identification and characterisation of TAs was initially achieved using expression libraries of human tumours probed with autologous serum (SEREX) (Jager 2007). This led to the detection of anti-TA T cell responses in cancer patients (Jager 2007). Ideally, the antigen should only be found in the cancer cell and not expressed in any normal, healthy tissues to prevent autoimmunity. Tumour antigens can be broadly categorized into tumour-specific antigens (TSA) expressed only in cancer and tumour-associated antigens (TAA) with expression in cancer and other tissues.

The success of prophylactic vaccination against HPV with Cervarix (Monie et al. 2008) and Gardasil (Schiller et al. 2012) vaccines has raised the possibility of HPV as an attractive vaccine target post-infection. The HPV-derived oncoproteins E6 and E7 are cancer specific and also non-self (King et al. 2015). HPV infection causes cervical, ano-genital and head and neck squamous cell carcinoma with a large unmet clinical need and >500.000 deaths annually from HPV-driven malignancies worldwide (Warnakulasuriya 2010). DNA vaccination targeting E6 and E7 proteins has demonstrated induction of robust T cell responses followed by regression of precancerous cervical disease in a randomized phase II clinical trial (Trimble et al. 2015).

The idiotypic Ig of B cell malignancies arising as a result of variable Ig gene rearrangements and somatic hypermutation is unique for every B cell and is an excellent example of a TSA (McCarthy et al. 2003). Its identification several decades ago was followed by clinical targeting with vaccination (McCarthy et al. 2003; Villanueva et al. 2011). More recently novel technologies have allowed rapid manufacturing of these personalized vaccines for clinical application (McCann et al. 2015; Bendandi et al. 2010; Tuse et al. 2015). Mutated proteins such as K-Ras and p53 are another type of TSA (Abrams et al. 1996). These have been of interest as cancer targets for many years but have been difficult to deliver (Carbone et al. 2005).

Further advances in deep sequencing methods have permitted a rapid identification of novel antigens or neoantigens that arise from tumour-specific mutations (Schumacher and Schreiber 2015; Vigneron et al. 2013). Because these neoantigens arise within the tumour itself, the specific T cells are unlikely to have been subjected to central tolerance. In theory this means that there will be a wider T cell repertoire with a higher affinity for pMHC, which would be advantageous for any cancer vaccine. However, they are likely to be subjected to peripheral tolerance mechanisms. A surprising finding has been that neoantigenic peptides are not only restricted by MHC I but can also be MHCII restricted (Schumacher et al. 2014; Tran et al. 2014; Kreiter et al. 2015; Zanetti 2015). For only a small minority of

these neoantigens T cell reactivities can be detected. This is presumably because the majority of reactive T cells have been tolerised in the periphery. Furthermore, owing to the fact that these novel sequences arise from within the tumour itself, they will rarely be shared among patients. Melanoma, colon, glioma and breast cancer are the examples of tumours where neoantigens have been found in humans (Schumacher et al. 2014; Tran et al. 2014; Linnemann et al. 2015; Kreiter et al. 2015). Both the financial and practical implications of 'personalized' cancer immunotherapy are a significant consideration, but intensive international efforts are underway to find technical solutions to this challenge. An important question also is how many of these mutated epitopes need to be targeted by one successful vaccine. The most likely scenario will be that a small number of targets may be enough to kick-start the immune system before 'epitope spreading' occurs (el-Shami et al. 1999; Ribas et al. 2003). Future experimental evidence will shed light on these important considerations.

TAA are expressed by both cancer and normal tissues and include cancer-testis antigens (CTAs), differentiation antigens and overexpressed antigens (reviewed in Buonaguro et al. 2011; Fratta et al. 2011). These antigens have received considerable attention in vaccine development over the years driven by the desire to vaccinate cohorts of patients in a consistent way. Where TAA are expressed in multiple types of tumour and in significant proportions or even in all patients with one cancer type, an off-the-self vaccine can be generated. The first approved cancer vaccine Provenge is the DC-based vaccine targeting TAA prostatic acid phosphatase (PAP) and is a successful example of this approach (Sonpavde et al. 2012). Several vaccines that target TAAs (hTERT, Her2, Muc1) are in late stage clinical trials with promising results (Acres et al. 2015; Quoix et al. 2015; Rochman 2015) (NCT02301754, NCT02327468, NCT02293707).

1.3 Activation of Relevant Immune Anticancer Mechanisms

Recognition of TAs by induced immune mechanisms is essential for cancer clearance. TAs are present on the cell surface, as soluble factors or located in the cytosol. Cell surface antigens such Her2-3, Id Ig, mammaglobin A (Tiriveedhi et al. 2014; Zuo et al. 2009), xCT (Lanzardo et al. 2016) and others can be recognized by antibody which in turn can induce several mechanisms of tumour elimination. Antibody-dependent cellular cytotoxicity, phagocytosis, direct antibody-induced apoptosis or cancer cell growth inhibition are the major mechanisms by which antibody causes tumour destruction (Weiner et al. 2009). Passive monoclonal antibody therapies targeting antigens on cancer cells have been very successful in the clinic demonstrating that this immune arm is effective for cancer elimination. Induction of potent antibody by active vaccination in clinical testing is equally desirable (Damodaran and Olson 2012). Vaccine CIMAvax-EGF targets a soluble

antigen epidermal growth factor (EGF), the ligand for the EGF receptor which is overexpressed on lung cancer cells. This vaccine has been developed to induce neutralizing antibody against EGF to block its tumour growth-promoting effect (Rodriguez et al. 2010). Patients with high antibody responses had a remarkably better outcome than the control group of patients with IIIB/IV stage non-small cell lung carcinoma (Neninger Vinageras et al. 2008; Quaratino et al. 2014). From early 2016 patients in several centres in Europe and Asia are being recruited to test the vaccine in a phase III clinical trial for non-small cell lung carcinoma (NCT02187367).

The majority of antigens that have attracted attention so far, however, are intracellular proteins and hence will not be targeted by antibody. Immune attack relies instead on short, usually 9-mer, antigenic peptides presented in the context of MHC class I. These can be targeted by CD8+ cytotoxic T cells which will recognize the peptide MHC I complex and directly kill the target cell through perforins/granzyme or Fas-mediated mechanisms (Stevenson et al. 2013; Stevenson et al. 2004a). Most approaches to developing cancer vaccines have focused on activating this immune mechanism (Stevenson et al. 2013). A minority of cancers are MHCII positive including B cell lymphomas and some epithelial tumours. Here it is possible to direct targeting to peptides presented in the MHCII context by CD4 T cells (Savelyeva et al. 2001); (Savelyeva et al. 2003). Unlike the traditional helper function of CD4 T cells to CD8+ and B cells, cytolytic function of CD4+ T cells is less well defined. In this case, CD4 T cells are of the Th1 subset and direct killing is mediated through mechanisms involving Fas/FasL (Ju et al. 1994). Killing can also be mediated through other members of TNF-receptor ligand family, i.e. TRAIL, the ligand for Apo2 (TRAIL-R2 and R3, also known as DR4 and DR5, respectively) (Dorothee et al. 2002; Thomas and Hersey 1998) and TWEAK with Apo3 (DR3) as a receptor (Kaplan et al. 2000). Perforin/granzyme-mediated cytotoxity against MHC class II positive tumours has also been documented (Dorothee et al. 2002).

There are however only a few known MHC II positive cancers which can be targeted by CD4 T cells directly through recognition of tumour-specific peptides. For MHCII negative tumours CD4+ Th1 cells characterized by secretion of IFN-γ and TNF-a, can engage the effectors of the innate immune system. Killing of myeloma cells by Th1-activated macrophages is an example of where this mechanism has been very well defined (Haabeth et al. 2014). Natural killer (NK) cells can be also be engaged through similar mechanisms (Perez-Diez et al. 2007). Reprogramming the tumour microenvironment by Th1 cells may also lead to reduction of Tregs (Kreiter et al. 2015). MHCII positive APC such as macrophages or B cells have been found to provide the antigen specificity link by presenting the antigen to CD4 T cells (Haabeth et al. 2014; Tran et al. 2014). These previously underappreciated Th1 cells have now been found in several human tumours and their role in antitumour immunity is rapidly growing and their antitumour functions have been exploited in adoptive T cell therapies in metastatic cancer (Tran et al. 2014).

2 Targeting Cancer with Vaccination

A number of vaccine delivery vehicles have been explored to target cancer. They have been described in detail elsewhere (Melief et al. 2015). Here we will focus on genetic vaccines and include novel approaches to vaccination we have been developing.

2.1 DNA Vaccines

DNA vaccines are becoming promising modalities for cancer owing to their recent clinical advances (Trimble et al. 2015; Kraynyak et al. 2015). As with conventional vaccines, DNA vaccination aims to induce specific and durable humoral and cellular responses against TAs. A DNA cancer vaccine consists of a plasmid backbone acting as the natural adjuvant, the encoded specific cancer antigen and a strong viral promoter, usually CMV, is included to drive expression of the encoded tumour antigen. The adjuvant properties of plasmid DNA are linked to activation through TLR9 as well as cytoplasmic sensors of DNA (reviewed in Rice et al. 2008 and Barber 2011). Several immune sensors of plasmid DNA located in the cytoplasm, including Absent In Melanoma (AIM2), IFN-γ-inducible 16 (IFI16), DAI/ZBP and DNA-dependent protein kinase (DNA-PK), have been identified (Paludan and Bowie 2013). They all can contribute to adjuvant properties of plasmid DNA and promote the immune response to the encoded antigen. DNA vaccines can be easily manipulated by means of genetic engineering and additional sequences to improve immunogenicity of the target antigen, i.e. cytokines, chemokines or, as discussed below, a sequence for induction of linked T cell help, can be incorporated.

2.2 Fragment C of Tetanus Toxin in Fusion DNA Vaccines

TA often display similar properties to that of other self-proteins and, used alone, fail to induce adequate T cell responses owing to a low immunogenicity resulting from tolerogenic pressure. The DNA fusion vaccine concept was developed to overcome this barrier. Initial studies with protein vaccines focused on conjugating lymphoma-derived Id Ig antigen to the foreign protein keyhole limpet hemocyanin (KLH) (George et al. 1988; McCarthy et al. 2003). Unfortunately this had little success; first, the complex polymeric structure of KLH made it impractical within DNA vaccines and second, KLH induced a predominantly Th2 response, which we now know is not favourable for treating cancer (Timmerman 2009).

The carboxy-terminal fragment C (FrC) of tetanus toxin (TT) is a single polypeptide of approximately 50 kD. It is highly immunogenic and lacks the ability of whole TT to paralyze neuromuscular transmission, and hence lacks its toxicity

(Neubauer and Helting 1981; Cook et al. 2001). No undesirable clinical effects have been reported following its introduction into humans (Calvo et al. 2012).

Our laboratory first demonstrated the successful use of FrC for provision of linked CD4+ T cell help for poorly immunogenic cancer antigens in lymphoma (Spellerberg et al. 1997; King et al. 1998). We found that lymphoma-derived idiotypic (Id) Ig antigen by itself was unable to induce sufficient T cell help to drive the anti-Id antibody response. Protective anti-Id antibody was induced when we used a DNA fusion vaccine design consisting of V_H and V_L of Id Ig antigen assembled as scFv through a flexible linker and genetically linked to FrC (Id-FrC) (King et al. 1998). The Id-FrC fusion DNA vaccine was able to engage the non-tolerant CD4+ T cell repertoire available for FrC, to provide T cell help to Id-specific B cells to produce antibody (Fig. 1). Remarkably the vaccine was not only effective in the lymphoma model but the same design also generated protection against myeloma, which is Id surface-low/negative and hence not readily susceptible to targeting by antibody. The effectors which operated in this case were Id-specific CD4 Th1 cells capable of clearing Id Ig surface negative myeloma cells. Again only the Id-FrC fusion vaccine was effective; Id DNA vaccine without FrC failed to induce protection (King et al. 1998). We concluded that the Id-FrC design provided effective linked T cell help to both B cells and to weaker CD4 Th1 cells (Fig. 1). The study not only highlighted the requirement of foreign T cell help for antibody induction but also provided means of induction of CD4 Th mediated protective responses to weak cancer antigens. The latter was likely to involve the help-to-helper mechanism when DC activated by strong Th response are capable of activating weaker Th subsets in a similar way to how T-cell help operates for CTL induction (Gerloni et al. 2000).

The substantial immunological response achieved with this relatively simple constructed FrC fusion vaccine, led to early phase clinical trials of DNA Id-FrC fusion vaccines in patients with follicular lymphoma and multiple myeloma (MM). In the lymphoma study anti-Id antibody responses were seen in 38 % of patients (Rice et al. 2008) but problems with assessment of the efficacy of the vaccine arose because of the fact that the vaccine itself was patient-specific and therefore inter-patient comparisons could not be made. For MM we recently reported a Phase I clinical trial of an Id-FrC fusion DNA vaccine in patients with complete or partial responses following high-dose chemotherapy and autologous stem cell transplantation (McCann et al. 2015). The vaccine administered at least 6 months post-transplant was able to induce vaccine-specific CD4 T cell responses (McCann et al. 2015). Both FrC- and Id-specific CD4 T cells were detected in 29 % of patients, with 43 % of patients responding to FrC alone. These results are promising for a number of reasons. First, Id-FrC DNA vaccine can induce Id-specific CD4+ Th1 reactivity in the face of previous data indicating that patients with advanced MM shift towards a Th2 response (Yi et al. 1995). Second, the study demonstrated immune responses were observed even in patients previously treated with regimens reported to decrease the ability to mount an immunological response to vaccinations (Arrowood and Hayney 2002). Additionally, the study confirmed that Id-specific Th1 cells were not deleted in patients who, while they had a low burden disease at

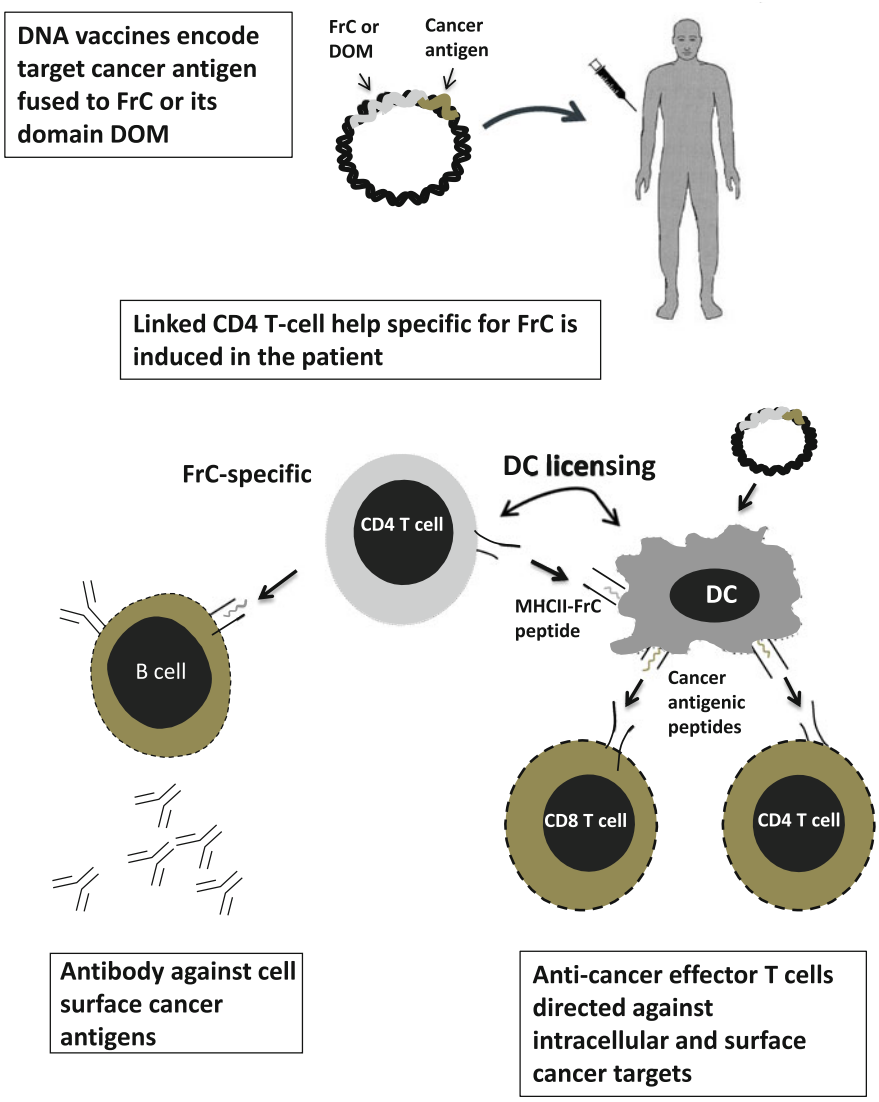

Fig. 1 Linked CD4+ T cell help induced by DNA vaccines encoding FrC or its domain DOM fused to target cancer antigen generates multiple immune mechanisms for cancer attack in patients. The key mechanism is induction of foreign FrC-specific T cell help which through licensing of dendritic cells (DC) leads to activation of CD8 and CD4 T cells specific for target cancer antigen. FrC-specific T cells also provide help to B cells to induce antibody against surface cancer targets

vaccination (with serum paraprotein below 50 g/L), previously had much higher levels of paraprotein. Deletion of Id-specific T cells (Bogen 1996) appears not to be an issue in our cohort therefore. Our data support that idiotypic vaccination is a valid concept for immunotherapy in this patient cohort.

2.3 The p.DOM-Epitope Design to Induce CTL Responses

As mentioned above most TA are intracellular and upon presentation in MHC I can be targeted by CD8 T cells. DNA vaccines are capable of inducing potent CD8+ T cell responses (Stevenson et al. 2013; Stevenson et al. 2004a). Lasting and efficient immunological responses require CD4 T cell help and FrC was expected to provide this. For cytotoxic responses however, a potential problem was identified. The natural CD8+ T cell response against viruses appears highly focused on only a few MHC Class I-binding epitopes, due to the phenomenon of immunodominance (Yewdell and Bennink 1999). This focus meant that delivery of cancer antigens via, for example, pox viral vectors, may fail due to competition from strong viral epitopes (Yewdell 2010). Failure has in fact been demonstrated in a clinical trial of an MVA-delivered melanoma antigen, where immune responses apparently focused on vector peptides rather than on the desired tumour peptides (Smith et al. 2005). We wondered therefore, whether FrC would also present strong epitopes for CD8 T cell attack and whether the immune response would focus on those rather than on the cancer-derived sequences. Indeed competitive epitopes were found in the C-terminal portion of FrC (Rice et al. 1999) therefore its subdomain, DOM was created through deletion of that portion (Rice et al. 2001). The DOM DNA fusion vaccine design decreased the potential competition from the carrier-derived MHC Class I-binding peptides (Rice et al. 2002). Tumour-derived peptide sequences have then been fused to the 3'-terminus of DOM to create the p.DOM-epitope design (Stevenson et al. 2004a). Using this design, we successfully demonstrated induction of high levels of epitope-specific CD8+ T cells in several preclinical models (Rice et al. 2008).

2.4 Clinical Experience with the p.Dom-Epitope Design

2.4.1 Induction of Immune Responses

Translation of preclinical studies of DNA vaccines to human patients initially appeared difficult. A hurdle for DNA cancer vaccines was the difficulty in scaling the amount and volume of the vaccine from mice to human subjects (Buchan et al. 2005). Since these initial attempts, there are now various methods to overcome this hurdle. Electroporation (EP) has emerged as a clinically viable method for DNA vaccination of cancer patients. The procedure is to pass an electric current across the tissue site at the same time as injection, or very soon after. The result is an increase in DNA transfection levels and therefore, antigen expression. Local inflammation was also increased through the electrical damage and also the activation of multiple sensors for dsDNA within the cell (Paludan and Bowie 2013). As a result, more antigen-presenting cells are attracted to the vaccination site, (Ahlen et al. 2007; Kraynyak et al. 2015) and significant immune responses can be induced even in large mammals.

Our own early experience evaluating immunogenicity of the p.Dom-PSMA epitope in patients with prostate cancer demonstrated low anti-DOM IgG antibody responses after i.m. delivery of DNA without EP (Low et al. 2009). In contrast, delivery of the vaccine with EP demonstrated significant levels of anti-DOM antibody and the responses were still seen after 18 months of follow up (Low et al. 2009). PSMA peptide-specific (PSMA27) responses were detected in 16 out of 30 vaccinated patients while 29 patients had DOM-specific Th1 responses. The clinical effects were visible as a significant increase of PSA doubling time in vaccinated patients (Chudley et al. 2012).

Clinical evaluation of a p.DOM-epitope fusion DNA vaccine without electroporation was carried out in 27 cancer patients in a multicenter clinical study (McCann et al. 2016). Patients were treated either with measurable disease after the failure of conventional treatment or in radiological remission. The vaccine targeted the HLA-A*0201 binding peptide CAP-1 from the carcinoembryonic antigen (CEA) linked to DOM. Both cellular responses against the CAP-1 peptide and humoral response against DOM were seen in patients without and with measurable disease. Mass-spectrometry confirmed that CAP-1 was expressed by cells in normal colon, primary colorectal tumour and metastatic colorectal tumour. ELISPOT and flow cytometry showed CAP-1 specific T cells in the peripheral blood confirming the immunogenicity of the vaccine. The vaccine was overall safe and well tolerated, but 48 % of patients reported diarrhoea. Interestingly, the bowel toxicity was linked to a better clinical outcome and immunological responses. Patients who reported diarrhoea were found to have higher DOM-specific responses with longer duration. Among patients with advanced cancer those with diarrhoea lived almost three times as long as those without diarrhoea, although the difference did not reach significance in this small cohort (McCann et al. 2016).

2.4.2 Understanding the Immunodominance Following p.DOM-Epitope Vaccine Delivery to Patients

One consideration we had in mind when designing the p.DOM-epitope vaccines was that the DOM portion should engage CD4 T cell help without inducing its own CD8 T cell responses. This was achieved by preserving Th epitopes and removing potential competitive CD8 epitopes including those restricted by HLA-A2 (Rice et al. 2001). Several studies in humanized HHD mice that are transgenic for the human HLA-A2 demonstrated lack of competition from DOM sequences and induction of robust CD8 responses to the chosen epitope (Joseph-Pietras et al. 2010; Campos-Perez et al. 2013).

Following DNA EP administration of p.DOM-epitope from WT-1 tumour antigen (WT1.37) to HLA-A2+ leukaemic patients, we were able to demonstrate induction of WT1.37-specific CD8+ CTL ex vivo. In parallel, using an overlapping peptide library to evaluate CD4 and CD8 responses to the DOM portion, we found not only robust CD4 T cell responses but unexpectedly also CD8 responses. When we next monitored the WT1 epitope and DOM CD8 T cell responses in patients over

time we found simultaneously induced responses to both WT1 and DOM epitopes. Reactivity to a new HLA-A2-restricted epitope from DOM was detected in patients, but there was no antigenic competition with the tumour-derived epitope (manuscript submitted). These findings were retrospectively confirmed in patients vaccinated with DOM-PSMA27 with prostate cancer. Our data demonstrate that in a cancer setting there is an opportunity to deliver more than one epitope simultaneously, with a possibility of induction of a broader CTL immunity directed to multiple peptides.

2.5 *Preexisting Immunity to TT Immunity and Performance of Vaccines Containing TT-Derived Fragments*

One challenge faced by clinicians seeking to use TT-derived vaccines is the presence of preexisting antibodies to FrC, which might slow the induction of subsequent immunity when FrC is used as a carrier (Forconi et al. 2002). Such antibodies are highly prevalent across most patient populations, owing to the successful adoption of prophylactic vaccination against *C. tetani* and to the use of conjugate vaccines where TT acts as carrier for the bacterial polycaccharide (Miller et al. 2011). Our data suggest that antibody to DOM does not cross-react with those against TT protein (Low et al. 2009). It appears likely that for the humoral arm of the immune system DOM is perceived as a neoantigen, even after prior exposure to prophylactic TT vaccine. In contrast, preexisting TT-specific CD4 T cell immunity recognizes the epitopes in DOM sequence. This T cell memory can lead to accelerated responses to DOM-containing vaccines.

Interestingly pre-vaccination with TT has been recently observed to have unexpected enhancing effects on the subsequent efficacy of dendritic cell cancer vaccines in patients with glioblastoma. Preconditioning of the vaccination site with the conventional TT/DT vaccine led to improved migration of dendritic cells and improved patient survival. This phenomenon was mediated by CD4+ T cells induced by the conventional vaccine as their depletion led to significant decrease of the effect when the phenomenon was modelled in mice (Mitchell et al. 2015). This unexpected effect of CD4 T cell help is not fully understood but thought to be mediated at least in part by the pro-inflammatory chemokine CCL3. Combination of TT-specific linked T cell help through FrC or DOM-containing vaccines with preconditioning of the vaccination site might lead to further improvement of TA-specific cancer vaccines.

2.6 *Novel Vaccination Approaches Based on FrC Linked T Cell Help*

We have recently developed a novel FrC-based vaccine to target Her2+ve cancers via antibody. Her2 (ERBB2) is an excellent target as it is overexpressed on many

solid cancers and its expression is associated with poor prognosis (Berchuck et al. 1990; Slamon et al. 1987). Passively transferred Mab Herceptin targeting Her2 has demonstrated efficacy in the clinic; a second monoclonal antibody pertuzumab has recently been approved (Hudis 2007; Swain et al. 2015).

However induction of potent anti-Her2 antibody by vaccination in patients is also attractive. To drive antibody responses to Her2 we used a novel truncated Her2 protein fragment ED44 which includes III and IV domains of Her2 (Chotprakaikiat et al. 2016). The ED44 fragment induced high levels of protective antibody against native Her2 in non-tolerant wild type mice. However in the tolerant preclinical model of spontaneous Her2 driven breast cancer, ED44 was effective only when it was conjugated to FrC. In this case, we demonstrated that conjugation not only significantly increased the levels of anti-Her2 antibody but led to increase of antibody affinity and additionally, broadened the spectrum of antibody isotypes. IgG2a, the most effective isotype for inducing ADCC and cancer attack through phagocytosis, was only induced by the FrC conjugate vaccine. These qualitative differences in the antibody response led to improved survival of mice with spontaneous metastatic Her2+ve breast cancer. We also demonstrated that FrC-induced T cell help was required for powerful antibody induction and could not be compensated for by TLR4 ligand adjuvant monophosphoryl lipid A. The latter is able to stimulate B cells directly and is a licensed adjuvant which is able to increase the antibody levels against targets from infectious diseases (Einstein et al. 2009). In our study in the tolerant setting it could increase the total levels of anti-Her2 antibody, however the antibody affinity was not increased. Importantly there was also a failure to induce ADCC and phagocytosis engaging IgG2a. Overall, despite the increase of total anti-Her2 IgG there was a minimal improvement in protection in the tolerant model in contrast to the FrC conjugate vaccine which induced a significant protection. This study was the first to demonstrate that foreign-linked T cell help induced through conjugation to FrC could not only impact the levels of antibody induced but also on the quality of the antibody against cancer targets.

2.7 mRNA Vaccines

mRNA vaccines are emerging as a promising vaccine modality and in the last five years clinical trials in metastatic melanoma, renal cell carcinoma, prostate cancer and other types of cancers targeting mutated and unmutated TA have been carried out (reviewed in Kreiter et al. 2011; Sahin et al. 2014). The idea behind mRNA vaccination is to inject RNA encoding TA, generated in vitro. The RNA is modified to improve stability and translation (reviewed in Kreiter et al. 2011) and additional means allowing to improve intracellular delivery are used including gene gun, electroporation, lipids and others (reviewed in Geall et al. 2013). Combining mRNA with liposomes also allows additional protection against nucleases and is becoming a preferred delivery method. After transfection the antigen is transcribed and presented to the immune system. Since RNA is the ligand for TLR7 and 8 (Diebold

et al. 2004; Kariko et al. 2005) the vaccine is self-adjuvanting similar to DNA vaccines. Additional RNA sensors such as RNA-dependent protein kinases, in the cytoplasm could be also involved in the immune recognition of mRNA vaccines (Cohen-Chalamish et al. 2009). In addition to mRNA vaccines against TA, more recently mRNA vaccines have been used to deliver multi-epitope vaccines carrying a string of T cell epitopes from neoantigens identified in the tumour through the 'mutanome' approach (Boisguerin et al. 2014; Kreiter et al. 2015).

2.8 Plant Viral Particles: A Novel Platform for Induction of Immunity Against Cancer

Plant viral particles (PVP) are promising vaccine platforms for TAs. They are simultaneously able to provide linked T cell help through the viral coat protein (CP) and adjuvant usually through plant virus-derived genomic ssRNA (Jobsri et al. 2015). Additionally PVP are structurally analogous to virus-like particles and hence, factors such as dense antigen display and the particulate structure also contribute to their excellent immunogenicity (Lebel et al. 2015). Similar to ssRNA of human viruses and their synthetic analogues, native PVP ssRNA is a TLR7 ligand and hence serves as an adjuvant similar to RNA vaccines (Diebold et al. 2004; Jobsri et al. 2015). Individual epitopes or small fragments containing targeted epitopes can be linked to PVP both genetically and chemically. For longer antigenic fragments biotin-streptavidin or chemical linkage have been used (Lebel et al. 2015; Jobsri et al. 2015). Antigens linked to PVP mimic the structure of PVP and hence inherit the immunogenic properties of PVP. We have demonstrated these principles for the weak antigen of lymphoma, the idiotype (Jobsri et al. 2015). When conjugated to PVP idiotype induced potent protective anti-Id antibody responses superior to the 'gold standard' Id-KLH vaccine. Not only were the antibody levels higher but the induced linked CP specific T cell help was largely of Th1 bias in contrast to Th2 inducing KLH. The ability of PVP-based vaccines alone to induce strong Th1 bias has been also explored for induction of CD8+ T cells against cancer TAA. Specific CD8+ T cell responses and protection in a transplantable melanoma model was demonstrated using PVP-epitope vaccine (McCormick et al. 2006a, b). Further research is required to uncover the full potential of these powerful vaccine modalities for targeted cancer immunotherapy.

3 Concluding Remarks

DNA vaccines incorporating linked T cell help targeting conventional tumour antigens have demonstrated the ability to induce anti-tumour responses in patients with evidence of clinical benefit. This approach is promising for induction of

diverse antitumour effector mechanisms in patients. Further extension of this approach to the newly discovered mutanome-derived neoantigens might offer an alternative approach to targeting cancer. Further work aiming to increase the potency of the induced responses through combinational approaches with checkpoint antibody inhibitors should benefit the cohorts of patients which are currently unresponsive to the checkpoint inhibitors alone.

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mRNA Cancer Vaccines—Messages that Prevail

Christian Grunwitz and Lena M. Kranz

Abstract During the last decade, mRNA became increasingly recognized as a versatile tool for the development of new innovative therapeutics. Especially for vaccine development, mRNA is of outstanding interest and numerous clinical trials have been initiated. Strikingly, all of these studies have proven that large-scale GMP production of mRNA is feasible and concordantly report a favorable safety profile of mRNA vaccines. Induction of T-cell immunity is a multi-faceted process comprising antigen acquisition, antigen processing and presentation, as well as immune stimulation. The effectiveness of mRNA vaccines is critically dependent on making the antigen(s) of interest available to professional antigen-presenting cells, especially DCs. Efficient delivery of mRNA into DCs in vivo remains a major challenge in the mRNA vaccine field. This review summarizes the principles of mRNA vaccines and highlights the importance of in vivo mRNA delivery and recent advances in harnessing their therapeutic potential.

Contents

Authors contributed equally to this work.

C. Grunwitz (✉) · L.M. Kranz (✉)
BioNTech RNA Pharmaceuticals GmbH, An der Goldgrube 12,
55131 Mainz, Germany
e-mail: christian.grunwitz@biontech.de

L.M. Kranz
e-mail: lena.kranz@biontech.de

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1 Introduction

There was not much enthusiasm for mRNA in the scientific community back in the 1990s, and the emergence of messenger RNA (mRNA)-based therapeutics was hard to foresee. Although first reports of plasmid DNA (pDNA) and mRNA vaccines appeared around the same time (Tang et al.1992; Martinon et al. 1993), pDNA dominated the field for over a decade and the prevailing doctrine described mRNA as expensive, difficult to manufacture and notoriously unstable. Thus, 25 years ago, mRNA appeared to be anything but a worthwhile platform for the development of a new class of drug products.

Having experienced the difficulties and hurdles accompanied with large-scale production in a controlled environment, initial assumptions about the easy and cost-efficient production of pDNA were gradually disproven. Simultaneous improvements in the production, modification and stabilization of mRNA have nurtured the recognition of mRNA as a potential alternative. During the last decade, mRNA became increasingly recognized as a versatile tool for the development of new innovative therapeutics. Especially for vaccine development, mRNA is of outstanding interest as it maintains the key characteristics of pDNA, but with a superior safety profile. Similar to pDNA, mRNA vaccines lack MHC haplotype restriction, unlike peptide vaccination strategies. But in contrast to pDNA, mRNA does not require nuclear localization for translation of the encoded antigen. Moreover, it represents the minimal vector enabling transient protein expression and does not harbor the risk of insertional mutagenesis or permanent genomic alteration. Consequently, mRNA demonstrates inherent safety features optimal for therapeutic use.

Since 2008, numerous clinical trials have been initiated (Table 1) and consistently demonstrated the feasibility and suitability of mRNA vaccines for cancer treatment. One major challenge in the field is the efficient delivery of mRNA into DCs in vivo, as it significantly determines the quality and quantity of the desired immune response. This review summarizes the principles of mRNA vaccines and highlights the importance of optimal in vivo delivery for harnessing their therapeutic potential.

Table 1 mRNA cancer vaccine trials

Sponsor/Collaborator	Indication	Delivery route/format	Status	Reference/Clinical trial identifier
University Hospital Tuebingen CureVac	Melanoma	Intradermal/mRNA + GM-CSF	Completed	Weide et al. (2008)
University Hospital Tuebingen CureVac	Melanoma	Intradermal/protamine-complexed mRNA + GM-CSF, ± KLH	Completed	Weide et al. (2009) NCT00204607
University Hospital Tuebingen CureVac	Renal cell carcinoma	Intradermal (mRNA + GM-CSF)	Completed	Rittig et al. (2011, 2016)
CureVac	Nonsmall cell lung cancer	Intradermal/self-adjuvanted mRNA	Completed	Sebastian et al. (2011) NCT00923312
BioNTech RNA Pharmaceuticals	Melanoma	Intranodal/mRNA	Completed	NCT01684241
University of Florida CureVac	Prostate cancer	Intradermal/self-adjuvanted mRNA	Completed	NCT00906243
CureVac	Prostate cancer	Intradermal/self-adjuvanted mRNA	Completed	Kübler et al. (2015) NCT00831467
CureVac	Prostate cancer	Intradermal/self-adjuvanted mRNA	Ongoing	NCT01817738
BioNTech RNA Pharmaceuticals	Melanoma	Intranodal/poly-neo-epitopic mRNA	Ongoing	NCT02035956
BioNTech RNA Pharmaceuticals	Melanoma	Intravenous/liposome-formulated mRNA	Ongoing	Kranz et al. (2016) NCT02410733
BioNTech RNA Pharmaceuticals Seventh Framework Program	Triple Negative Breast Cancer	Intravenous/liposome-formulated mRNA	Recruiting	NCT02316457
University Hospital Southampton BioNTech RNA Pharmaceuticals Seventh Framework Program	HPV-induced cancers	Intravenous/liposome-formulated mRNA	Ongoing	EudraCT: 2014-002061-30

2 Concept of mRNA Vaccines

Induction of T-cell immunity is a multi-faceted process: antigen acquisition (active uptake, viral infection), antigen processing and presentation, and immune stimulation. The effectiveness of any vaccine intended to raise long-lasting virus- or tumor-specific T-cell responses, may it be peptide/protein, nucleic acids or cell-based, fully relies on making the antigen(s) of interest available to professional antigen-presenting cells, especially DCs. Their ability to convert antigenic information and accompanying danger signals into a format that can be recognized by T cells and that provides instructions about the type and extent of response is pivotal for vaccination purposes. The significance of DCs in mounting robust and long-lasting T cell immunity is well established and is reflected by the widespread use of ex vivo manipulated DCs for vaccination and FDA-approval of Sipuleucel-T in 2010.

mRNA vaccines are faced with another barrier. The mRNA molecule itself is not the pharmacologically active product but rather a vector encoding the relevant message. In contrast to peptide/protein vaccines, a crucial intermediate step is needed before the antigenic information can be made available to T cells: translation. The fundamental principle of mRNA therapeutics is 'hijacking' the cell's translational machinery to produce pharmacologically active proteins. In order to do so, synthetic mRNAs follow the blueprint of naturally occurring, mature and processed mRNA, and are exposed to the same regulatory mechanisms. Minimal structural requirements are a 5′ cap, a poly(A) tail, and an open reading frame (ORF) coding for the protein of interest, with a start codon in Kozak surrounding and flanked by a 5′ and 3′ untranslated region (UTR). Production in a cell-free system via in vitro transcription of a linearized pDNA or PCR product using a bacteriophage RNA polymerase is followed by DNase digestion and purification by standard methods (precipitation, bead-based methods, and chromatography).

Unlike cell-based vaccination where mRNA is electroporated directly into the cytoplasm of DCs ex vivo, direct mRNA vaccination faces various barriers that need to be overcome in order for this specialized molecule to perform as intended. These barriers can be summarized as (i) barriers encountered on the way from the injection site to the cell population of interest, i.e., DCs (extracellular barriers), (ii) barriers regarding active mRNA uptake by the target cell (endocytic barriers), and (iii) barriers affecting endosomal escape and mRNA release into the cytoplasm (intracellular barriers) (Fig. 1).

3 Antigen Acquisition

3.1 Uptake and Endosomal Release

Once mRNA has reached the close neighborhood of DCs (by any delivery route), the subsequent steps of cellular uptake and, importantly, access to the cytoplasm,

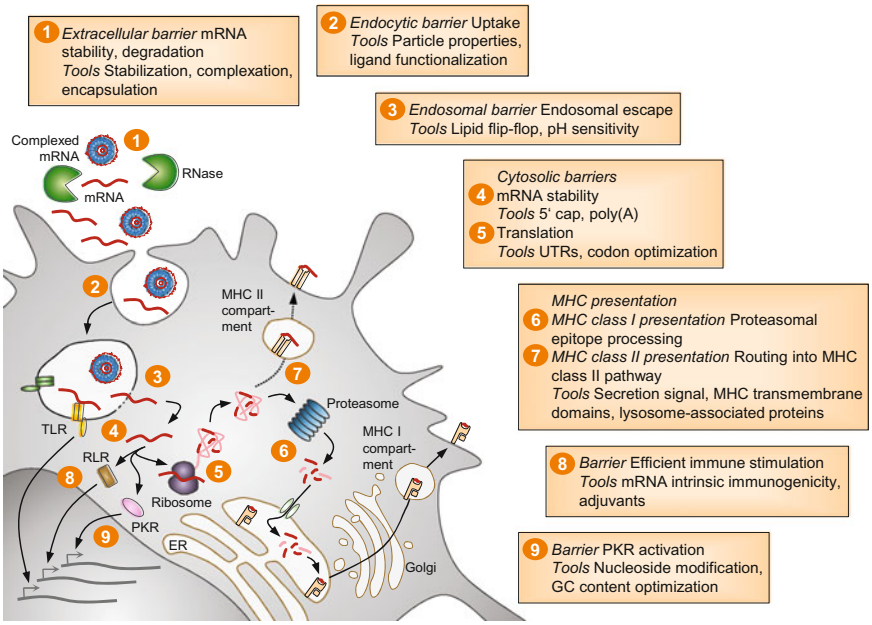

Fig. 1 Antigen-specific immunity, mRNA-specific barriers, and tools to evade them

are critical. Nonlipid nanoparticles may by highly protective of their cargo and easily coupled to targeting ligands, yet they are often confronted with the problem of efficient mRNA release once inside the cell. Incorporation of destabilizing or pH sensitive polymers is an option, although convincing vaccination studies have so far not been reported. Lipid-based nanoformulations, however, have a long record of being used for transfection of negatively charged biomolecules such as DNA, RNA, and oligonucleotides in vitro, and have been thoroughly investigated. The cationic lipids DOTMA and DOTAP have been the most extensively used transfection reagents due to their ability to facilitate membrane fusion (Felgner et al. 1987; Crook et al. 1998). Spontaneous fusion with cell membranes seemingly precludes its use for targeted in vivo applications, however, using these lipids in negatively charged complexes seems to prevent spontaneous, unspecific membrane fusion (Kranz et al. 2016), presumably due to electrostatic repulsion. Naked mRNA as well as negatively charged DOTMA or DOTAP containing mRNA-lipoplexes ($\sim$400 nm) have been shown to be internalized via macropinocytosis (Kranz et al. 2016; Diken et al. 2011), a mechanism constitutive in DCs as well as macrophages that covers a broad range of particle sizes (100 nm to 5 μm). Phagocytosis, restricted to macrophages, mainly deals with particles larger than 500 nm up to 10 μm, and clathrin- or caveolin-mediated endocytosis with particles smaller than 120 nm (Hirota and Terada 2012).

The exact nature of endosomal escape remains elusive, but depending on the formulation, a variety of concepts were proposed. DOPE, a zwitterionic phospholipid widely used in combination with cationic lipids as a helper lipid, is known to destabilize endosomal membranes (Zhou and Huang 1994; Farhood et al. 1995), as is cholesterol. For nucleic acid lipoplexes and especially those containing DOTAP and DOPE, a flip-flop mechanism has been proposed, where the positively charged lipids of the entrapped nanoformulations interact with anionic lipids present in the cytoplasm-facing endosomal monolayer. Lateral diffusion of the anionic lipids into the lipoplex generates neutrally charged ion pairs, leading to the displacement of nucleic acids from these cationic lipids and their release into the cytoplasm (Zelphati and Szoka 1996). Importantly, the lipid composition has a significant impact on the stability of the resulting mRNA-liposome complex, and the transfection rate.

3.2 Translation

When the interest into the use of mRNA as a gene delivery platform was eventually sparked, exploration of structural elements has led to engineered mRNA molecules highly optimized for stability and translational efficiency. pDNA templates encode all structural elements of functional mRNA except the 5′ cap, and sometimes the poly(A) tail.

The 5′ cap influences recognition by the translation initiation factor eIF4E and is the focus of numerous studies. Capping is achieved by the addition of a dinucleotide with a 5′-5′ triphosphate linkage during in vitro transcription, and is quite inefficient: Conventional caps (m^7GpppG) compete with GTP, and are incorporated into mRNA in both orientations, resulting in two RNA populations, Gpppm^{7}GpN, and m^7GpppGpN (Pasquinelli et al. 1995) (N: first template-encoded nucleotide). Consequently, one-half of the mRNA is improperly capped and will not be recognized by the translation initiation complex. The introduction of anti-reverse cap analogs (ARCA) solved the issue of reverse incorporation by the elimination of the 2′ or 3′ hydroxy group (to prevent nucleophilic attack by the RNA polymerase) through substitution of the 2′ or 3′ hydroxy group with a methoxy group ($m^{7,2'O}$GpppG and $m^{7,3'O}$GpppG, respectively) (Stepinski et al. 2001). Replacing a nonbridging oxygen in the phosphate moiety by sulfur resulted in ARCAs with improved resistance against 5′ to 3′ decay (β-S-ARCA), where one of the resulting ARCA diastereomers was superior over the other in terms of translational efficiency (Kuhn et al. 2010). Very recently, dithiodiphosphate (2S) analogs were introduced with high affinity for eIF4E and improved resistance against decapping enzymes (Strenkowska et al. 2016).

Both UTRs critically affect mRNA half-life and can serve as negative regulators as demonstrated for the integration of destabilizing AU-rich elements (Barreau et al. 2005) and miRNA binding sites (Fang and Rajewsky 2011). UTRs of human α- and

β-globin have been most widely incorporated (based on historical use rather than substantial evidence), and provide options for improvement, for instance as reported by Holtkamp et al. (2006).

Translational efficiency can be further enhanced by replacing rarely used codons with frequent, synonymous codons. Although extensively described in the literature, coding sequence optimization appears to be a rather empirical process the necessity of which should be carefully evaluated case by case (reviewed in Mauro and Chappell 2014). A major caveat of codon optimization in the context of RNA vaccination is the elimination or introduction of potent cryptic T cell epitopes generated by ribosomal frameshifting or internal initiation of transcription (Saulquin et al. 2002; Schwab et al. 2003).

mRNA stability and protein expression are also improved by increasing the length of the poly(A) tail until around 120 adenosines (Holtkamp et al. 2006), presumably, because the formation of a circular structure through poly(A) binding proteins protects from decapping and deadenylating enzymes. 3′ extension should be avoided (unmasked poly(A) tail), for instance by pDNA linearization with type IIS restriction enzymes (Holtkamp et al. 2006). Encoding the poly(A) tail in the template vector is beneficial for its therapeutic use, yielding products with reproducible tail lengths compared to the enzymatic two-step method.

3.3 Antigen Processing and Presentation

After translation of the mRNA in the cytosol, the encoded protein will eventually be degraded into peptides by the proteasome, routed into the endoplasmic reticulum (ER) for loading onto MHC class I, and via the secretory pathway shuttled to the cell surface for presentation to CD8+ T cells. In contrast to peptide/protein vaccines, mRNA vaccines deliver antigen directly into the cytosol, facing the hurdle of intracellular proteins usually not entering the MHC class II pathway very efficiently (autophagy is one mechanism), with peptide loading taking place in MHC class II-containing secretory vesicles. In order to make mRNA-encoded protein eligible for MHC class II presentation, trafficking signals of endosomal or lysosomal proteins residing in MHC class II processing compartments have been fused to the encoded antigen, such as lysosome-associated membrane protein-1 (LAMP-1) (Bonehill et al. 2004; Bonini et al. 2001; Su et al. 2002), the chaperone calreticulin, MHC class II-associated invariant chain (Bonehill et al. 2003), and HIV TAT protein transduction domain (Kim et al. 2008). Kreiter et al. incorporated the secretion signal and the transmembrane as well as cytosolic domains of MHC class I at the N and C terminus, respectively, which improved both CD4+ and CD8+ T cell responses (Kreiter et al. 2007, 2008). Antigen routed into the secretory pathway may be reinternalized, and subsequently, enter the MHC class II presentation pathway, or, in the case of cross-presenting DCs, be processed for MHC class I presentation.

4 Immune Stimulation

T cell activation does not come easy, and presentation of antigen on MHC alone does not make a vaccine. Instead, the expression of costimulatory signals on the surface of antigen-presenting DCs needs to be actively induced and recognized by T cells, in the presence of a polarizing cytokine environment provided by mature DCs. Insufficient immune stimulation leads to the opposite effect, i.e., T cell deletion, anergy or tolerance towards the tumor. Adjuvants such as aluminum salts (alum), monophosphoryl lipid A or toll-like receptor (TLR) agonists stimulate 'danger signals' to provide immunostimulation when peptide or protein vaccines with low intrinsic immunogenicity are used. Synthetic mRNA, however, is recognized by pattern recognition receptors (PRRs) and functions per se immunostimulatory (Karikó et al. 2004; Ishii and Akira 2005; Reis e Sousa 2004). Ligation of endosomal TLR3, TLR7 or TLR8 with endocytosed mRNA in immune cells triggers the production of type I interferon (IFN), a master regulator of several inflammatory cytokines, Th cytokines, costimulatory molecules, chemokines, and receptors thereof. While TLR3 is activated by double-stranded RNA (dsRNA) (Alexopoulou et al. 2001) including double-stranded secondary structures (Karikó et al. 2004), TLR7 and 8 signal in response to ssRNA (Reis e Sousa 2004; Heil et al. 2004). Soluble cytoplasmic receptors trigger the expression of type I IFN and proinflammatory factors similar to TLRs (Yoneyama and Fujita 2007). The natural ligand for the retinoic acid-inducible gene-I (RIG-I) is short RNA with blunt-ended double-stranded base pairing and an uncapped 5′ triphosphate end (Hornung et al. 2006; Schlee et al. 2009), while activation of melanoma differentiation-associated antigen 5 (MDA5) has been reported to require much longer dsRNA than RIG-I (at least 2 kb) (Pichlmair et al. 2009), or viral mRNA lacking 2′-O-methylation (Züst et al. 2011).

In contrast, activation of dsRNA-recognizing protein kinase R (PKR) should be avoided, as triggering this signaling cascade would interfere with mRNA translation (Sahin et al. 2014). Incorporation of naturally occurring modified nucleosides, such as 2-thiouridine, N6-methyladenosines, 5-methylcytidine, or pseudouridine can suppress PKR activation (Anderson et al. 2010) by not forming dsRNA (2-thiouridine, N6-methyladenosine) or reducing dsRNA contaminants during in vitro transcription (5-methylcytidine, pseudouridine; Katalin Karikó, unpublished). HPLC purification alone was observed to abolish PKR activation. It has to be noted, however, that nucleoside modification, sophisticated purification as well as sequence optimization by maximizing GC content (Thess et al. 2015) are indeed valid methods for increasing translation, but come at the cost of immune activation. Consequently, caution should be taken when designing optimal mRNA, to fit it to the intended use: for cancer therapy, better immune activation may outweigh translation, whereas immune activation would be detrimental for other approaches such as protein replacement (Karikó et al. 2012), where high protein production is the primary aim.

Beneficial activation of combined innate and adaptive immunity has been recently reported for the systemic administration route. Systemic application of lymphoid-targeted mRNA formulated with liposomes induced strong upregulation of costimulatory molecules CD40 and CD86 on DCs (Kranz et al. 2016). Interestingly, maturation of DCs was not confined to the portion of DCs taking up mRNA, but was observed for the majority of splenic DCs, and especially for CD8α + DCs. Expression of early activation marker CD69 was similarly induced on all CD4+ and CD8+ T cells, in addition to NK cells. Concomitant type I IFN secretion by pDCs and macrophages led to considerable type I IFN serum levels, which was essential for generating this fundamentally stimulating environment, for T cells to differentiate into cytolytic effectors [confirmed by Broos et al. (2016)], and for the elimination of established tumors.

In this context, it is important to note that the carrier itself may be immunostimulatory to some extent, or may even be designed specifically for this purpose. DOTMA/DOPE or DOTMA/cholesterol liposomes are by themselves capable of inducing low systemic type I IFN when administered intravenously at high amounts (unpublished observation by the authors), and incorporation of LPS directly into the lipid shell of mRNA nanoformulations can improve the antitumoral response after subcutaneous injection, presumably due to generating inflammation at the injection site (Oberli et al. 2016). Protamine-stabilized mRNA constitutes a stronger danger signal compared to naked mRNA in vitro (Scheel et al. 2004, 2005). Intranodal administration [where type I IFN production is confined to the respective lymph node (unpublished observation by the authors)] has been demonstrated to profit from co-administration of mRNA-encoded active TLR4, CD40L, and CD70 (TriMix) which further improved antitumoral immunity (Van Lint et al. 2012). Other compounds reported to boost mRNA vaccine efficacy are the mTOR inhibitor rapamycin, increasing the quantity and quality of the memory pool (Diken et al. 2013), and Fms-like tyrosine kinase 3 ligand (FLT3L), which expands pDCs in the lymph node and promotes T cell homing into tumors (Kreiter et al. 2011).

5 Delivery

5.1 Delivery Route and Format: The Combination Matters

The modifications described above affect the mRNA molecule itself and become relevant once the mRNA molecule has entered the cytoplasm. With the mRNA molecule itself optimized, delivery has become the dominating field of mRNA research. Despite more than 20 years of exploring nucleic acid delivery and broadening our understanding of what these barriers are and how to tackle them, efficient targeting of mRNA into DCs specifically in vivo remains a problem. Apart from naked mRNA delivery, the field focused on complexation or encapsulation of mRNA to avoid excessive extracellular degradation and promote enhanced uptake by DCs. For mRNA in vivo delivery, lipid-based carrier systems have been used the

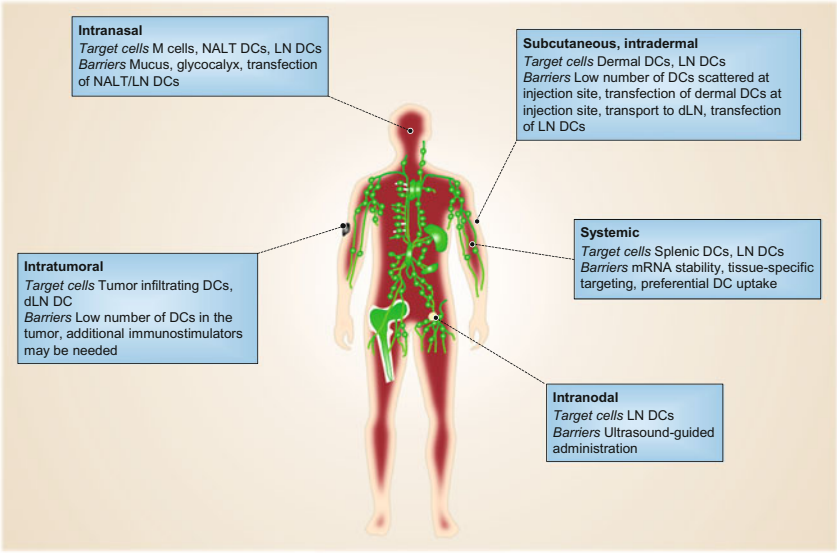

Fig. 2 mRNA delivery routes and associated barriers. *d* Draining. *LN* Lymph node. *NALT* Nasal-associated lymphoid tissue

most, building on extensive research on DNA gene transfer [reviewed in Phua et al. (2014), Phua (2015)], and among these also commercially available transfection reagents (such as Lipofectin, Lipofectamine, RNAiMAX, and Stemfect) have demonstrated in vivo transfection of DCs.

Two main factors are decisive when trying to solve the problem of delivery: delivery route (the way/site of entry into the body), and delivery format (naked, stabilized, adsorbed, complexed, encapsulated, etc.) (Fig. 2). We have learned that these two factors are interconnected, and need to be considered as a system. Each delivery route (subcutaneous, intradermal, intratumoral, intranasal, intranodal, intravenous) has to cope with its specific barriers, and these barriers will determine the adequate delivery format. In other words, a rational combination of delivery route and format should be the underlying motivation when designing vaccination concepts. Obtaining reasonable immune responses with a certain format and a certain delivery route does not necessarily mean that a certain delivery route is per se the better route, or that the format used will equally outperform other formats when injected at a different site.

5.2 Subcutaneous and Intradermal Injection

Although secondary lymphoid organs seem to be the obvious target tissues, many studies have focused on injection at distant sites, such as subcutaneous and

intradermal application. Vaccination at these sites relies on the immediate presence of dermal DCs ready to take up antigen and migrate actively to the draining lymph node, or on passive transport to draining lymph nodes. Whereas both options are relevant for intradermal application, the subcutaneous space between the skin and underlying muscle tissue does not contain cells, impeding access to dermal DCs and making this vaccination strategy almost entirely dependent on the trafficking efficiency through the lymphatics to draining lymphoid tissue. Both naked and lipid-formulated mRNA injected s.c. lead to transfection of cells at the injection site, with naked mRNA outperforming lipid-formulated mRNA regarding translational efficiency (Phua et al. 2013). Induction of antigen-specific T cells in response to both formats was observed (Martinon et al. 1993; Pollard et al. 2013), although neither of them has been reported to transfect nodal cells (Phua et al. 2013; Kreiter et al. 2010). In contrast, a study using slightly negatively charged lipid nanoformulations of roughly 70–100 nm reported high and lasting translation at the injection site, much superior to naked mRNA, as well as in CD11c+ cells in draining lymph nodes (whether transfection occurred only at the transfection site or also directly in the node was not evaluated), leading to delayed growth of B16 melanoma (Oberli et al. 2016).

Intradermal vaccination with improved access to dermal DCs was predominantly advocated and advanced by the University of Tubingen spin-off biotech company CureVac. The discovery that antigen-specific T cell immunity was elicited after intradermal injection of naked or protamine-stabilized mRNA (Hoerr et al. 2000) initiated further characterization of the influence of protamine on stabilization and immune stimulation (Scheel et al. 2004, 2005; Carralot et al. 2004), and eventually led to the development of RNActive, a mixture of naked and protamine-stabilized mRNA. While the naked mRNA compound ensures translation of the antigen, the protamine-stabilized compound is poorly translated but functions as a TLR7 ligand (Fotin-Mleczek et al. 2011). This vaccine is internalized at the injection site predominantly by macrophages, neutrophils, and DCs, of which the majority take up both naked and protamine-stabilized mRNA, but uptake of naked mRNA is preferred (Kowalczyk et al. 2016). In addition, Selmi et al. showed naked mRNA was translated in the dermis rather than the epidermis (no translation), internalized mostly by dermal DCs independent of langerin expression, and that macropinocytosis by these cells was required for mounting T cell immunity (Selmi et al. 2016).

Induction of relevant immune responses in response to injection into nonlymphoid tissues seems to rely heavily on active cellular transport from the site of injection to draining lymphoid tissue. So far, seemingly contradictive data have been published about the capability of stabilizing polymers or nanoparticle formats to outperform naked mRNA, owing to the differences in particle characteristics. However, while naked mRNA will remain dependent on cellular transport as it may not stay intact during lymphatic trafficking, nanoformulations harbor great potential: Promoting efficient access to the lymphatics and increasing antigen availability to a larger reservoir of DCs may be achieved by rational design of carrier features

such as particle size, charge, and colloidal stability. Achieving this would make distant injection an even more attractive delivery route for mRNA vaccines.

5.3 Intranasal Administration

The nasal cavity harbors lymphoid tissue, the nasal-associated lymphoid tissue (NALT) which is directly located under a thin layer of nasal epithelium intertwined with microfold (M) cells. These M cells can translocate particles from the epithelium to the NALT (Miller et al. 2007). Intranasal delivery takes advantage of this transport system to reach either NALT-resident DCs in close neighborhood with T cells or to drain passively from the NALT area to cervical lymph nodes through the lymphatics. The mucus layer covering the glycocalyx of the epithelium and the glycocalyx itself represent barriers for particle permission but also offer 'points of attack' for particle design. Promoting adhesion to the mucus may be one way to enhance uptake, while increased retention time should not come at the cost of impaired movement through the mucus. Small- to medium-sized lipid-formulated mRNA or mRNA adsorbed on pegylated core-shell nanoparticles were reported to elevate and prolong antigen expression when positively charged compared to naked mRNA (Phua et al. 2013; Su et al. 2011), while others find also negatively charged particles capable of transportation through the nasal epithelium (Rajapaksa et al. 2010), despite adjacent negatively charged glycocalyx. Pegylation may be one way to increase movement through the mucus, and larger particle sizes seem to be beneficial as well (Lai et al. 2009). Phua and colleagues have been doing intensive research on intranasal administration as a noninvasive needle-free mRNA cancer immunotherapy and were able to demonstrate for the first time antigen-specific immunity and delayed progression of subcutaneous tumors (Phua et al. 2014). Interestingly, these results were confined to positively charged lipid-formulated mRNA and were not achieved with naked mRNA, which is in line with previously reported lower antigen expression in response to naked mRNA.

Intranasal mRNA administration has proven to be a convenient method for antigen delivery to NALT DCs and induction of antitumoral immunity, provided that the mRNA is formulated. Further improvement of access to the NALT by facilitating the passage through the nasal epithelium through rational modification of particle properties seems feasible and holds promise for a noninvasive, broadly applicable tumor vaccine.

5.4 Intratumoral Injection

Tumor infiltrating DCs are in a unique position to prime T cells relevant for antitumoral immunity and aid to revert the 'corrupted' immunosuppressive tumor microenvironment. Using intratumoral mRNA administration, these DCs can be

provided with (i) antigenic information to activate/re-activate antigen-specific T cells already present in the tumor, (ii) immunostimulatory molecules that trigger migration of tumor antigen-loaded DCs to draining lymph nodes, and/or (iii) immunomodulatory compounds (cytokines, decoy receptors, TLR agonists, antibodies, checkpoint inhibitors, etc.) fostering in situ vaccination and/or sustain T cell responses.

Intratumoral delivery to DCs has been most advanced by Thielemans and colleagues [reviewed in Van der Jeught et al. (2015)]. Their studies demonstrate that naked mRNA is taken up predominantly by cross-presenting CD8α+ DCs and that these cells can reactivate T cells at the tumor site, as well as migrate to the draining lymph node when in vivo transfected with TriMix (Van Lint et al. 2016). They further showed in a proof-of-concept study, that mRNA-encoded secreted proteins were able to lift some of the pressure off immune cells by reducing MDSC suppression, stimulating DCs and activating T cell lysis, which enhanced delay of tumor growth when combined with PD-1 blockade (Van der Jeught et al. 2014). Less immune-infiltrated tumors may not be quite as susceptible to intratumoral delivery; for these tumors, increasing the half-life of mRNA through stabilization or formulation may be an option. Nevertheless, utilizing DCs not only as antigen presenters but also as transient producers of mRNA-encoded immunomodulators at site and thus shapers of the tumor environment is another asset of mRNA immunotherapy.

5.5 Intranodal Injection

Injection directly into lymph nodes circumvents anatomical and physiological barriers associated with DC availability on site and migration/passive transport to draining lymphoid tissue, as discussed for intradermal and subcutaneous injection. A large DC reservoir at immediate disposal and close proximity between injected compound, target cell and immune effectors presumably supports rapid uptake in high numbers and minimizes loss of bioactive compound due to extracellular degradation, which is critical for naked, unprotected mRNA. Sahin and colleagues followed up on this hypothesis and described in 2010 that intranodal delivery of pharmacologically optimized naked antigen-encoding mRNA elicited potent antitumor immunity (Kreiter et al. 2010), and that mRNA was internalized and translated by lymph node resident conventional and cross-presenting CD8α+ DCs through macropinocytosis (Diken et al. 2011). Another study by Thielemans et al. confirmed the potency of this delivery route and format in additional tumor models (Van Lint et al. 2012). Expression and presentation of the encoded antigen in a costimulatory environment initiated by the mRNA itself induced protective immunity unattainable by subcutaneous, near-nodal or intradermal administration (Diken et al. 2011; Kreiter et al. 2010). Intranodal delivery is certainly not the most practical or comfortable route of injection, yet regarding immunological and antitumor responses, direct targeting of the lymphatic system seems to be the preferable route of injection for naked mRNA, compared to injection at distant sites.

5.6 Systemic Injection

The delivery problem is most apparent when it comes to intravenous administration. Precise and efficient delivery of mRNA-encoded antigen to secondary lymphoid compartments still constitutes a major challenge. How to stabilize mRNA during transit, how to avoid interaction with serum proteins (risk of aggregation) and at the same time guarantee release into the cellular interior? How to reach predominantly a certain tissue or cell type? Naked mRNA is instantly degraded and does not leave a lot of options for directed delivery. What is needed is a carrier for protection and direction. Lipid-based nonviral delivery systems have been exploited for efficient pDNA transfer since the late 1980s. Spleen and especially liver are favored for intravenous particle delivery as they can be reached through sinusoids and their large capillary endothelial fenestrations, where blood flow is slower and extravasation easier. The cells readily taking up particles are white pulp resident cells and hepatocytes, respectively. The majority of particles distributes to the liver, but mRNA is poorly translated (Kranz et al. 2016; Li et al. 1998) (presumably degraded by Kupffer cells), and even less mRNA is translated by splenic DCs. As it was repeatedly shown in vitro that positively charged pDNA-lipid complexes yielded much higher transfection efficiencies than neutral or negatively charged particles, mostly positively charged pDNA-LPX were investigated for their gene transfer capability in vivo. Upon intravenous injection of these particles, researchers found high reporter gene expression in the lungs (Canonico et al. 1994a, b; Lee et al. 1996; Liu et al. 1997), and although some expression was observed in the spleen when decreasing the positive charge of the complex, a systematic analysis of the charge-biodistribution relationship was not seriously pursued at that time. Instead, developments focused almost stringently on functionalization of liposomes and other nanoparticles with molecular ligands to reroute pDNA or mRNA nanoparticles to lymphoid tissues and DCs (Phua 2015; Tacken et al. 2007; Mitragotri et al. 2014). Intravenous delivery of positively charged lipid mRNA formulations produced from Stemfect transfection reagent also led to antigen expression in the spleen, lungs and liver (Phua et al. 2013). However, Midoux and colleagues demonstrated that mRNA encoding MART1 complexed with cationic histidylated PEG-polylysine (PEG-HpK) and further encapsulated in histidine-containing HDHE/cholesterol liposomes (so-called lipopolyplexes), delayed melanoma progression in a prophylactic setting (Mockey et al. 2007). Another study from the same group, this time using PEG-HpK-mRNA complexes encapsulated with mannosylated liposomes, demonstrated increased antigen expression in splenic DCs compared to nonmannosylated particles (no data was provided regarding transfection of nonDCs). However, therapeutic efficacy was not tested (Perche et al. 2011).

In principle, induction of tumor-specific immunity is feasible via this delivery route, but the transfection efficiency of DCs still appeared far too low to be of therapeutic relevance. Very recently, Sahin and colleagues reported that delivery of mRNA can be achieved without the need of highly functionalized, complex and

costly delivery formats. By systematic in vivo assessment of antigen expression delivered from mRNA-lipoplexes made from different cationic lipids and uncharged helper lipids, they discovered that the mRNA-to-lipid ratio of lipid-formulated, tumor antigen-encoding mRNA determined the biodistribution of mRN-lipoplexes, and that a slightly negative particle net charge was the key feature for targeting DCs in vivo (Kranz et al. 2016) (confirmed with the commercial transfection reagent RNAiMAX [unpublished and Broos et al. (2016)]. Besides the primary target spleen, they managed to transfect CD11c+ cells in the bone marrow, an important tissue for T cell memory homing, and surprisingly, antigen expression was also detectable in the lymph nodes which for the major portion did not originate from blood-borne infiltrating cells. Expanding the targeted DC reservoir this way, strong effector and memory CD8+ and CD4+ T cell immunity was elicited against viral, mutant neo-antigens or self-antigens, and progressive tumors in therapeutic mouse models of melanoma, colon carcinoma and human papillomavirus (HPV)-associated cancer were rejected. Toxicities have not been observed in mice and cynomolgus monkeys, and a first-in-human clinical study for melanoma is currently ongoing.

When drawing our attention particularly to clinically approved drugs, it is remarkable that despite enormous experimental efforts, only for a limited number of drugs nanoformulations are used. These are nonfunctionalized and composed of materials with a long record of medicinal use. Notably, almost all of the approved drug nanoformulations are composed of well-known biomaterials (e.g., phosphatidylcholine, cholesterol and phosphatidylethanolamine), solely relying on passive targeting. Having the complexity of scale-up processes, GMP production and the regulatory framework in mind, it appears that newly developed mRNA vaccine formulations should be as simple as possible and only as complex as necessary to serve their ultimate purpose—protection of mRNA, fostering efficient engulfment by professional antigen-presenting cells and enabling efficient antigen processing and presentation.

6 Conclusion

Nowadays the great flexibility of mRNA is well appreciated. In the vaccine field, first clinical studies are completed and several others ongoing. Strikingly, all of these studies have proven that large-scale GMP production of mRNA is feasible and concordantly report a favorable safety profile of mRNA vaccines. By now, multiple mRNA vaccine projects are at late clinical stage of development. Undoubtedly, the efficient delivery of mRNA to specific organs, tissues or cell types is a major obstacle in the field. The current strategy of evaluating and optimizing formulations for nucleic acid delivery is highly debatable and it needs to be noted that isolated in vitro studies are not expedient. The studies mentioned above highlight that especially for systemic targeting, in vitro transfection efficiencies of specific cell types are not particularly helpful for predicting effectiveness in vivo,

since in vitro studies cannot, by any means, foresee distribution behavior in complex organisms. There is already a plethora of published studies reporting highly functionalized and complex particles with an outstanding transfection rate in vitro. In spite of it all, only a handful of formulations is applicable in vivo. By now several studies have shown that characteristics, such as negative surface charge and a particle size larger than 200 nm, foster uptake by DCs while reducing unspecific uptake by nontarget cells. The potential of lipid-based mRNA vaccines for cancer immunotherapy has long been acknowledged, but it was not until recently that discoveries in this field have brought significant advances.

Beside vaccines, mRNA is taking hold in other fields, underpinning its role as a multipurpose platform. This is reflected by a continuously increasing number of new companies on the scene (Table 2). Notably, the medicinal portfolios of these

Table 2 Company overview: mRNA vaccines and mRNA-based therapeutics

Company	Research focus	Industry partners	Location	Year of foundation
Arcturus Therapeutics	Protein replacement Rare diseases	Ultragenyx Pharmaceutical	San Diego, USA	2013
BioNTech RNA Pharmaceuticals	Cancer Infectious disease	Genentech Sanofi-Aventis Bayer Healthcare	Mainz, Germany	2008
CureVac	Infectious disease Cancer	Boehringer Ingelheim Sanofi Pasteur Acuitas Therapeutics In-cell-Art	Tuebingen, Germany	2000 (Weide et al. 2008)
eTheRNA Immunotherapies	Cancer Infectious disease		Niel, Belgium	2013
Ethris	Rare diseases Protein replacement Regenerative medicine	Shire Pharmaceuticals	Planegg, Germany	2009
Eukarÿs	Protein replacement		Évry, France	2010
GlaxoSmithKline	Infectious disease		Brentford, UK	2000
In-cell-Art	Infectious disease Protein replacement	Sanofi Pasteur CureVac	Nantes, France	2005
Moderna[a]	Cardiometabolic disease Cancer Rare diseases Infectious disease	AstraZeneca MSD Sharp & Dohme Alexion Vertex	Cambridge, USA	2010
Novartis	Infectious disease		Basel, Switzerland	1996
PhaseRX	Protein replacement Rare diseases		Seattle, USA	2006
Silence Therapeutics	Protein replacement		London, UK	1994

[a]Including all Moderna proprietary venture companies

companies lean towards another high impact field: gene therapy with a focus on rare genetic disorders. While mRNA vaccines very much depend on efficient immunostimulation and less on translational yield per cell, the opposite is desired for gene therapy. For such purposes, modified 'stealth' mRNA is optimized for low immunogenicity and high translation since high protein yield is the primary aim. In 2016, AstraZeneca and Moderna filed the first-in-human clinical trial (NCT02935712) using modified mRNA encoding vascular endothelial growth factor-A (VEGF-A).

Given that the mRNA field is moving beyond 'simple' vaccines, challenges associated with tissue- or cell-specific targeting still need to be faced. In cases where high protein yield in the circulation is the goal, the specificity of the producing cell type will be less critical, and passive targeting strategies might be sufficient. Nevertheless, in cases such as genome engineering (e.g., CRISPR/Cas9) and genetic reprogramming, selective mRNA delivery will have a detrimental impact on therapy safety and might be only feasible by introducing new mRNA formulations with active targeting components.

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The Use of Anti-CD40 mAb in Cancer

Marcus Remer, Ann White, Martin Glennie, Aymen Al-Shamkhani and Peter Johnson

Abstract Immunomodulatory monoclonal antibody (mAb) therapy is at the forefront of developing cancer therapeutics with numerous targeted agents proving highly effective in selective patients at stimulating protective host immunity, capable of eradicating established tumours and leading to long-term disease-free states. The cell surface marker CD40 is expressed on a range of immune cells and transformed cells in malignant states whose signalling plays a critical role in modulating adaptive immune responses. Anti-CD40 mAb therapy acts via multiple mechanisms to stimulate anti-tumour immunity across a broad range of lymphoid and solid malignancies. A wealth of preclinical research in this field has led to the successful development of multiple anti-CD40 mAb agents that have shown promise in early-phase clinical trials. Significant progress has been made to enhance the engagement of antibodies with immune effectors through their interactions with Fcγ receptors (FcγRs) by the process of Fc engineering. As more is understood about how to best optimise these agents, principally through the fine-tuning of mAb structure and choice of synergistic partnerships, our ability to generate robust, clinically beneficial anti-tumour activity will form the foundation for the next generation of cancer therapeutics.

Contents

M. Remer (✉) · A. White · M. Glennie · A. Al-Shamkhani · P. Johnson
Cancer Sciences Unit, Faculty of Medicine, University of Southampton, Southampton General Hospital, Southampton SO16 6YD, UK
e-mail: m.e.remer@soton.ac.uk

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1 Introduction

A revolution in cancer immunotherapy has recently emerged with the development of novel immunomodulatory antibodies, classified as immunostimulatory agonist mAb or checkpoint blocking mAb. Noteworthy clinical trial response rates and survival outcomes have been described with anti-CTLA-4 and anti-PD-1 mAb checkpoint blockers across a range of predominantly solid tumours, such as lung cancers and melanoma, in heavily pretreated patients with advanced disease associated with very poor prognoses. The fully human IgG1 anti-CTLA-4 mAb ipilimumab was approved by the FDA in March 2011 after several large phase III trials demonstrated prolonged overall survival (OS) in advanced melanoma (Hodi et al. 2010; Robert et al. 2011). Metastatic melanoma has been historically associated with particularly poor outcomes, with 2-year survival being between 10 and 20 % prior to immunotherapy (Falkson et al. 1998; Middleton et al. 2000). The addition of ipilimumab to the conventional cytotoxic agent dacarbazine improved the median duration of best overall response (ORR) from 8.1 to 19.3 months (Robert et al. 2011). However, 2-year OS was 24.2 % with ipilimumab monotherapy (Hodi et al. 2010). Essentially, the survival benefit was being driven by a small subpopulation of roughly 10 % who were achieving significant prolonged long-term survival. The emergence of the fully human IgG4 anti-PD-1 mAb nivolumab has enabled 1-year and 2-year OS to reach 62 and 43 %, respectively, in melanoma with an ORR of 31 % (Topalian et al. 2014). The combination of ipilimumab with nivolumab produced an additive effect with an ORR of 53 % and 1- and 2-year OS of 85 and 79 %, respectively (Wolchok et al. 2013; Sznol et al. 2014). These achievements are matched with other anti-PD-1 mAbs, such as pembrolizumab (formerly lambrolizumab) with an ORR of 52 % in its highest dosing cohort in metastatic melanoma (Hamid et al. 2013), and pidilizumab for relapsed/refractory diffuse large B cell lymphoma (DLBCL) achieving an ORR of 51 % (Armand et al. 2013). Immunomodulatory

antibody research has garnered interest in the ability to stimulate immune responsiveness to induce robust anti-tumour immunity.

Although less developed than the antagonistic immune checkpoint blocking mAb, the agonistic immunostimulatory mAb also shows great promise as cancer therapeutics (Melero et al. 2007; Lee et al. 2013). One target under active investigation is the costimulatory receptor CD40. CD40 is a member of the tumour necrosis factor (TNF) superfamily of receptors and ligands (Smith et al. 1994) that are critical mediators of multiple physiological processes involved in immune cell function and apoptosis. CD40 is a 277-amino acid, 45–50 kDa transmembrane glycoprotein that is principally expressed on antigen-presenting cells (APCs) (Clark and Ledbetter 1986; Hart and McKenzie 1988; Schriever et al. 1989). B lymphocytes, dendritic cells (DCs), and monocytes/macrophages are professional APCs that constitutively express CD40 where it acts as a key regulator of humoral and cellular immunity (Grewal and Flavell 1998). CD40 is also expressed on a plethora of non-immune cells including endothelial cells, epithelial cells, haematopoietic progenitors, and platelets (Galy and Spits 1992; Yellin et al. 1995). CD40 expression was first described on normal and malignant B cells and human urinary bladder carcinomas (Paulie et al. 1985). Subsequently, it has been described on virtually all B cell malignancies (e.g. non-Hodgkin's lymphoma) and numerous solid epithelial tumours (e.g. breast, lung, ovary, melanoma, and bladder). The CD40 ligand (CD40L), originally termed CD154, is primarily expressed by activated CD4+ T helper (Th) lymphocytes and is transiently induced under inflammatory conditions (Armitage et al. 1992).

Agonistic anti-CD40 monoclonal antibody (mAb) therapy is an emerging immunotherapy with much promise of offering clinical benefit through its role in activating a variety of immune cells to generate effective anti-cancer immune responses. Extensive research has provided convincing evidence that CD40 signalling regulates tumour survival and is critical in tumour antigen presentation to the immune system. Early pivotal studies demonstrated that anti-CD40 antibodies can be used to eradicate lymphomas in murine models and provide protection against tumour rechallenge through rapid induction of anti-tumour cytotoxic T cells that occurs independently of CD4+ T cell help (French et al. 1999; Todryk et al. 2001; Nowak et al. 2003). CD40-activated DCs can overcome tolerance to restore antigen-specific cytotoxic T lymphocyte (CTL) responses in mice depleted of CD4 + T cells (Ridge et al. 1998). Depletion of CD8+ T cells abrogates anti-tumour immunity, exposing the central role of CTL-mediated tumour eradication in solid tumours (Todryk et al. 2001).

The application of cancer immunotherapy strategies to target CD40 aims to effectively boost anti-tumour immunity and abrogate the multitude of cancer host immune evasion mechanisms. Tumours proliferate and metastasise by escaping immune surveillance by a variety of means including down-regulation of antigen processing and presentation by reduced expression of essential costimulatory molecules e.g. B7 family members, loss of MHC or tumour antigens, and deletion of T cells which lack the optimal activation signals (Chen et al. 1992; Restifo et al. 1993; Gajewski et al. 2006). A key mechanism by which CD40 stimulation

improves immune recognition is through upregulation of adhesion and costimulatory molecules on the tumour cell surface. Whilst in normal B cells, CD40 signalling augments proliferation, differentiation, and survival, it induces apoptosis in many transformed cells in vitro and in vivo, such as breast carcinomas and aggressive lymphomas (Costello et al. 1999; Ziebold et al. 2000). To date, there are a number of existing anti-CD40 mAb that have been developed and are undergoing early-phase clinical trials as monotherapies and in combination with conventional chemotherapy. Anti-CD40 mAb therapy enhances natural endogenous CD40/CD40L interactions facilitating CD40-conditioned DCs to generate antigen-specific effector CD8+ CTLs leading to tumour destruction.

2 Normal Physiological Role of CD40

Contact-dependent CD40-CD40L cross-linking mediates a broad spectrum of systemic immune and inflammatory responses including T cell-dependent humoral responses, B cell proliferation, differentiation, adhesion and survival, memory B cell and germinal centre development, immunoglobulin class switching and affinity maturation, and interleukin (IL)-6 secretion (Noelle et al. 1992; Tsubata et al. 1993; Klaus et al. 1994; van Kooten and Banchereau 2000; Dadgostar et al. 2002). The critical role of CD40 in humoral immunity is demonstrated by the inability of immunoglobulin class switching, formation of germinal centres, and failure to develop B cell memory in individuals with X-linked hyper-IgM syndrome, whereby a defective CD40L gene leads to a breakdown of crosstalk between B and T cells (Hill and Chapel 1993). Such individuals are markedly immunocompromised and susceptible to opportunistic infections. A similar phenotype is seen in mice engineered to have defective CD40 or CD40L genes, with the mutant mice being unable to mount non-IgM responses (e.g. IgG, IgA and IgE) to thymus-dependent antigens and dysfunctional germinal centre formation, suggesting ineffective memory B cell development (Kawabe et al. 1994; Xu et al. 1994). These mice were capable of generating T cell-independent responses to thymus-independent antigens, highlighting the essential role of CD40 in T cell-mediated B cell immunity. Aside from the setting of genetic immunodeficiency, the consequences of CD40 signalling are best understood when appreciated in the context of the cell type expressing CD40 and the characteristics of the microenvironment in which it operates.

The key functions of CD40 are mediated through its expression on APC. CD40 is expressed on B cells from the early pro-B stage through to the terminally differentiated secreting plasma cell or memory B cell (van Kooten and Banchereau 2000). B cell activation through CD40 enables clonal proliferation and differentiation into antibody-secreting plasma cells and memory B cells although distinct biological effects are dependent on the stage of B cell maturation. CD40L has been shown to preferentially direct the terminal differentiation of germinal centre B cells to become memory B cells rather than plasma cells (Arpin et al. 1995). In addition,

CD40-stimulated B cells have been shown to prolong memory B cell lifespan in vitro through upregulation of telomerase activity (Hu et al. 1997).

On DCs, CD40 signalling contributes to CD4+ helper function and enables functional maturation of DCs. Licensed DCs upregulate expression of costimulatory ligands, such as CD80 (B7-1), CD86 (B7-2), and CD70, and downregulate inhibitory molecules, such as programmed cell death ligand (PD-L1) (Kurts et al. 2010). Through cross-priming, these licensed mature DCs process and present exogenous antigens in bound complexes with MHC I molecules to naive CD8+ T cells, mediating their inauguration into effector CTLs with potent cytotoxic potential and capacity for memory differentiation (Schoenberger et al. 1998; Heath and Carbone 1999). CD40 signalling amplifies the cytotoxic response and prevents tolerance induction or energy.

CD40 stimulation on macrophages enhances APC function promoting cytokine release (IL-12) and upregulating costimulatory molecules (CD80 and CD86) to augment T cell responses (Kurts et al. 2010). In addition, in vitro CD40 activation on macrophages leads to the production of the inflammatory cytokine TNF-α, nitric oxide, and TNF-related apoptosis-inducing ligand (TRAIL), which mediates macrophage cytotoxic function (van Kooten and Banchereau 1997; Griffith et al. 1999; Klimp et al. 2002). CD40-activated macrophages and monocytes can exert direct pro-apoptotic anti-tumour effects enabling them be become tumouricidal with the self-generation of the pro-inflammatory cytokine IFN-γ, in turn leading to further macrophage activation (Alderson et al. 1993; Buhtoiarov et al. 2005).

3 CD40 Intracellular Signalling

CD40 engagement with the multimeric extracellular CD40L initiates receptor trimeric clustering leading to a conformational change and CD40 localisation to membrane lipid rafts that recruit cytoplasmic adapter molecules known as TNF receptor-associated factors (TRAFs) to distinct binding sites on the CD40 cytoplasmic tail (McWhirter et al. 1999). The TRAF trimers initiate rapid protein phosphorylation by recruiting specific tyrosine and serine/threonine kinases that activate well-characterised signal transduction pathways, which include the phosphoinositide 3-kinase (PI-3K), nuclear factor-κB (NF-κB), p38/mitogen-activated protein kinase (MAPK), and c-Jun-NH2-kinase (JNK)/stress-activated protein kinase (SAPK) pathways (Berberich et al. 1994, 1996; Ren et al. 1994; Li et al. 1996). These pathways modulate targeted gene expression through activation of selective transcription factors which regulate apoptosis, cell cycle progression, survival, cytokine production, expression of costimulatory molecules, and sustained MHC/peptide complex presentation (Quezada et al. 2004; Vonderheide 2007). CD40 intracellular signalling is cell-type specific, resulting in differential signalling between B cells and DCs, controlled at the level of TRAF selection, leading to diversity of downstream pathway recruitment, and thereby resulting in distinctive phenotypes, such as B cells with enhanced affinity maturation (Ahonen et al. 2002).

Ligation of CD40/CD40L on DCs activates the NF-κB pathway whose gene product includes the anti-apoptotic protein BCL-2 in human DCs and Bcl-X_L in mice, responsible for DC maturation and survival (Quezada et al. 2004). DC maturation enables prolonged antigen presentation to cognate T cells, which in turn become activated, differentiate and undergo clonal expansion (Miga et al. 2001).

In the context of malignancy, high-grade B cell non-Hodgkin's lymphoma (NHL) cells show constitutive expression of NF-κB due to the sustained presence of an assembled large scaffold-like signalling structure called a signalosome within the membrane lipid raft (Pham et al. 2002). This unique signalling mechanism is initiated through autochthonous or ectopic production of CD40L, enabling the neoplastic B cell to bypass external accessory signals required by normal B cells. The lymphoma cells become autonomous, self-sustaining with continuous cell-cycling growth. The CD40 signalosome represents a therapeutic target with in vitro studies demonstrating disappearance of the CD40 signalosome, downregulation of NF-κB expression, growth inhibition, and lymphoma cell death with anti-CD40 and anti-CD40L antibody therapy (Pham et al. 2002).

4 CD40 Expression in Cancer

CD40 expression has been identified on a wide range of tumour cells, comprising almost 100 % of B cell neoplasms and approaching 70 % of solid malignancies (Vonderheide 2007). CD40 expression is likely to reflect pre-existent expression on non-transformed precursor cells. CD40 is also expressed on the majority of multiple myeloma (MM) cells, in contrast to low expression levels of CD20 in MM (Pellat-Deceunynck et al. 1994). Examples of epithelial tumours expressing CD40 include breast, lung, ovarian, melanoma, renal, cervical, nasopharyngeal, and bladder malignancies (Thomas et al. 1996; Wingett et al. 1998; Cooke et al. 1999; Sabel et al. 2000; Gallagher et al. 2002).

4.1 Effect of CD40 Activation in Malignancy

The effect of CD40 pathway activation in B cell malignancies is heterogenous, with the effect of inducing apoptosis and inhibition of cell growth in some xenograft tumour models and B cell lines in vitro (Funakoshi et al. 1994; Planken et al. 1996). This anti-tumour effect is seen in high-grade NHLs, such as Burkitt's lymphoma or EBV-driven lymphomas. Anti-CD40 mAb augments human B cell responses through enhanced secondary IgG antibody responses and can prevent lymphomatous transformation of EBV-positive cells, which would otherwise occur spontaneously (Funakoshi et al. 1995; Murphy et al. 1999). When combined, these early studies showed that anti-CD40 mAb could promote normal B cell function and is selective in inhibiting malignant cells. Growth inhibition is presumed to be a

function of activation-induced cell death (AICD)—apoptosis, cell cycle arrest or necrosis mediated by Fas/FasL interactions that negatively regulate activated T cells to maintain peripheral tolerance. CD40 activation upregulates surface expression of the death receptor Fas leading to pro-apoptotic triggering (Wang et al. 1997). CD40 pathway activation mediates AICD in aggressive lymphomas through upregulated expression of Bax, a pro-apoptotic member of the Bcl-2 gene family, which translocates to the mitochondria mediating mitochondrial outer membrane permeabilisation, cyctochrome c release, and activation of caspases (Hengartner 2000; Szocinski et al. 2002). In contrast, CD40 stimulation promotes malignant transformation, tumour proliferation, and lymphomagenesis in other lymphoma subtypes, particularly low-grade indolent lymphomas, such as follicular NHL, CLL, and hairy cell leukaemia (Fluckiger et al. 1994; Voorzanger-Rousselot et al. 1998; Castillo et al. 2000; Homig-Holzel et al. 2008; Kusam et al. 2009). This differential outcome to CD40 activation in B cell malignancies may correlate with the stage of B cell maturation at which transformation occurred.

Gene expression analysis has identified the pre-existing status of CD40 signalling in lymphoma cells as an important predictor of response to anti-tumour CD40 therapy (Burington et al. 2011; Shi and Dornan 2012). Sensitivity to stimulatory anti-CD40 mAb (SGN-40) is associated with p53 mutation and BCL6 expression with higher levels of intrinsic DNA damage. NHL cell lines with constitutively activated CD40 signalling were more resistant to anti-CD40 mAb (SGN-40) whilst cells with inactive CD40 were more sensitive to SGN-40. A 15-gene expression signature panel has been validated using DLBCL tumours demonstrating a majority (88 %) of the 39 specimens predicted to be resistant to CD40 activation failed to respond to SGN-40 (Burington et al. 2011).

Similarly to lymphoid malignancies, CD40 activation in MM also produces differential responses with studies reporting both MM proliferation and apoptosis/growth arrest in response to CD40 ligation (Teoh et al. 2000). CD40L upregulates IL-6 secretion, a paracrine and autocrine growth factor, which has been shown in some studies to negatively modulate the disease and effect prognosis (Urashima et al. 1995). Anti-CD40 mAb is able to specifically block this effect reducing IL-6 secretion. IL-6 activity is regulated by the cell cycle regulatory protein p53, expressed by the TP53 gene—'the guardian of the genome'. CD40 activation directly modulates p53-dependent cell cycle regulation in MM cell lines and can induce either proliferation or growth arrest depending on p53 status (Teoh et al. 2000).

In metastatic melanoma, CD40 expression is downregulated during melanoma progression enabling tumour escape, with a differential level of expression seen in the primary tumour to that of the metastases (Thomas et al. 1996). CD40 expression is only seen in a minority of cell lines derived from metastases. The net result of CD40 activation in immunogenic metastatic melanoma is tumour-specific CTL-mediated cell lysis and apoptosis (von Leoprechting et al. 1999). The upregulation of MHC class I molecules, enabling tumour antigen recognition, is thought to be responsible for the enhanced susceptibility of specific CTL-mediated destruction.

Ligation of CD40 in CD40-expressing breast carcinomas cell lines leads to increased MHC class II expression resulting in growth inhibition and Fas-mediated

apoptosis (Wingett et al. 1998). Early in vivo studies demonstrated this growth inhibition and prolonged survival through AICD, with the administration of soluble recombinant human CD40L in SCID mice challenged with human breast tumours (Hirano et al. 1999). The significantly higher expression level of CD40 in breast tumour cells compared to normal breast epithelial cells has been demonstrated in both nuclear and cytoplasmic CD40 expression (83 versus 30 % and 53 versus 30 %, respectively; where $n = 181$), with improved survival associated with cytoplasmic CD40 expression (Slobodova et al. 2011).

5 Antibody Mechanism of Action

The goal of immunostimulatory antibodies is indirect tumour cell eradication by triggering immune receptors to stimulate an immune response against cancer cells (Melero et al. 2007). Agonist antibodies provide a therapeutic strategy to boost anti-tumour immunity that can augment natural immune responses or as potential adjuvants alongside cancer vaccines. Anti-CD40 mAb has several potential mechanisms of action, which include tumour eradication through recruitment of immune effectors and antibody-dependent cell-mediated cytotoxicity (ADCC), cell signalling to induce direct apoptosis or growth arrest, and crucially, through licensing of APCs to stimulate an anti-cancer immune response. Cross-linked anti-CD40 mAbs can trigger CD40 on APCs to prime effector CTLs, release pro-inflammatory cytokines such as IL-2, and indirectly activate NK cells (Turner et al. 2001). The different mechanisms of anti-CD40 mAb therapy were not identified simultaneously. Initial development was driven by an understanding that direct apoptotic signalling on CD40+ tumours could represent an alternative target in rituximab-resistant lymphoid malignancies. Direct apoptotic signalling targets CD40-expressing cells and is a non-immune-mediated mechanism of tumour killing. Interest in targeting the tumour indirectly, through immune stimulation to generate an anti-tumour response, has led to the emergence of new clinical trials and shaped the field of agonist anti-CD40 mAb therapy.

5.1 Direct Apoptotic Signalling

Early studies demonstrated that direct targeting of CD40+ tumours by ligation of anti-CD40 mAb or soluble recombinant CD40L induced pro-apoptotic signalling and growth arrest (Funakoshi et al. 1994; von Leoprechting et al. 1999; Eliopoulos et al. 2000; Ghamande et al. 2001). This has been observed in various lymphoid and solid tumours (Pellat-Deceunynck et al. 1996; Wang et al. 1997; Hirano et al. 1999). Cell growth could be inhibited by specific anti-CD40 mAb both in vitro and in vivo in Burkett's lymphoma cell lines (Funakoshi et al. 1994). A total of 40–60 % growth inhibition was achieved in the Daudi BL cell line in vitro.

No inhibition was seen in the Raji BL line with soluble anti-CD40 mAb. However, cross-linking the anti-CD40 mAb with a secondary antibody led to significant growth inhibition. This effect was further characterised highlighting a possible direct mechanism by demonstrating that growth inhibition could still be maintained despite the concurrent use of mAb against the Fc receptor which blocks ADCC (Funakoshi et al. 1996). This was in contrast to anti-CD20 mAb which despite greater efficacy than anti-CD40 mAb, its effect was fully abrogated by inhibiting ADCC. Nonetheless, the growth inhibitory effect of anti-CD40 mAb is primarily mediated through Fc receptor-dependent mechanisms, namely ADCC, as opposed to direct apoptotic signalling. The direct anti-tumour activity of anti-CD40 mAb is not the same for all CD40-expressing tumours, with CD40+ melanoma cell lines being insensitive to direct targeting with the immunostimulatory anti-CD40 IgG2 mAb CP-870,893 (Kalbasi et al. 2010).

5.2 T Cell-Dependent Cytotoxicity

Anti-CD40 mAb send a 'licence to kill' signal by activating CD40-expressing APCs, which in turn cross-primes tumour-specific CTLs in the tumour, peripheral blood and spleen, capable of eradicating even CD40-negative solid tumours in vivo (Todryk et al. 2001; van Mierlo et al. 2002). Thus, a key cellular target of anti-CD40 mAb is a CD40-expressing DC which becomes activated upon stimulation leading to CTL cross-priming and migration from the periphery to draining lymph nodes. This results in increased tumour antigen presentation in tumour-draining lymph nodes.

In preclinical murine studies in vivo, agonistic anti-CD40 mAbs (rat anti-mouse IgG2a FGK45 and 3/23) produced robust therapeutic efficacy leading to the complete eradication of multiple CD40+ B cell lymphomas and were resistant to tumour rechallenge with the same lymphoma (French et al. 1999). Depletion studies confirmed that treatment is CD8+ T cell dependent through APC activation, occurring in the absence of CD4+ T cell help. Efficacy was also dependent on a sufficient tumour burden of viable lymphoma cells, achieved through either larger inoculum of tumour cells or delayed antibody administration to permit tumour growth pre-treatment. The hypothesis to explain this phenomenon was the requirement of sufficient tumour-associated antigen to prime CD40–activated APCs. These findings were reproduced in follow-up studies that confirmed that anti-CD40 mAb therapy could eradicate B cell lymphomas even after widespread dissemination throughout secondary lymphoid organs, liver, and bone marrow (Tutt et al. 2002). Anti-CD40 mAb therapy led to an early rapid NK cell cytotoxic response; however, upon NK cell depletion, this effect was not required for therapeutic efficacy, which again was solely CD8+ CTL dependent—both in the primary response and in the secondary response with tumour rechallenge. This suggests that CD8+ T cell memory provides long-term protection; however, this does decline over time.

5.3 T Cell-Independent Cytotoxicity

A considerable body of research has focused on anti-CD40 mAb ability to directly kill CD40-expressing tumour (e.g. via ADCC) or though T cell-mediated cytotoxicity; however, CD40 ligation has been shown to activate innate immune cells such as NK cells and macrophages which are capable of anti-tumour activity in their own right. One group showed that anti-CD40 ligation indirectly activated NK cells through secretion of Th1 cytokines (IL-12 and IFN-γ), as opposed to via ADCC and that the anti-tumour response was reduced by NK cell depletion in murine melanomas, colon adenocarcinomas, and neuroblastomas (Turner et al. 2001). Furthermore, anti-CD40 mAb administration after CD8+ T cell depletion produced an anti-tumour effect, attributed to NK cell-mediated cytolysis. Other immune mechanisms of action, independent of T cell-mediated cytotoxicity, include tumouricidal macrophages that induce the depletion of tumour stroma leading to tumour breakdown and subsequent regression, as described in pancreatic tumours (Beatty et al. 2011). This is thought to be a key mechanism of action behind the strong agonistic anti-CD40 mAb CP-870,893. Macrophages act as APCs and express CD40, which can be directly activated by anti-CD40 mAb to secrete IFN-γ and mediate tumour cell destruction, as described in melanomas both in vitro and in vivo (Buhtoiarov et al. 2005). Finally, CD40-activated follicular B cells may function both as APCs and antibody-secreting plasma cells directed at tumour-specific antigens after protective immunity was demonstrating against mesotheliomas in vivo (Jackaman et al. 2011).

5.4 Recruitment of Immune Effectors

Therapeutic cancer antibodies are capable of utilising various effector mechanisms to mediate cytotoxicity in order to achieve efficacy. These include ADCC, complement-dependent cytotoxicity (CDC), and antibody-dependent cell-mediated phagocytosis (ADCP). ADCC is the process where non-specific cytotoxic effector cells (NK cells and macrophages/monocytes) ubiquitously express membrane-bound FcγRs that cross-link with the Fc region of the IgG antibody molecule, which is attached to a specific target cell, i.e. CD40-positive lymphoma cell. FcγRs provide the physical connection between the specificity of the adaptive immune system and the potent innate immune system that executes cytotoxic effector responses. FcγR binding increases the metabolic activity of the cytotoxic cell facilitating the release of cytoplasmic lytic enzymes, granzymes, perforin-containing granules and TNF that induce cell lysis of the antibody-targeted cell. FcγRs are also present on DCs with activatory signalling resulting in cross-presentation of antigens to CD8+ CTLs, CD4+ Th cells, and Treg cells that recognise MHC-restricted peptides triggering activation of various effector functions, cross-signalling, and regulation of peripheral tolerance.

A substantial body of research has investigated the critical interactions between the Fc portion of therapeutic antibodies and FcγRs of immune cells. The characteristics of these interactions, including the antibody isoforms, glycoform, variants, binding affinity, avidity, structural motifs, orientation, and immobilisation are important determinants of the amplitude of the cytotoxic effector response generated in addition to the duration of the response is maintained. Manipulation of these factors through Fc engineering has significantly increased the binding affinities for activating FcγRIIIa and FcγRIIa, giving rise to optimised ADCC and ADCP. It is important to note that human FcγRs are not fully analogous with their orthologous murine counterparts. For instance, the affinity of the human FcγRs for the different IgG isotypes is significantly lower than is the case in mice. The use of mouse models to investigate the mechanisms governing immune regulation and the therapeutic potential of novel antibodies remains invaluable; however, these effects are not always directly transferable to humans.

5.5 Fcγ Receptor Interactions

There are two functional classes of FcγRs—activating receptors (human FcγRI, FcγRIIa,c, and FcγRIIIa,b) and their murine orthologues (FcγRI, FcγRIII, and FcγRIV) and inhibitory receptors (FcγRIIb) (Nimmerjahn and Ravetch 2008). Differences in their molecular structure confer distinct antibody binding affinities and cellular distribution, acting as discriminators of their functional capacity. Intracellular signalling of antibody-bound FcγRs is regulated in activatory receptors via an immunoreceptor tyrosine-based activation motif (ITAM) comprised of a specific amino acid sequence within the intracellular tail of the receptor. Phosphorylation of tyrosine kinases induce an activatory signalling cascade with a functional outcome determined by the type of cell expressing the FcγR. Inhibitory FcγRIIb signalling is regulated via an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain. Phosphatase enzymes that remove phosphate groups from tyrosine residues inhibit FcγR signalling. A fundamental aspect of the FcγR system is the coexpression of both activatory and inhibitory receptors on an individual cell regulating signalling thresholds for activation. The ratio of activating-to-inhibiting receptor binding (A/I ratio) has been shown to predict in vivo mAb activity and determines isotype dominance (Nimmerjahn and Ravetch 2005). The exceptions to this rule are B cells which only expresses the low-affinity inhibitory FcγRIIb, regulating BCR activation, and NK cells which only express the activating FcγRIII. With the exception of the high-affinity FcγRI, all other receptors bind with low-medium affinities, which reduce binding of background monomeric antibody molecules thereby preventing non-specific triggering of pro-inflammatory responses.

The Fc portion on the constant region of therapeutic antibodies comprises of one of the four IgG isotypes in humans (IgG1–IgG4) and mice (IgG1, IgG2a, IgG2b, and IgG3). The differential specificity and binding affinity of IgG isotypes for each FcγR, which is different between humans and mice, entrench a high degree of

complexity at the interface of FcγR interactions. This complexity is compounded by many nuanced caveats woven into the FcγR system. In humans, IgG1 and IgG3 are the most pro-inflammatory isotypes, whilst in mice, IgG2a and IgG2b are the most pro-inflammatory with greatest activity (Nimmerjahn and Ravetch 2006). Mouse IgG1 antibodies have an A/I ratio less than one, which suggests their activity is critically influenced by the inhibitory FcγRIIb (Nimmerjahn and Ravetch 2005). As a result, mouse IgG1 preferentially binds to inhibitory FcγRIIb conferring minimal ADCC activity. By contrast, mouse IgG2a and IgG2b antibodies had much higher A/I ratios (70 and 7, respectively) and are therefore under less control by the inhibitory FcγRIIb. Mouse IgG2a is the most potent mouse IgG isotype in mediating ADCC due to its efficient engagement with activating FcγRIV. These factors are important considerations in the design of therapeutic antibodies, especially when human antibodies have been derived from murine antibodies. That withstanding, the A/I ratios between the human IgG isotypes are different, relative to one another, with human IgG1 mAb binding with a high A/I ratio to a broad range of FcγRs, thereby augmenting ADCC. By contrast, human IgG2 mAb has minimal FcγR binding other than to an allelic variant of FcγRIIa and does not potentiate ADCC. Allelic variants or polymorphisms of activatory FcγRs can give rise to differences in A/I ratios and hence alter the affinity for certain antibody isotypes. Analysis of FcγR polymorphisms demonstrated that differences in molecular structure modulate antibody–receptor binding affinity, characterising the strength of ADCC. NHL patients carrying the FcγRIIIa allotype resulted in high affinity for anti-CD20 IgG1 rituximab, leading to improved clinical efficacy compared with the low-affinity allelic variant (Cartron et al. 2002).

Therapeutic antibodies that deplete target cells via ADCC demonstrate enhanced tumour protection in FcγRIIb-deficient mice in xenograft models of breast cancers treated with trastuzumab (Clynes et al. 2000). ADCC was abrogated in mice deficient in activating Fc receptors resulting in loss of anti-tumour activity. Agonistic FcγRIIb-specific mAbs have been shown to induce programmed cell death in vitro (Williams et al. 2012) but failed to produce tumour protection in vivo due to receptor internalisation on target cells, in addition to consumption by host cells (Williams et al. 2013).

Pivotal studies addressing the role of isotype selection of anti-CD40 mAb have highlighted the key role of the inhibitory receptor FcγRIIb in regulating antibody agonist activity (Li and Ravetch 2011; White et al. 2011). In the first pivotal study, rat anti-mouse CD40 mAb (3/23) with engineered mouse IgG1 or mouse IgG2a Fc constant regions were given alongside immunisations with OVA to mice adoptively transferred with OVA-specific CD4+ and CD8+ T cells enabling an OVA-specific T cell response to be interrogated (White et al. 2011). 3/23 mouse IgG1 vaccination with OVA produced a dramatic increase in anti-OVA Ab levels and expansion of peripheral and splenic CD4+ and CD8+ T cells, indicating that the IgG1 isotype was able to induce both humoral and cell-mediated immune stimulation. Conversely, 3/23 mouse IgG2a was not an immunostimulator, being unable to induce an Ab or CD8+ T cell response. In addition, the mouse IgG1 Ab and parental rat IgG2a activated APCs (splenic DCs) in vitro and in vivo as evidenced by increased

expression of CD70 (the TNF receptor ligand expressed on APCs—which provides T cell costimulation, via it receptor CD27) and induced robust B cell activation, proliferation, and prosurvival intracellular signalling, consistent with strong agonistic activity. This was again in contrast to the mouse IgG2a Ab. Experiments involving activatory and inhibitory FcγR knockout mice demonstrated that mouse IgG1 anti-CD40 mAb increased anti-OVA Ab responses and splenic B cell proliferation in wild-type mice and mice lacking activatory FcγRs. In mice lacking the inhibitory FcγRIIb, mouse IgG1 failed to produce anti-OVA Ab responses and failed to stimulate B cell proliferation. Mouse IgG2a anti-CD40 mAb produced only low-level B cell proliferation in wild-type and knockout mice. These findings indicate that engagement with the inhibitory FcγRIIb is responsible for the agonistic ability of the anti-CD40 mAb whilst activatory FcγRs are not required. Therefore, the choice of antibody Fc isotype, which confers differing binding affinities to inhibitory FcγRIIb, has a critical role in determining overall anti-CD40 mAb activity.

In the second pivotal study, a mouse model was set up that enabled the interrogation of an OVA-specific CD8+ T cell response activated by OVA presentation on a DC receptor (DEC205-positive cells) triggered by a chimeric antibody with fused human IgG1 Fc region [DEC-OVA(hIgG1)] (Li and Ravetch 2011). This mAb alongside an agonistic anti-CD40 mAb was then injected into mice whose FcγRs were selectively knocked out or manipulated to provide insights into relationships between FcγR and the final T cell response generated, via a CD40-licensed DC. Mice lacking in activating FcγRs were still able to produce OVA-specific T cells upon OVA-targeting and anti-CD40 mAb stimulation. In contrast, mice lacking the inhibitory FcγRIIb were unable to generate OVA-specific T cell responses with anti-CD40 mAb. With the addition of the immunostimulant Toll-like receptor 3 (TLR3) agonist, poly I:C, OVA-specific T cells were activated indicating the critical requirement of agonistic anti-CD40 mAb to engage with inhibitory FcγRIIb to generate an antigen-specific CD8+ T cell response. This finding demonstrated a potential mechanism to enhance the efficacy of agonistic anti-CD40 mAb by manipulating its Fc region to optimise binding with inhibitory FcγRIIb. Anti-CD40 mAb with Fc isotypes that preferentially bind the inhibitory FcγRIIb (mouse IgG1 and human IgG1) led to expansion, activation and effector activity (IFN-γ secretion), whilst mouse IgG2a, which has 10-fold less affinity to inhibitory FcγRIIb failed to augment a T cell response. A human IgG1 variant with enhanced affinity for inhibitory FcγRIIb further potentiated the T cell response. The anti-tumour activity of these modified anti-CD40 mAb were investigated in an OVA-expressing CD40-negative melanoma mouse model with either mouse FcγRIIb (in wild-type mice) or human FcγRIIb (in humanised transgenic mice). T cell-mediated anti-tumour activity was significantly enhanced by Fc isotypes that preferentially bind the inhibitory FcγRIIb (mouse IgG1 and human IgG1). These findings were replicated in a CD40-negative B cell lymphoma model in the absence of immunisation with a tumour-expressed antigen, with the additional demonstration of resistance to tumour rechallenge at 10 weeks indicating the presence of a memory T cell response. These findings show that T cell-dependent anti-tumour immunity is optimised by anti-CD40 mAb that preferentially binds the inhibitory

FcγRIIb. However, anti-CD40 mAb activity also functions via ADCC, which is driven by activating FcγRs on innate immune effectors (NK cells and monocytes/macrophages) in response ligation with CD40 on CD40-expressing cells. These two mechanisms would appear to be in conflict with one another with respect to which Fc isotype choice is most effective, i.e. one that favours inhibitory FcγRs vs. activatory FcγRs. When the above model was repeated in two CD40+ tumour models, treatment with anti-CD40 mAb enhanced for ADCC (mouse IgG2a) significantly depleted peripheral CD40+ cells but only led to a minor survival advantage in one CD40+ tumour. This was in stark contrast to anti-CD40 mAb enhanced for inhibitory FcγRIIb binding (mouse IgG1), which significantly expanded peripheral CD8+ T cells, resulting in growth arrest in one CD40+ tumour model and long-term survival in the other. The interpretation of these findings is that T cell-dependent cytotoxicity mediated via CD40-induced APC licensing is a more potent anti-tumour effect than ADCC mediated via the recruitment of immune effectors.

The immunostimulatory activity of anti-CD40 mAb is driven by antibody cross-linking mediated by FcγRIIb, which is located on neighbouring cells to antibody-ligated CD40+ cells. Cross-linking of anti-CD40 mAb results in enhanced CD40 receptor clustering and activation, which mimics the effect of endogenous multimeric CD40L (Li and Ravetch 2013). Furthermore, it is the FcγRIIb bioavailability —level of surface expression and its biodistribution that is important for cross-linking and hence in vivo activity, rather than intracellular signalling effects or binding affinity (White et al. 2011). Significantly, the binding affinities of specific FcγR do not directly correlate with their ability to induce anti-CD40 mAb antibody cross-linking. The key immune cells expressing FcγRIIb are most likely DCs and B cells. Mouse DCs express both activating and inhibiting FcγRs whilst B cells exclusively express FcγRIIb. This could suggest that B cells are more important determinants of anti-CD40 mAb activity, through activation of humoral responses and as APCs, stimulating cellular T cell-mediated responses.

Recently, White et al. demonstrated anti-CD40 mAb isotype being a critical determinant of therapeutic activity, correlating with the degree of FcγRIIb engagement, with mouse IgG1 conferring long-term tumour protection in a syngeneic B cell lymphoma model, in contrast to the inactive mouse IgG2a (White et al. 2014). In contrast with direct-targeting anti-CD20 mAb, complete responses were achieved in large established tumours with relatively small doses of agonistic anti-CD40 mAb, highlighting the effectiveness of immunostimulation. Anti-tumour immunity was dependent on FcγRIIb engagement on the tumour cell itself and not on host cells. This dependence on FcγRIIb may in part reflect its high expression levels on B cells, facilitating the critical cross-linking needed for agonistic activity, since therapeutic efficacy can be reproduced when cross-linking is mediated through upregulated activatory FcγRs or chemically induced independent of FcγR expression. That withstanding, transcoengagement with FcγRIIb successfully mediates receptor cross-linking, whilst barring ADCC-induced depletion of CD40+ targets cells and promoting T cell-mediated tumour protection. These findings are supported by data showing that cross-linking anti-CD40 mAb FGK45 (rat anti-mouse IgG2a) is

required to induce B cell activation in vitro and that optimal agonistic activity is FcγRIIb dependent in vitro (Richman and Vonderheide 2014).

Most human anti-cancer mAb are IgG1 isotypes, intentionally selected because of their ability to augment cytotoxic activity with stronger binding to activating FcγR and higher A/I ratios. However, in mice, IgG2a isotypes most share these functional characteristics and represent the appropriate choice when cytotoxic activity is required. Nonetheless, mouse IgG1 isotypes serve as the most potent anti-CD40 mAb agonists for the reasons described. These differences in FcγR coengagement are unique to mouse IgG1/IgG2a and rat IgG2a isotypes and do not exist in human Fc isotypes, particularly with reference to the preferential binding of mouse IgG1 to FcγRIIb. Fc engineering would be a strategy to generate human IgG1 anti-CD40 mAb that have the greatest affinity for FcγRIIb to promote agonistic activity (Vonderheide and Glennie 2013). However, the assumption is that the superior cross-linking ability of FcγRIIb is the same in humans as mice. Furthermore, there may be an ultimate trade-off between enhanced adaptive immunity, principally T cell effector responses, and cytotoxicity mediated by innate immune mediators.

5.6 Fc Engineering

The goal of Fc engineering is to manipulate coengagement between an antibody Fc region and FcγRs on immune effector cells. FcγRIIb-targeted Fc engineering has been shown to potentiate apoptosis and increase anti-tumour activity (Li and Ravetch 2012, 2013; Tobinai et al. 2012).

Considerable progress has been made in generating optimised antibody Fc binding affinity for activating FcγRs, principally FcγRIIIa and FcγRIIa, resulting in enhanced ADCC and ADCP (Desjarlais et al. 2007; Stavenhagen et al. 2007). Antibodies which have been successfully Fc engineered include anti-CD20 mAb, rituximab; anti-Her2/neu mAb, trastuzumab; anti-EGFR mAb, cetuximab; anti-CD52 mAb, alemtuzumab; anti-CD30 mAb, and anti-CD19 mAb (Lazar et al. 2006; Horton et al. 2008). Fc engineering of anti-CD40 mAb that optimises ADCC has been shown to enhance anti-tumour immunity, which is primarily mediated through FcγR-dependent mechanisms (Horton et al. 2010). The Fc-engineered humanised anti-CD40 antibody (XmAbCD40) possessed increased binding affinity for activating FcγRIIIa producing significantly increased NK cell-mediated ADCC, whilst only modestly enhanced FcγRIIa binding produced a smaller increased macrophage-mediated ADCP. XmAbCD40 resulted in almost complete tumour regression in Ramos lymphoma xenograft models in vivo. With a smaller tumour load given intraperitoneally, in contrast to intravenous administration, XmAbCD40 therapy completely eradicated all tumours (n = 15), whilst rituximab eradicated none (P = 0.000076). Despite the anti-tumour effect of XmAbCD40 being statistically significant compared to the non-Fc-engineered anti-CD40 mAb IgG1 analogue, the relative difference was not dramatic with the IgG1 analogue still able to induce

tumour shrinkage. Understanding what these results might mean for human patients is not straightforward due to the inherent differences between the human and mouse FcγR systems. XmAbCD40 enhanced binding to mouse-activating FcγRI and FcγRIV, which are expressed on mouse DCs and macrophages but not NK cells. NK cells exclusively express activating FcγRIII in humans and mice. The enhanced binding of XmAbCD40 to mouse FcγRIII or human FcγRIIIa, expressed on NK cells, is much higher in humans relative to mice, suggesting that NK cell-mediated ADCC is of greater importance in humans than mice. Conversely, in mice, macrophage-mediated cellular immunity could be more important with another study demonstrating that the anti-tumour activity of the anti-CD40 mAb SGN-40 occurs via FcγR coengagement on macrophages that mediate ADCP (Oflazoglu et al. 2009). Fc engineering has the potential to optimise Fc-FcγR interactions such that the ability to recruit immune effectors is of greater consequence than the cell surface expression level of the target antigen or antibody epitope specificity.

6 Developed Anti-CD40 mAbs with Early Clinical Trial Data

To date, three agonistic and one antagonistic anti-CD40 mAb (SGN-40, CP-870,893, ChiLob 7/4, and lucatumumab, respectively) have been investigated in early human clinical trials in a range of lymphoid and solid tumours, as monotherapies and in combination with other agents (Tables 1, 2, 3, 4 and 5). These antibodies show marked heterogeneity of activity ranging from strong agonism to antagonism with dose-escalated infusions administered at doses as low as 0.01 mg/kg and as high as 16 mg/kg. The agonistic strength of anti-CD40 is determined either by their ability to stimulate B cell proliferation or their ability to activate B cells through upregulation of costimulatory molecules and cytokine secretion (Carpenter et al. 2009; Rakhmilevich et al. 2012). Concerns about potential toxicity due to autoimmune reactions, severe cytokine release syndrome (CRS), hyper-immune stimulation syndrome leading to AICD, thromboembolic disease (since CD40 is expressed on endothelial cells and platelets), tumour proliferation or angiogenesis have not been realised in early-phase clinical trials in a clinically relevant way. The incidence of severe CRS was observed with the most potent agonist, CP-870,893, in 11 % of patients when administered with weekly dosing; however, this was manageable with no cases of hospitalisation (Ruter et al. 2010). Severe autoimmune reactions, which are associated with approved immunomodulatory antibodies (e.g. anti-CTLA-4 mAb, ipilumumab), have not been observed. Transient, asymptomatic, but occasionally dose-limiting, transaminitis, and thrombocytopenias have been observed but with no suggestion of liver failure, bleeding or disseminated intravascular coagulation (Vonderheide et al. 2007; Advani et al. 2009; Furman et al. 2010; Hussein et al. 2010; Bensinger et al. 2012). Overall, toxicity of

Table 1 Summary of phase I trials of SGN-40 anti-CD40 mAb therapy

Author	Patient characteristics	Anti-CD40 mAb	Treatment arms (n)	Adverse events (n)		Antidrug antibodies (%)	Response (n)	
Hussein et al. (2010) Phase I USA NCT00079716 Completed	44 Patients Relapsed/ Refractory advanced MM	SGN-40 Dacetuzumab Humanised mAb Murine CDRs Weak Agonist Fc Region IgG1	**Single-agent SGN-40** 11 cohorts; 1 cycle 0.5–8 mg/kg IV Day 1 0–8 mg/kg Day 4 0.5–16 mg/kg Days 8, 15, and 22 0–16 mg/kg Day 29 MTD 12 mg/kg with steroid premedication	6 DLTs Commonest AEs: • Fatigue 57 % Headache 43 % • CRS 1st dose 41 % • Transaminitis 41 % • Lymphopenia 27 % • Nausea 23 % • Anaemia 21 %	≥G3 SAEs (18 %): • G4 Aseptic meningitis 1 patient • G4 Hyperviscosity syndrome 1 patient • G3 Thrombocytopenia 7 % • G3 anaemia, headache, hypercalcaemia, neutropaenia, renal failure 5 %	1 Patient HAHA Low titre	**OR** Best response PD	**0/44** SD 9/44 (20 %) 5/44 (115)
Advani et al. (2009) Phase I USA NCT00103779 Completed	50 Patients Relapsed/ Refractory NHL (DLBCL 42 %) (FL 24 %) (MCL 20 %) (MZL 6 %) (SLL/CLL 2 %) (Others 6 %)		**Single-agent SGN40** 6 Cohorts; 1–2 cycles 1 mg/kg IV Day 1 0–2 mg/kg Day 4 2–4 mg/kg Day 8 3–8 mg/kg Days 15, 22 and 29 MTD not reached	Commonest AEs: • Fatigue 34 % • Pyrexia 22 % • Headache 20 % • Inflammatory eye disorders 12 %	≥G3 SAEs (26 %): • Lymphopenia 18 % • Hyperuricaemia 14 % • Malignant neoplasm progression 10 % • Anaemia 6 % • Pleural effusion 6 % • Thrombocytopenia 4 % • Cardiotoxicity 4 %	None detected	**OR** CR PR SD PD	**6/50 (12 %)** 1/50 (2 %) 5/50 (10 %) 13/50 (26 %) 29/50 (58 %)

(continued)

Table 1 (continued)

Author	Patient characteristics	Anti-CD40 mAb	Treatment arms (n)	Adverse events (n)		Antidrug antibodies (%)	Response (n)	
Furman et al. (2010) Phase I USA NCT0028310 Completed	12 Patients Relapsed CLL Prior purine-analogue containing regimen		**Single-agent SGN40** 4 Cohorts; 1–2 cycles 1–4 mg/kg IV Days 1, 4 and 8 3–8 mg/kg Days 15, 22 and 29 of cycle 1 and Days 1, 15, 29 and 43 of cycle 2 MTD not reached	1 DLT Commonest AEs: • Fatigue 50 % • Headache 33 % • Anorexia 25 % • Conjunctivitis 17 %	≥G3 AE: • G3–4 Thrombocytopenia 17 % • G3 Neutropaenia 8 %	None detected	**OR**	**0/12**
							Best response	SD 5/12 (42 %)
							PD	7/12 (58 %)

MM multiple myeloma; *CDR* complimentarity-determining region; *DLT* dose-limiting toxicities; *MDT* maximum-tolerated dose; *AE* adverse event; *SAE* serious adverse event; *CRS* cytokine release syndrome; *HAHA* human anti-human antibodies; *γ-GTP* γ-glutamyl transpeptidase; *OR* objective response; *CR* complete response; *PR* partial response; *SD* stable disease; *PD* progressive disease; *NHL* non-Hodgkin's lymphoma; *DLBCL* Diffuse large B cell lymphoma; *FL* follicular lymphoma; *MCL* mantle cell lymphoma; *MZL* marginal zone lymphoma; *SLL* small lymphocytic lymphoma; *CLL* chronic lymphocytic lymphoma; *VTE* venous thromboembolism; *PE* pulmonary embolus; *RCC* renal cell carcinoma

Table 2 Summary of Phase I trials of CP-870,893 anti-CD40 mAb therapy

Author	Patient characteristics	Anti-CD40 mAb	Treatment arms (n)	Adverse events (n)		Antidrug antibodies (%)	Response (n)	
Vonderheide et al. (2007) Phase I Completed	29 Patients Advanced solid tumours: Melanoma 52 % NSCLC 17 % Sarcoma 10 % Cholangioncarcinoma 7 % Breast cancer 3 % + others	CP-870,893 Human mAb No murine sequences Agonist Fc Region IgG2	**Single-agent CP-870,893** Redosing for responders 6 dose levels (mg/kg): 0.01, 0.03, 0.06, 0.1, 0.2, 0.3 MTD 0.2 mg/kg	3 DLT Commonest AEs: • G1-2 CRS 55 %	≥G3 AE: G3-4 Lymphopenia 45 % G3 Electrolyte disturbance 24 % G3 Transaminitis 10 % <1 %: G3 Pericardial effusion, G3 Pneumonia, G4 Seizure, G3 Headache, G4 PE, G3 Bone pain	None detected	**OR**	**4/29 (14 %)**
							Best response	PR 4/29 (14 %) (Melanoma)
							SD	7/29 (24 %)
							PD	18/20 (62 %)
Ruter et al. (2010) Phase I Completed	27 Patients Advanced solid tumours: Melanoma 41 % Breast cancer 11 % Mesotheioma 7 % RCC 7 % + others (13 in total)		**Single-agent CP-870,893** Weekly administration 4 dose levels (mg/kg): 0.05, 0.1, 0.2, 0.25 MTD 0.2mg/kg	2 DLT (CRS, urticaria) Commonest AEs: • CRS any grade (56 %)	≥G3 AE: G3 Abdominal pain 7 % G3 CRS 11 % G4 PE 1 patient G3 Autoimmune diabetes 1 patient	None detected	**OR**	**0/27**
							Best response	SD 7/27 (26 %)
							PD	20/27 (74 %)

NSCLC Non-small cell lung cancer; *RCC* renal cell carcinoma; *VTE* venous thromboembolism; *PE* pulmonary embolus

Table 3 Summary of phase I/II trials of Lucatumumab anti-CD40 mAb therapy

Author	Patient characteristics	Anti-CD40 mAb	Treatment arms (n)	Adverse events (n)		Antidrug antibodies (%)	Response (n)	
Bryd et al. (2012) Phase I NCT00108108 Completed	26 Patients Symptomatic CLL Relapsed/ refractory to fludarabine-based regimen(s)	Lucatumumab (HCD122) Human Ab Strong Antagonist Fc Region: IgG1	**Single-agent Lucatumumab** Weekly ×4 5 dose levels (mg/kg): 0.3, 1.0, 3.0, 4.5, 6.0 MTD 3.0 mg/kg	4 DLT (fat enzyme elevation): Commonest AEs Chills (54 %), Nausea (46 %), Hypotension (35 %), Arthralgia, Pyrexia (27 %), Diarrhoea, Vomiting, Fatigue (23 %)	≥G3 AE: Elevated lipase 19 % Neutropaenia 11 % Dyspnoea, elevated amylase, pneumonia 8 %	None detected	**OR**	**1/26 (3.8 %)**
							Best response	PR 1/26 (3.8 %)
							SD	17/26 (65 %)
							Mean duration	76 days (range 29–504)
							PD	5/26 (19 %)
Bensinger et al. (2012) Phase I NCT00231166 Completed	28 Patients Relapsed/ Refractory MM		**Single-agent Lucatumumab** Weekly ×4 for 1–2 cycles 4 dose levels (mg/kg): 1.0, 3.0, 4.5, 6.0 MTD 4.5 mg/kg	3 DLT Commonest AEs: Infusion reactions (71 %) Chills (54 %), Hypoalbuminaemia, Hyperglycaemia (75 %), Hypophosphataemia (50 %), Transaminitis (43 %), Elevated lipase, Hypokalaemia (39 %), Hyponatraemia (36 %), Elevated Creatinine, Headache, Hypotension, Pyrexia (32 %), Fatigue (29 %)	Commonest ≥G3 AE: Lymphopenia (93 %), Leucopenia (25 %), Anaemia (21 %), Neutropaenia (18 %), Thrombocytopenia (11 %), Elevated lipase (11 %), Hypophosphataemia (11 %)	n/a	**OR**	**1/28 (4 %)**
							Best response	PR 1/28 (4 %)
							SD	12/28 (43 %)
							Mean duration	61 days (range 17–798)
							PD	10/28 (36 %)

(continued)

Table 3 (continued)

Author	Patient characteristics	Anti-CD40 mAb	Treatment arms (n)	Adverse events (n)		Antidrug antibodies (%)	Response (n)	
Fanale et al. (2014) Phase Ia/II NCT00670592 Completed	111 Patients Relapsed/ Refractory lymphomas FL (19 %) DLBCL (30 %) MALT (6 %) MCL (11 %) HL (33 %)		**Single-agent Lucatumumab** Weekly ×4 of 8 week cycle Retreatment for ≥SD 2 dose levels: 3–4 mg/kg MTD 4 mg/kg	6 DLT Commonest AEs: Chills (39 %) Pyrexia (34 %) Fatigue (25 %) Nausea (23 %) Dyspnea (22 %)	Commonest ≥G3 AE: Lipase increased (25 %)	n/a[a]	**FL OR**	**7/21 (33 %)**
							FL CR	1/21 (4.8 %)
							FL PR	6/21 (28.6 %)
							FL SD	11/21 (52 %)
							FL PD	3/21 (14 %)
							DLBCL OR	**4/34 (11.8 %)**
							DLBCL CR	2/34 (5.9 %)
							DLBCL PR	2/34 (5.9 %)
							DLBCL SD	11/34 (32 %)
							DLBCL PD	15/34 (44 %)
							HL OR	**5/37 (13.5 %)**
							HL CR	0/37
							HL PR	5/37 (13.5 %)
							HL SD	12/37 (32 %)
							HL PD	14/37 (37.8 %)
							Best response MCL SD	5/12 (41.7 %)

DLBCL diffuse large B cell lymphoma; *FL* follicular lymphoma; *HL* Hodgkin lymphoma; *MALT* mucosa-associated lymphatic tissue lymphoma; *MCL* mantle cell lymphoma

[*] anti-lucatumumab antibodies were measured however no results provided

Table 4 Summary of combination therapy trials with anti-CD40 mAb therapy

Author	Patient characteristics	Anti-CD40 mAb	Treatment arms (n)	Adverse events (n)		Antidrug antibodies (%)	Response (n)	
Fayad et al. (2011) Phase IIb NCT00529503 Terminated	151 Patients with refractory DLBCL Futility analysis lead to early trial termination as response rate was not improved with SGN-40	SGN-40 Dacetuzumab Humanised mAb Murine CDRs Weak Agonist Fc Region IgG1	**R-ICE +/− SGN-40** SGN-40 2–8 mg/kg IV; D-1, 3, 8, 15 of Cycle 1, and D1, 8, 15 of Cycles 2, 3 Rituximab 375 mg/m^2 IV; D-2 of Cycle 1, and D1 Cycles 2, 3: Etoposide 100 mg/m^2 IV; D1-3 of Cycles 1–3: Carboplatin AUC = 5 mg/mL min IV; D2 of Cycles 1–3 Ifosfamide 5 g/m^2 24 h. IV; D2 of Cycles 1–3				R-ICE + SGN-40	ORR 64 % CR 36 %
							R-ICE alone	ORR 68 % CR 42 %
Beatty et al. (2013) Phase I NCT00711191 Completed	22 Patients Advanced chemotherapy-naive pancreatic ductal adenocarcinoma	CP-870,893 Human mAb No murine sequences Agonist Fc Region IgG2	**Gemcitabine + CP-870,893** CP-870,893 0.1–0.2 mg/kg D3 Gemcitabine 1000 mg/m^2 IV weekly ×3 4-week cycles CP-870,893 MTD 0.2 mg/kg	1 DLT (stroke) Commonest AEs: CRS (85 %) Fatigue (85 %) Transaminitis (85 %) Hyperglycaemia (85 %) Anaemia (77 %) Hypocalcaemia (77 %) Nausea (77 %) Lymphopenia (54 %) Vomiting (46 %) Pyrexia (46 %) Constipation (46 %)	Commonest ≥G3 AE: Lymphopenia (23 %) Hyperglycaemia (18 %) Neutropaenia (14 %) CRS 1 patient Stroke 1 patient	n/a	**OR**	**4/22 (19 %)**
							Best response	PR 4/22 (19 %)
							SD	11/22 (50 %)
							Median PFS	5.2 months [95 % CI, 1.9–7.4]
							Median OS	8.4 months [95 %CI, 5.3–11.8]
							1-year OS	28.6 %

(continued)

Table 4 (continued)

Author	Patient characteristics	Anti-CD40 mAb	Treatment arms (n)	Adverse events (n)		Antidrug antibodies (%)	Response (n)	
Vonderheide et al. (2013) Phase I NCT00607048 Completed	32 Patients Advanced solid tumours: Melanoma 78 % + others (7 in total)	CP-870,893 Human mAb No murine sequences Agonist Fc Region IgG2	**Paclitaxel + Carboplatin + CP-870,893** Paclitaxel 175 mg/m^2 IV D1 Carboplatin AUC6 IV D1 CP-870,893 0.1–0.2 mg/kg IV D3 OR D8 21-day cycles MTD 0.2 mg/kg	2 DLTs Commonest AEs: Fatigue (81 %) Peripheral neuropathy (46 %) CRS (42 %) Neutropaenia (38 %)	SAEs G5 intracranial haemorrhage 1 patient G4 Febrile neutropaenia 1 patient	None detected	**OR**	**6/30 (20 %)**
							Best response	PR 6/30 (20 %)
							Mean duration	75 days [50–96]
							SD	12/30 (40 %)
							PD	12/30 (40 %)
Forero-Tores et al. (2013) Phase Ib NCT00655837 Completed	33 Patients Relapsed/Refractory DLBCL 61 % Transformed 30 % Follicular G3 9 %	SGN-40 Dacetuzumab Humanised mAb Murine CDRs Weak agonist Fc Region IgG1	**SGN-40 + Rituximab + Gemcitabine** SGN-40 4–12 mg/kg IV; Days 1, 4, 8, 15 and 22 of Cycle 1 and Days 1, 8 and 15 of Cycles 2–8. Rituximab 375 mg/m^2 IV; Days 2, 8, 15 and 22 of Cycle 1 and Day 1 of Cycles 2–8. Gemcitabine 1000 mg/m^2 IV; Days 1 and 15 of all Cycles	Commonest AEs: CRS (61 %) Nausea (42 %) Fatigue (36 %) Thrombocytopenia (36 %) Headache (30 %)	SAEs (45 %) Commonest ≥G3 AE: Thrombocytopenia (36 %) Hypokalaemia (18 %) Neutropaenia (15 %) Pneumonia (15 %) G3 Cardiomyopathy 1 patient G5 Pneumonitis 1 patient	None detected	OR	14/30 (47 %)
							CR	6/30 (20 %)
							PR	8/30 (27 %)
							Median PFS	25 weeks [95 % CI,15–36.6]

NSCLC non-small cell lung cancer; *RCC* renal cell carcinoma; *VTE* venous thromboembolism; *PE* pulmonary embolus

Table 5 Active trials with anti-CD40 mAb therapy at the time of writing (epub 5/2/2015)

Anti-CD40 mAb	Study type	Tumour type	Cotreatment	Sample size	Clinical trials.gov identifier
Chi Lob 7/4	Phase I	NHL	None	30	NCT01561911
CP-870,893	Phase I	Metastatic melanoma	Tremelimumab	32	NCT01103635
	Phase I	NHL	Rituximab	22	NCT00556699
	Phase II	NHL	None	46	NCT00435916
Dacetuzumab (SGN-40)	Phase I	MM	Lenalidamide and Dexamethasone	38	NCT00525447
	Phase Ib	MM	Bortezomib	18	NCT00664898
Lucatumumab (HCD122)	Phase I	NHL	Bendamustine	50	NCT01275209

NHL non-Hodgkin's lymphoma; *MM* multiple myeloma

anti-CD40 mAb has been acceptable, including in combination with multi-agent chemotherapy.

Immune pharmacodynamic analysis of patients treated with the IgG1 chimeric agonistic anti-CD40 mAb—ChiLob7/4 failed to produce an augmented response in combination with DC-activating TLR ligands (LPS and CpG) (Chowdhury et al. 2014). Notably, the IgG2 fully human agonistic anti-CD40 mAb—CP-870,893 has produced augmented B cell activation in combination with CpG TLR9 stimulation (Carpenter et al. 2009). Similarly, the cytokines TNF-α and IL-6, associated with a classical cytokine storm, were not detected in ChiLob 7/4-treated patients but have been in CP-870,893-treated patients (Vonderheide et al. 2007). These differences suggest that the antibody FcγR isotypes are important determinants of biologic activity and the potential for toxicity. Equally, the choice of antibody IgG isotype is critical to its function with anti-CD40 IgG2 mAb (CP-870,893) principally mediating its effects via DC and macrophage activation, whilst anti-CD40 IgG1 mAbs (SGN-40, ChiLob7/4) principally augment ADCC and CDC (Vonderheide and Glennie 2013). Interestingly, FcγR, both activating and inhibiting, bind IgG1 with greater affinity than IgG2 isotypes, with the inhibitory FcγRIIb binding to IgG2 with the lowest affinity compared to all other Fc isotypes (Bruhns et al. 2009; Bruhns 2012).

6.1 Dacetuzumab (SGN-40)

SGN-40, developed by Seattle Genetics Inc., is a humanised anti-CD40 IgG1 mAb with murine complementarity-determining regions (CDRs) that target CD40. Through multiple discrete mechanisms, SGN-40 is capable of inducing apoptosis in cultured NHL cells in vivo by augmenting effector CTL responses, ADCC, ADCP,

and direct apoptotic signalling (Law et al. 2005; Oflazoglu et al. 2009). The humanised SGN-40 was developed from the chimeric anti-CD40 mAb SGN-14, which itself was derived from the murine mAb S2C6 (Francisco et al. 2000). Both murine and humanised antibodies recognise the same epitope. In contrast to other anti-CD40 mAbs, SGN-14, and SGN-40 are weak or partial agonists (K_d of ~1 nmol/L) for resting B cells and do not block CD40/CD40L interactions in vitro. Preclinical studies have identified immune effector functioning via ADCC and direct apoptotic signalling via caspase-3 activation as key mechanisms responsible for SGN-40 anti-tumour activity. The precise pathway leading to caspase-3-mediated apoptosis has not been elucidated, with neutralising anti-FasL antibodies failing to suppress apoptosis of lymphomatous B cells treated with SGN-40, suggesting a Fas-independent pathway. The combination use of SGN-40 with cycloheximide (CHX), a protein synthesis inhibitor, sensitises MM cell lines to CD40-mediated apoptosis (Tai et al. 2004). Specifically, SGN-40 led to down-regulation of the IL-6 receptor and upregulation of multiple cytotoxic TNF ligands (FasL, TRAIL, and TNF-α). Utilising multiple death signals results in apoptosis mediated by several pathways that activate specific caspases. CHX blocks the anti-apoptotic protein FLIP (FLICE (FADD-like IL-1β-converting enzyme) inhibitory protein). These findings suggested a role for combination therapy with SGN-40. Proliferation assays have shown that SGN-40 does not stimulate resting B cells and is therefore unlikely to lead to an expansion of normal circulating haematopoietic CD40+ cells (Law et al. 2005).

The first-in-human phase I trial examining SGN-40 was conducted in 44 patients with recurrent/refractory advanced MM that had a median number of 5 previous systemic therapies (Table 1) (Hussein et al. 2010). This multicentre trial utilised a 3 + 3 conventional study design with patient cohorts receiving weekly or initial biweekly SGN-40 with sequential intrapatient dose escalation for 4–5 weeks in total. The dosing range was broad between 0.5 mg/kg weekly ×4 up to 8–16 mg/kg weekly ×5, with a corresponding C_{max} between 12.1 and 636 μg/mL. Treatment was generally well tolerated, 41 % developed CRS associated with first infusion, which reduced with subsequent treatments and steroid premedication. 6 patients experienced dose-limiting toxicity (DLT), no objective responses (OR) were observed and the best clinical response was stable disease (SD) in 20 %, which was unrelated to dose. 12 % of patients had 1-level improvement in ECOG performance status. The maximum-tolerated dose (MTD) was 12 mg/kg/week with dose escalation and steroid premedication.

The phase I trial of SGN-40 in relapsed/refractory NHL subtypes was performed in 50 patients using a dose escalation schedule over 5 weeks with a maximum weekly dose of 8 mg/kg (Advani et al. 2009). 13 patients (26 %) achieving stable disease or better went on to receive a second cycle of 4 infusions of SGN-40. The MTD was not established at the doses tested, and there was no dose-response relationship, with responses seen at 3 mg/kg and higher. The ORR was 12 % with one DLBCL patient achieving a durable CR for greater than a year. Two patients who discontinued the study due to adverse events went on to have delayed and durable CRs. Variable degrees of elevation were observed in the concentration of

plasma cytokines IL-1β, IL-6, IL-10, and TNF-α in response to SGN-40. The degree of CD40 expression was not correlated with clinical response. SGN-40 was generally tolerable with adverse events being less frequent in cycle 2 suggesting tolerability with extended therapy.

A small 12-patient phase I trial of SGN-40 was conducted in patients with CD40-positive CLL with a median of 4 prior systemic therapies (range 2–11) (Furman et al. 2010). The study design was similar to other SGN-40 phase I trials with 3 patient cohorts following intrapatient dose escalation of 3–8 mg/kg/week for up to 9 weeks (equivalent to 2 cycles). The aim of this design was to reduce first dose CRS, alongside paracetamol/antihistamine premedication. No SAEs were observed, Grade 4 thrombocytopenia was the only DLT, and the MTD was not reached. There were no objective responses with the best clinical response being SD in 42 %. The study authors postulated that prior immunosuppressive therapies and the burden of disease are possible factors that negatively affected efficacy to SGN-40. Based on these modest results, the investigators documented that phase II monotherapy of SGN-40 in CLL would not be pursued.

Overall, SGN-40 is well tolerated with mild side effects, unrelated to dose, which most commonly reflect mild, self-limiting CRS symptoms (e.g. headache, fatigue, fever, etc.) or non-infectious inflammatory eye disorders such as conjunctivitis and blepharitis. Drug-related side effects such as conjunctivitis and mild asymptomatic transaminitis are likely to reflect CD40 expression on conjunctiva and hepatic Kuppfer cells, respectively. The efficacy of SGN-40 as a monotherapy is modest across the tumour types tested.

6.2 CP-870,893

CP-870,893 (Pfizer) is a fully human anti-CD40 IgG2, kappa, mAb that binds human CD40 with sub-nanomolar affinity (K_d of 0.4 nmol/L) augmenting DC costimulatory molecule expression and cytokine secretion. CP-870,893 has poor complement activation and minimal FcR binding on effector cells due to its IgG2 isotype and does not block CD40L binding (Bedian et al. 2006). Recent data have confirmed that CP-870,893 does not elicit ADCC or CDC in $CD40^{hi}$ cancer cell lines in vitro and is not dependent on FcγR interactions or antibody cross-linking for it biologic activity (Richman and Vonderheide 2014). Thus, its mechanism of action is not to kill by direct targeting and cell deletion, but rather through indirect activation of DCs and macrophages (Kalbasi et al. 2010; Vonderheide and Glennie 2013). Agonist activity was determined as CP-870,893 upregulates MHC Class II, CD80, CD86, CD23, and ICAM-1 surface molecules when incubated with human whole blood, with an EC50 of 5–50 ng/ml. High levels of IL-12, a T cell stimulating factor, were secreted when human monocyte-derived DCs were stimulated with CP-870,893. In vitro and primate studies with CP-870,893 demonstrated activation of APCs, a rapid depletion in circulating B cells, upregulated B cell surface markers and enhanced immunogenicity of CD40-positive tumour cells

susceptible to eradication by human CTLs. One hypothesis for the rapid but transient reduction of circulating B cells is that CP-870,893 increases expression of the adhesion molecule ICAM-1 and chemokine CCR7, driving B cells into the tumour and secondary lymphoid organs (Carpenter et al. 2009). However, no lymphadenopathy or acute splenomegaly was seen in the phase I trial (Vonderheide et al. 2007). Single intraperitoneal injections of CP-870,893 into CD40-positive human tumours in SCID beige mice induced growth arrest of B cell lymphomas, the breast carcinoma BT-474, and the prostate tumour PC-3 (Gladue et al. 2006). Additional in vitro studies further demonstrated the agonist ability of CP-870,893 with activation of both naive CD19+ and memory CD19+CD27+ B cells triggering the expression of immunostimulatory molecules (CD86, CD70, CD40, and MHC class I and II), with B cells acting as conditioned APCs leading to activation of reactive T cells with effector cytokine secretion (IL-2 and IFN-γ) (Carpenter et al. 2009). The half-life of CP-870,893 was found to be less than 24 h in two human studies. Tumour regression was seen in combination with a sub-optimal dose of cisplatin chemotherapy. Modest objective anti-tumour activity was seen in the first phase I trial (Table 2); however, weekly dosing resulted in little efficacy which may reflect immune overstimulation and subsequent relative immunosuppression (Vonderheide et al. 2007; Ruter et al. 2010).

The first human phase I clinical trial of CP-870,893 monotherapy was performed in 29 patients with advanced solid malignancies, which included melanoma, non-small cell lung cancer (NSCLC), sarcoma, cholangiocarcinoma, and several others (Vonderheide et al. 2007). Six single-dose levels were explored between 0.01 and 0.3 mg/kg, with repeat dosing available for responders. Three patients experienced DLT with a MTD established at 0.2 mg/kg. Pharmacodynamic testing showed a rapid but transient, dose-dependent reduction in circulating CD19+ B cells and up to 10-fold increase in CD86 costimulatory molecule expression. CP-870,893 was well tolerated, with the commonest adverse event being mild transient CRS associated with elevated serum TNF-α and IL-6 in some patients. Normal serum tryptase levels were used to indicate that CRS was not an anaphylactic or allergic reaction. Two melanoma patients developed widespread vitiligo, suggestive of T cell-dependent autoimmunity. The best responses were seen in four patients with advanced melanoma achieving partial responses, one of which continued to respond beyond 14 months from first dose and has been described separately as achieving complete remission after 5 years (Vonderheide and Glennie 2013).

Due to the encouraging results of CP-870,893, a second phase I trial was conducted with weekly administration in 27 patients with multiple advanced solid tumours (Ruter et al. 2010). The weekly dosing schedule was designed according to the pharmacodynamics profile of CP-870,893 with effects such as transaminitis resolving within 1 week from administration. Overall, good tolerability was observed, with an MTD of 0.2 mg/kg, the same established in the initial phase I trial and defined by a similar incidence of CRS with typical transient self-limiting symptoms such as rigours, fever, and chills. Grade 3 CRS was used to describe

prolonged or recurrent symptoms; however, no patients required hospitalisation. Lymphocytes, monocytes, and platelets were transiently depleted, returning to normal 24–48 h after infusion of CP-870,893; however, platelet recovery was slower. This reflects CD40 expression on these cell populations. Elevated liver enzymes returned to baseline prior to the next infusion. The best response observed was SD in 26 %, seen at all dosing levels, with a median time to progression of just over 4 months in these patients. Weekly dosing of CP-870,893 is feasible; however, no objective clinical responses were observed. Similar to previous in vitro and in vivo data, there was upregulation of B cell costimulatory and adhesion molecules, consistent with B cell activation, which was sustained above baseline levels and observed with each dose. There was inter-patient variation of baseline peripheral T cell populations; however, 50 % of patients tested post-treatment had marked depletion of CD4+ and CD8+ T cell subsets with inconsistent functional capability as determined by IFN-γ and perforin expression, respectively. These findings are consistent with mouse melanoma models where anti-CD40 mAb treatment alone accelerated depletion of tumour-specific CD8+ T cells producing a negative effect of cancer therapy (Kedl et al. 2001). These tumour-specific CD8+ T cells could be rescued when anti-CD40 mAb was given with selective tumour antigen vaccination. In another study, mice immunised with agonistic anti-CD40 mAb with IL-2 or IL-15 (an important cytokine for memory T cell development) had significant primary anti-tumour response but subsequently developed marked CD4+ T cell apoptosis with impaired secondary memory responses, succumbing to secondary tumour challenge (Berner et al. 2007). Despite the ability of CP-870,893 to activate B cells with APC capacity, the induction of specific anti-tumour T cells was not observed. The study authors concluded that the lack of clinical responses observed may reflect to frequent dosing of CP-870,893, leading to immune hyperstimulation with counterproductive peripheral T cell depletion and persistent B cell stimulation. This is supported by the immunosuppressive effect seen with daily agonistic anti-CD40 mAb administration used to treat chronic autoimmune inflammatory rheumatoid arthritis in mouse models (Mauri et al. 2000).

6.3 ChiLob 7/4

ChiLob 7/4 is an IgG1 chimeric agonistic anti-CD40 mAb (K_d of 0.2 nmol/L) developed by the University of Southampton. The phase I trial of ChiLob 7/4 monotherapy is currently ongoing (Table 5), involving a dose-escalation study design of 4 weekly IV infusions at a range from 0.5 to 240 mg/dose in 28 CD40-positive patients with a range of solid (n = 26) and lymphoid malignancies (DLBCL; n = 2) (Johnson et al. 2010, 2013). Preliminary data reported the best clinical responses observed have been stable disease, seen in 11 of the first 21 patients, with one mesothelioma patient achieving prolonged disease stabilisation

after receiving a single 1.6 mg initial dose. The same patient was retreated after more than 3 years with a 240 mg dose achieving ongoing stable disease in excess of 17 months. ChiLob 7/4 was tolerable, with infusion reactions prevented by steroid premedication and dose-limiting Grade 3 hepatic transaminitis observed in two patients at 240 mg single dose. Human anti-chimeric antibody (HACA) responses were common at low doses (5–50 mg) but absent at higher doses (160–240 mg), corresponding with dose-dependent partial depletion of peripheral blood CD19+ B cells. Activation of innate immune effectors, NK cells and monocytes, was detected by elevated CD54 expression at doses of 160 mg or above, but there was no consistent change in T cell markers suggestive of activation.

Alongside the phase I study, investigators monitored the effects of ChiLob 7/4 on the activation of myeloid dendritic cells (mDC1), plasmacytoid dendritic cells (pDC) and cytokine profiles by performing ex vivo whole-blood stimulation assays on the peripheral blood of study participants (Chowdhury et al. 2014). When ChiLob 7/4 was cross-linked with a goat anti-human IgG Fcγ-specific antibody, it was able to upregulate CD83 on pDCs and mDC1s in a dose-dependent manner, indicating early dendritic cell activation. Similarly, increases in various Th1 cytokines (IL-12, MIP1a, MIP1b, IL-8, IL-6, and TNF-α) were detected with increasing concentration of Fc receptor cross-linked ChiLob 7/4. The activation capacity of ChiLob 7/4 to induce dendritic cell maturation and migration, as evidenced by the upregulation of the chemokine receptor CCR7, was quantified by comparison with known DC-activating TLR ligands. When cross-linked, ChiLob 7/4 showed near-equivalent levels in activating DCs when compared to the potent TLR ligand LPS, but occurred more rapidly, with mDC1 CCR7 upregulation detectable after 4 h. A similar cytokine profile was generated with cross-linked ChiLob 7/4 to that of LPS; however, IL-6 expression was significantly lower with ChiLob 7/4 suggesting an overall lower pro-inflammatory response. The fact that ChiLob 7/4 has a human IgG1 Fc portion and relatively low binding affinity to inhibitory FcγRIIb, thus limiting the opportunity for cross-linking, may be suggestive that IgG1 is not the ideal isotype to exploit its immunostimulatory activity.

In vivo effects of ChiLob 7/4 on circulating DCs and cytokine expression were investigated through sequential blood sampling of study participants. Two of the cytokines that are frequently associated with a cytokine storm (TNFa and IL-6) were not detectable in samples from ChiLob 7/4-treated patients. However, this may reflect missed detection due to time point sampling or steroid-induced cytokine suppression. Overall, stronger CD40 stimulation was required to trigger cytokine production than DC activation. Despite the heterogenous patient population producing a mixed pattern of DC activation kinetics, similarities were observed with higher ChiLob 7/4 doses in the ex vivo analysis with artificial cross-linking. This suggests that some degree of antibody cross-linking may be occurring in vivo after administration with the soluble non-cross-linked drug. The biologic activity of agonistic anti-CD40 mAb is dependent on FcγR cross-linking which provides a structural scaffold to enable CD40 signalling.

7 Antagonistic Anti-CD40 mAb

7.1 Lucatumumab (HCD122)

Lucatumumab (Novartis) is a fully humanised anti-CD40 IgG1 mAb that augments ADCC and blocks the ligation of CD40L with CD40. Lucatumumab binds to CD40 with high affinity (K_d of 0.5 nmol/L) has a slow 'off-rate' with preclinical studies showing lucatumumab to be a potent CD40L antagonist inhibiting signalling pathways, secretion of cytokines, differentiation, proliferation and survival (Luqman et al. 2008). Lucatumumab inhibits CD40L-mediated growth of normal B cells, NHL cells and CLL cells in vitro (Byrd et al. 2012). Lucatumumab, formerly named CHIR-12.12, binds to CD138-expressing MM lines, inhibiting PI3K/AKT, NF-κB, and ERK activation induced by CD40L, antagonising CD40L-induced proliferation and triggering lysis via ADCC in CD40-positive cells (Tai et al. 2005a, b). Lucatumumab was shown to have strong antagonistic activity in B cell CLL tumour cells causing ADCC mediated lysis more efficiently than rituximab in vitro, despite nearly sixfold higher CD20 cell surface binding sites compared to CD40. This effect was thought to be because lucatumumab, unlike rituximab, is not internalised but remains uniformly distributed on the cell surface and therefore more responsive to interact with NK cells or other effector cells that mediate ADCC (Luqman et al. 2008). Lucatumumab has potent activity in MM cells in vitro and xenograft models in vivo, significantly prolonging the survival in tumour-bearing mice with tumour regression seen in 63 % ($p < 0.01$) (Long et al. 2005).

A first-in-man phase I trial of lucatumumab monotherapy was conducted in 26 patients with symptomatic, fludarabine-experienced CLL with five dose cohorts between 0.3 and 6.0 mg/kg, receiving weekly infusions for 4 weeks (Table 3.) (Byrd et al. 2012). A MTD was established at 3.0 mg/kg due to four DLTs from asymptomatic elevated lipase/amylase levels with no clinical or radiological evidence of pancreatitis. This effect most likely represented CD40 expression within pancreatic tissue, a phenomenon previously reported (Vosters et al. 2004). Overall, lucatumumab had acceptable tolerability and a half-life of approximately 7 days. The best objective response was a nodular PR in one patient, whilst 65 % of participants achieved SD for a mean duration of 76 days (range 29–504 days). The study authors summarised these results as representing minimal single-agent activity, advising future studies to focus on combination approaches.

A phase I trial of lucatumumab monotherapy was performed in 28 patients with relapsed/refractory MM with four dose levels of 1.0–6.0 mg/kg in weekly infusions for 4–8 weeks (Bensinger et al. 2012). Three DLTs were observed establishing an MTD of 4.5 mg/kg. Numerous AEs were reported, frequently related to infusion reactions that were generally mild to moderate in severity. SAEs included haematological, biochemical and electrolyte disturbances with 14 % of participants discontinuing the study drug as a result of AEs. The ORR was 4 % due to one PR. 43 % of patients achieved SD, lasting on average just over 2 months. The study authors reflect on these modest results by highlighting that the study population was

heavily pretreated with a median of eight prior MM therapies and that the 11-week follow-up period may fail to document delayed responders. They postulate potential synergy could exist between lucatumumab and approved agents such as lenalidomide or bortezomib for MM.

A larger phase Ia/II trial of single agent lucatumumab was conducted in 111 patients with multiple NHL subtypes and HD (Fanale et al. 2014). The six DLTs consisted of asymptomatic elevated lipase and transaminitis resulting in a MTD of 4 mg/kg. The half-life at 4 mg/kg was approximately 4.5 days. SAEs occurred in 27.9 % with the most common being secondary to dyspnoea, fever, and chills. Efficacy was subtype specific with an ORR for MALT, FL, HD, and DLBCL of 42.9, 33.3, 13.5, and 11.8 %, respectively. Lucatumumab has overall modest activity as a single agent with several potential mechanisms of action; however, further investigation is required to establish an optimal treatment setting within a particular tumour subtype.

8 CD40 Combination Therapy

As with the monotherapy clinical trials, dacetuzumab causes direct killing of tumour cells whilst CP-870,893 exploits the immunostimulatory capacity of anti-CD40 activation. Overall, the combination studies demonstrate modest clinical efficacy; however, activity in pancreatic cancer by immune stimulation represents a role for immunotherapy in a cancer frequently associated with resistance to conventional cytotoxic chemotherapy. Combining immunotherapy with non-immunoablative chemotherapy has the aim of chemotherapy-induced tumour cell killing leading to increased tumour antigen available for cross-presentation, which when combined with immunomodulatory antibodies increases cross-priming of tumour-specific CTLs (Lake and Robinson 2005). Chemotherapy-induced apoptosis of large murine tumours loads APCs with tumour antigens and sensitises them to CD40 signalling. One study demonstrated that gemcitabine chemotherapy which induced apoptosis in established murine solid tumours (mesothelioma) enabled subsequent synergy with an anti-CD40 mAb (FGK45) leading to long-term tumour remission and resistance to tumour rechallenge indicative of established immunological memory (Nowak et al. 2003). Exogenous CD40 ligation was associated with both CD4+ and CD8+ T cell infiltration into the tumours. However, depletion studies demonstrated that unlike the pivotal role of CD8+ effectors, CD4+ cells were not necessary for tumour rejection.

Alternative approaches with combination studies aimed to increase direct killing of tumour cells through ADCC and direct apoptotic signalling. ADCC assays demonstrated synergy between anti-CD40 mAb (SGN-40) and rituximab showing an additive anti-tumour response with combination therapy (Lewis et al. 2008a, b). SGN-40 appears to chemosensitise lymphoma cells to augment enhanced ADCC with improved efficacy in combination with multi-agent cytotoxic chemotherapy regimens such as ICE (ifosfamide, carboplatin, and etoposide) and CHOP (cyclophosphamide,

doxorubicin, vincristine, and prednisolone), both with and without rituximab (Lewis et al. 2007). In vitro and xenograft models combining SGN-40 and rituximab demonstrated a synergistic effect in tumour cell apoptosis; however, SGN-40 did not potentiate rituximab-induced ADCC activity (Lewis et al. 2011). Both agents activated MAP kinase signalling with the oncoprotein BCL-6 being strongly downregulated by SGN-40 in each cell line, whilst this downregulation was cell line-specific with rituximab. SGN-40 alone induced expression of the pro-apoptotic proteins Fas and Tap63 (p53 family member) and rituximab partially blocked SGN-40-mediated induction of prosurvival BCL-xL expression.

An international phase IIb of 151 patients with refractory DLBCL was randomised to receive 3 cycles of R-ICE (rituximab, ifosfamide, cyclophosphamide, etoposide) + dacetuzumab (SGN-40) (8 mg/kg) versus R-ICE + placebo (Table 4) (Fayad et al. 2011). A futility analysis conducted after 112 patients were treated revealed a similar CR (36 % dacetuzumab vs. 42 % placebo) and ORR (64 vs. 68 %), and as a result, further enrolment was stopped. After approximately 1-year follow-up, there was a trend towards improved failure-free survival (FFS) (hazard ratio [HR] = 0.789) and OS (HR = 0.714) for dacetuzumab patients. There was a survival advantage in dacetuzumab patients who went on to receive subsequent autologous stem cell transplant (ASCT) (HR = 0.167, p = 0.008) with a 14 % reduction in death due to disease progression (25 % dacetuzumab vs. 39 % placebo). These data are based on results presented at the international conference of malignant lymphoma (ICML), Switzerland, which suggest a potential survival advantage with agonistic anti-CD40 mAb combination therapy despite no improvement in objective tumour response.

A phase I study investigated the role of CP-870,893 in combination with conventional gemcitabine in 22 patients with chemotherapy-naive advanced pancreatic ductal adenocarcinoma (Beatty et al. 2013). CP-870,893 with gemcitabine was well tolerated and associated with early evidence of efficacy with 4 PRs (ORR of 19 %). This is significant when compared to an ORR of just 5.4 % with gemcitabine monotherapy, and 1-year OS of 18 versus 28.6 % with combination therapy (Burris et al. 1997). Pancreatic cancers carry a particularly poor prognosis due to intrinsic chemotherapy resistance and a hostile tumour microenvironment characterised by the presence of tumour-associated macrophages and poor vascularity. Preliminary studies had demonstrated that the use of agonistic anti-CD40 mAb caused CD40-activated tumouricidal macrophages to rapidly infiltrate pancreatic tumours, causing targeted depletion of the tumour stroma (Beatty et al. 2011). Within the trial, combination therapy produced immune stimulation with transient depletion of B cells, increased inflammatory cytokines (IL-6, IL-8, IL-10) and increased B cell costimulatory molecules (CD86 and MHC class II receptor HLA-DR). This immune activation was transient and did not represent a biomarker of response. PET imaging demonstrated metabolic responses within the primary tumour in the majority of patients imaged; however, some patients developed concurrent disease progression of metastatic deposits, highlighting the heterogeneity of tumour response. Large studies are required to better assess anti-tumour efficacy with combination strategies in pancreatic cancer.

A phase I study investigated the role of CP-870,893 when combined with two chemotherapy agents, paclitaxel and carboplatin, in patients with advanced solid tumours (Vonderheide et al. 2013). CP-870,893 was either given on day 3 or 8 post chemotherapy. This scheduling was designed to establish the optimum time point after chemotherapy where released tumour-associated antigens were maximally present to provide an immunogenic stimulus. However, there was no significant difference in response or immune pharmacodynamics between the two schedules. The ORR was 20 % with best responses being PRs seen in patients with metastatic melanoma, renal cell carcinoma, prostate cancer, and ovarian cancer. Responses were observed in some patients who had previously progressed on platinum and taxane chemotherapy, highlighting that efficacy did not simply reflect chemotherapy activity. Toxicity analysis of the three drug combination was considered to be safe; however, one DLT was a fatal stroke occurring six days after the first CP-870,893 infusion. Immune pharmacodynamics analysis showed rapid depletion and activation of peripheral B cells and evidence of transient induction of melanoma-specific T cell responses in two patients tested. These findings were similar to those with CP-870,893 monotherapy leading to the conclusion that chemotherapy does not prevent immune activation. Encouragingly, the peripheral T cell depletion seen with weekly CP-870,893 dosing in the phase I study and preclinical study (Kedl et al. 2001; Ruter et al. 2010) was not observed with three weekly dosing alongside combination chemotherapy.

A phase Ib study investigated the combination of SGN-40, rituximab, and gemcitabine in 33 patients with relapsed/refractory DLBCL (Forero-Torres et al. 2013). The study population was described as predominantly elderly with a median age of 67 (range, 29–80). As mentioned earlier, preclinical studies had previously demonstrated synergy of anti-tumour activity with rituximab and SGN-40 in vivo (Lewis et al. 2008a, b, 2011). The combination of agents was generally well tolerated; however, a relationship between the one fatality and SGN-40 could not be excluded. The cause of the death was secondary to pneumonitis in a patient previously irradiated who received 20 infusions of SGN-40. The frequency of gemcitabine administration was reduced during the trial to overcome the development of thrombocytopenia. The ORR was 47 % with a CR of 20 %, median PFS of 25 weeks and median duration of response of over 6 months. All CRs had high CD40 expression levels (3+) whilst conversely CD40-negative expression was associated with non-response. There was no correlation between FcγRIIa and FcγRIIIa polymorphisms and objective response. The response rate observed was similar to an existing second line regimen with rituximab, gemcitabine, and oxaliplatin (R-GEMOX) (ORR 43 %) (Lopez et al. 2008); however, the CR rate was lower (20 vs. 34 %) and OS was not determined in the SGN-40 study to allow a comprehensive comparison. Long-term results of R-GEMOX demonstrated higher ORR of 57 and 37 % OS at 3.5 years as salvage therapy in transplant ineligible DLBCL patients (Corazzelli et al. 2009).

There are currently several ongoing combination trials involving anti-CD40 mAb with a mixture of chemotherapy and biological agents (Table 5). The study design and choice of agents to combine are based on preclinical studies to determine mechanisms of synergy between agents. For instance, two preclinical studies

of SGN-40 and lenalidomide in CLL and MM have shown pretreatment with lenalidomide sensitised MM cells to SGN-40-induced cell death in vitro (Tai et al. 2005a, b; Lapalombella et al. 2009). The combination induced direct apoptosis in MM and CLL and markedly enhanced ADCC of autologous CD40-expressing MM cells and primary CLL B cells. Lenalidomide facilitated IL-2-mediated activation of NK cells and induced more potent mediators of ADCC against target tumour cells.

9 Currently Active Trials

See Table 5.

10 Conclusion

It remains a major scientific goal to address the unmet need for improved anti-cancer therapies to reduce the high burden of morbidity and mortality associated with the many incurable or poorly responsive tumour types. The goal of cancer immunotherapy is to activate the host immune system in a targeted way in order to increase recognition and eradication of malignant cells. The challenge is daunting given the vast hurdles needed to be overcome from initial target identification and validation to preclinical investigation, through to early 'proof of concept' clinical trials, late-phase efficacy, safety, and economic evaluation to final approval. Nonetheless, the last few decades of incremental scientific and clinical progress in this field are coming to fruition with the remarkable results from several approved and emerging immunostimulatory antibodies and immune checkpoint blockers, such as those that target CTLA-4 and PD-1.

The key reason that targeting CD40 to augment protective anti-tumour immunity remains attractive and offers significant therapeutic potential is that it exerts its effects by a plethora of different mechanisms. In broad terms, these include indirect modulation of host immune cells through boosting APC function, activating tumour-specific T cells, NK cells, and tumouricidal macrophages, or direct pro-apoptotic signalling and through the recruitment of innate immune effectors via ADCC/ADCP. Early clinical trials have shown modest clinical activity in the absence of troublesome toxicity. If developed through well-constructed clinical trials, which will most likely involve innovative combinations of agents, with careful attention to scheduling and exploiting multi-agent synergy, anti-CD40 mAb therapy could be harnessed to treat a broad range of lymphoid and solid malignancies. Equally, the development of new anti-CD40 agents that capitalise on the many strengths of existing agents but can be engineered to exploit the importance of engagement with the inhibitory FcγRIIb on neighbouring immune cells to facilitate antibody cross-linking will further potentiate immunostimulatory activity and thus clinical efficacy. The interactions between anti-CD40 mAb and FcγRs are dependent on the nature of

the local immune environment and the anatomical location of the FcγRs. The realisation that coengagement with the inhibitory FcγRIIb is essential to the immunostimulatory activity of many anti-CD40 mAb has been a watershed development and stands in contrast to direct-targeting antibodies, which require activatory FcγR interactions to augment activity through cell deletion. The future of immunostimulatory antibody therapy is extremely bright and likely represents our greatest hopes of making a fundamental transformation to the natural course of the many currently incurable human cancers.

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