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Correlates of immune and clinical activity of novel cancer vaccines

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ABSTRACT

Cancer vaccines are solely meant to amplify the pool of type 1 cytokine oriented CD4+ and CD8+ T cells that recognize tumor antigen and ultimately foster control and destruction of a growing tumor. They are not designed to deal with all aspects of immune ignorance, exclusion, suppression and escape that are generally in place in patients with cancer and may prevent the T cells to enter the tumor or to exert their effector function. This simple fact prompted for a reappraisal of the many recent trials in which therapeutic cancer vaccines have been examined as monotherapy. In this review, I focus on trials examining therapeutic cancer vaccines at different stages of existing disease. The analysis of vaccine-induced immune responses and clinical activity of therapeutic cancer vaccines revealed four levels of evidence for vaccine efficacy. The lowest levels, reflect the many trials in which the strength of the tumor-reactive T cell response of vaccinated patients is associated with better clinical outcome or change in tumor marker. The highest levels indicate occasional regressions of tumors and metastases after vaccination or reflect a stronger clinical impact of vaccine in a randomized trial. A whole series of trials in which vaccine-induced tumor immunity correlates with the clinical impact of cancer vaccines in premalignant diseases, settings of low tumor burden or tumor regressions in patients with cancer, form an attest to the fact that cancer vaccines work. While the current number of true clinical responders in each cancer trial is too low for firm conclusions on immune correlates of clinical reactivity in cancer, extrapolation of the results from vaccinated patients with pre-cancers suggest a requirement of broad type 1 T cell reactivity.

1. Introduction

The immune system has an important role in the control of tumor outgrowth. There is the consensus that a strong Th1 cytotoxic microenvironment is associated with a more favorable prognosis and therapy responsiveness in many tumor type [1,2]. Harnessing the immune system to detect and destroy tumors has been a long-term goal in mankind since the 1891 report of Coley [3]. A number of effective strategies, including adoptive cell transfer [4,5] and immune checkpoint blockade [6,7], have been developed such that immunotherapy of cancer has become one of the pillars of modern cancer therapy in the clinic. The response rate to checkpoint therapy varies tremendously per cancer. Growing evidence indicates that patients lacking pre-existing tumor immunity are less likely to respond [8,9], suggesting that their immune system needs to be pre-sensitized to tumor antigens. Cancer vaccines are excellently suited for this job since they can amplify the pool of tumor-reactive T cells from the naive repertoire, reactivate existing tumor-specific T cells and are able increase the breadth and diversity of the tumor-reactive T cell response.

The key component of a vaccine is the antigen used to stimulate the immune system. Initial cancer vaccines were based on cancer cell lysates but the molecular identification and characterization of a the first gene reported to encode a defined tumor antigen that was recognized by tumor-killing CD8+ T cells, boosted the development of potential cancer vaccines [10]. Since then many suitable target antigens have been identified. Tumor antigens can be classified as tumor associated or tumor specific [11]. Many of the cancer vaccines developed aimed to increase T cell reactivity to self-proteins that are overexpressed, involved in tissue differentiation or which are expressed by tumor cells and immune privileged tissue such as the cancer-testis antigens. Together, they form the broad category of tumor associated antigens (TAA). The preference to use TAA in cancer vaccines was their broader applicability (e.g. multiple patients with same cancer, cancers of different types sharing antigen expression). There is accumulating

Abbreviations: AML, acute myeloid leukemia; CML, chronic myeloid leukemia; BCG, Bacillus Calmette-Guerin; CEA, carcinoembryonic antigen; CMV, cytomegalovirus; CR, complete response; DTH, delayed type hypersensitivity; DFS, disease free survival; GBM, glioblastoma multiforme; HER2, human epidermal growth factor receptor-2; HPV16, human papillomavirus type 16; HIF-1α, hypoxia-inducible factor-1α; IDO, Indoleamine 2,3 dioxygenase; IVS, in vitro stimulation; LDH, lactate dehydrogenase; MDS, myelodysplastic syndrome; MR, mixed response; MUC1, mucin-1; RECIST, response evaluation criteria in solid tumors; RFS, recurrence free survival; OS, overall survival; PD, progressive disease; PFS, progression free survival; PPV, personalized peptide vaccine; PR, partial response; PSA, prostate specific antigen; SD, stable disease; SLP, synthetic long peptide; TAA, tumor associated antigen; TIL, tumor infiltrating lymphocytes; TSA, tumor specific antigen

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evidence for the presence of spontaneously activated T cells recognizing self-proteins that are preferentially expressed by regulatory immune cells. Vaccines designed to stimulate T cell responses to these antigens indirectly target tumors by unleashing spontaneous tumor immunity. However, it turns out that given the self-nature of TAA, responding T cells are likely to suffer from some degree of central tolerance and are not truly tumor specific.

The group of tumor specific antigens (TSA) is formed by the proteins of oncogenic viruses that are expressed by transformed cells and by neoantigens generated as products of somatic mutations and frame shifts. Some of the evidence for the existence and important role of tumor specific antigens in tumor control dates back to the early days in tumor immunology, showing that experimental tumors arising in the skin after exposure to chemical carcinogens or ultraviolet (UV)light bear unique tumor-rejection antigens [12,13], whereas virus-induced tumors displayed viral proteins that functioned as such [14,15]. There is also data suggesting that a group of TAP-independent self-peptides, which are only expressed by cells deficient for the peptide transporter TAP, can act as tumor specific antigens [16,17]. The development and impact of preventive cancer vaccines has recently been excellently reviewed by Finn [18] and Spira et al. [19]. In this review, the focus is on therapeutic cancer vaccines, applied at different stages of existing disease. The mechanisms and components required to build effective therapeutic cancer vaccines and how to deliver them to patients, has been reviewed excellently by Hu et al. [20].

In the past, the results of cancer vaccines led to a too pessimistic view on their potential within the immunotherapy space [21,22]. This view was fueled by large phase III studies with negative outcomes and based on the misconception that immunotherapy was just a matter of replenishing the host with tumor-reactive T cells, whereas we now know that cancer immunity is influenced by a complex set of host, tumor and environmental factors [23]. Hence, while vaccines are only meant to amplify the pool of type 1 cytokine oriented tumor-reactive CD4+ and CD8+ T cells they were, in fact, expected to deal with all aspects of immune ignorance, exclusion, suppression and escape. Therefore, published trials should be appraised in the context of our current knowledge that the full clinical potential of therapeutic cancer vaccines can only be determined when appropriate co-treatments are provided that overcomes systemic and local immune suppression as well as immune exclusion [24]. In this review, a number of negative phase III trials are discussed in the context of today's knowledge of the tumor microenvironment. Then, a whole series of recent cancer vaccine trials is reevaluated with respect to their capacity to induce tumor immunity and the correlation of this immune response to clinical outcome. Keeping in mind that previously much optimism was based on surrogate endpoints rather than actual tumor regressions [21], four levels of evidence for vaccine efficacy on clinical outcome (Fig. 1) were distinguished.

It turns out that increases in functional tumor-reactive type 1 T cell responses and regression of lesions or metastases can be observed after vaccination in quite a number of trials. New studies will require investigators to address the reasons for successful regressions as this will lead the way for application of cancer vaccines under the best conditions.

2. Therapeutic vaccination and clinical outcome

2.1. Phase 3 trials with tumor-associated antigens failed for a reason

A series of phase 3 cancer vaccination trials have been reported in the last couple of years. None of them had a positive outcome. Considering the task cancer vaccines have, one should revisit these trials with the knowledge of today, rather than throwing the immunotherapeutic potential of cancer vaccines in the waste basket.

One large study evaluated 3 different HLA-A*0201 restricted melanoma peptides previously found to elicit a T cell response in 35% of the stage IV melanoma patients and of which the immune responders did show higher overall survival (OS) than the non-immune responders [25]. This resulted in a new randomized, placebo-controlled phase III study where 815 patients, 436 of which were HLA-A*0201, with completely resected stage IV melanoma or high-risk stage III were vaccinated with the peptide vaccine, GM-CSF or both, but no improved recurrence free survival (RFS) or OS was found [26]. Inspection of patient demographics teaches us that about 90% of the patients displayed absent or sparse infiltrate in their primary tumor. Such non-inflamed tumors have a low capacity to attract T cells, and therefore are not likely to respond to vaccination or other individual immunotherapies [23]. This in combination with the apparent low immunogenicity of the vaccine would allow only a very small percentage of patients to respond (10% of 35% makes 3,5% of patients) [25,26]. In view of our understanding with respect to the role of neoantigen-specific T cells in melanoma, targeting of TAA in melanoma is not expected to drive major clinical successes.

GV1001, targeting telomerase, was tested in randomized phase 3 trial of patients with pancreatic ductal adenocarcinoma to receive either gemcitabine/capecitabine chemotherapy or chemotherapy with sequential GV1001 or chemotherapy with concurrent GV1001 [27]. The immune response was tested in small part of the vaccinated group, and only measurable by proliferation after > 10 days of in vitro stimulation (IVS). A difference in response over background of > 1.8 was defined as positive. Still only 30% of the patients in the sequential group and 15% of the patients in the concurrent group showed a T cell proliferative response. In addition, only 12% and 20%, respectively, showed a positive delayed type hypersensitivity (DTH) response to the vaccine. There were no differences in survival [27]. Thus, the vaccine was able to induce a T cell response in a minority of patients. This tremendously lowers the number of patients that could show clinical reactivity. Furthermore, the choice to combine with chemotherapy was based on pre-clinical mouse models showing a positive effect of gemcitabine on immune suppressive cells [28,29] and with cancer vaccines [30]. However, recently it was shown that gemcitabine has an effect on a phenotypically defined population of myeloid derived suppressor cells (MDSC) in patients [31] but also that this particular population was not suppressive. Other MDSC phenotypes that were suppressive were not decreased by gemcitabine treatment [32]. Neither the impact of the immunosuppressive cells nor the influence of the tumor immune contexture was assessed within this trial.

IMA901, a vaccine consisting of 9 HLA-class I- and 1 HLA class II-restricted tumor-associated peptides, was tested in a phase III trial in patients with metastatic clear cell renal cell carcinoma [33]. Patients were either treated with sunitinib only or in combination with the vaccine. There were no differences in survival. In addition, the previously reported correlation between survival and the number of epitopes recognized [34,35] was not confirmed. An important finding within this study was the observation that the CD8+ T cell responses to the vaccine were 3-fold lower than previously observed in the phase 1–2 trials with this vaccine and type of patients. However, in these earlier trials sunitinib was not used. The reason to use sunitinib was based on mouse models showing that levels of regulatory T cells (Tregs) were reduced, it also has an established clinical effect in renal cell carcinoma. Studies in patients receiving sunitinib confirmed the reduction in Tregs, albeit small. Also, sunitinib treatment has been reported to reduce CD33+, HLA−DR− MDSC and CD15+, CD14− MDSC [36,37]. However, sunitinib may also affect other myeloid populations. The authors found a strong reduction in the number of monocytes after first round of sunitinib. This effect on monocytes was known and is the result of reduced hematopoiesis [38] but sunitinib also displays other negative effects such as the induction of IL-10 production by M1 macrophages [38]. Potentially, the strong effect on monocytes is also mediated on DC. This is currently unknown but would be expected and in combination with the modulatory effects on M1 macrophages it could explain why the T cell response is lower in

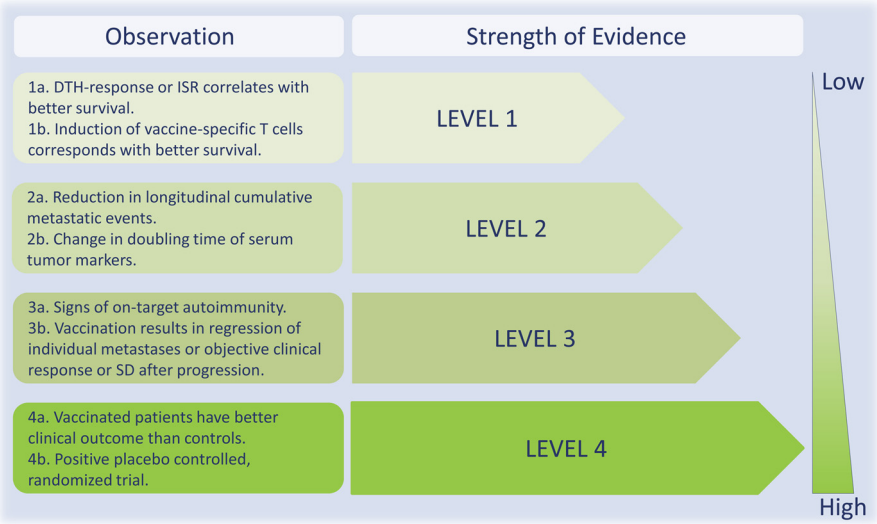

Fig. 1. The levels of evidence for vaccine efficacy. Cancer vaccine trials report vaccine-induced immune responses in the context of different clinical observations. The strength of this evidence for a true impact of the immune response on tumor growth can be considered low to high.

this trial. An important factor that in hind sight could explain failure or the trial is the existence of at least four molecular subtypes of clear cell renal cell carcinoma. The subtype associated with a strong inflammatory, Th1-oriented but suppressive immune environment is the least sensitive to sunitinib [39]. This means effectively, that only one subgroup of patients has an inflamed tumor allowing access of vaccine-induced T cells but it is not clear if these cells could resist the immune suppressive environment. Furthermore, patients with tumors of one of the other molecular subtypes are less likely to benefit from the vaccine but respond better to sunitinib, obscuring vaccine effects on survival.

Three phase 3 trials were performed in non-small cell lung cancer (NSCLC). The first was a placebo-controlled randomized study with tecemotide, a 25 amino acid long MUC-1 lipopeptide derived from the tandem repeat region of MUC-1. The vaccine was given as maintenance therapy to stage III unresectable NSCLC patients with objective responses or stable disease (SD) after chemotherapy [40]. Three days before first vaccination a low-dose cyclophosphamide was provided, based on a trial in breast cancer patients showing stronger immunity. In a preceding phase IIB trial with stage 3b and IV NSCLC patients it was shown that this regimen had a positive effect on survival in the subgroup of IIIB patients, hence the phase 3 trial was started. Interestingly, when the immune response is examined in that trial it turns out that only 16 of 78 patients tested displayed a MUC-1 specific T cell proliferative response, two of which had stage 3b disease [41]. No vaccine associated survival effects were seen in the phase 3 trial, which should not have come to a surprise in view of the low immunological response rate in stage 3b patients. The second study in stage 3/4 NSCLC patients was performed with an allogeneic tumor vaccine, comprising four TGFβ2 antisense gene modified (to prevent immune suppression and to increase immunogenicity) irradiated NSCLC cell lines, as maintenance therapy. No benefit was found [42]. In an earlier phase 2b, IFNγ Elispot reactivity to the allogeneic cell lines was found in 17 of 36 patients, the majority of which were patients with a tumor control of stable disease or better. However, also allogeneic HLA-specific antibodies were found in most of the SD patients, indicating that the T cell reactivity found is likely targeted to the HLA molecules present on these allogeneic tumor cells that are foreign to the patient rather than recognizing tumor antigens [43]. The third trial randomized placebo controlled phase 3 trial comprised a recombinant MAGE-A3 protein vaccine with AS15 immunostimulant. It was tested with or without chemotherapy in patients with stage IB, 2 and 3a MAGE-A3-positive NSCLC [44]. No vaccine effect was seen on disease free survival (DFS), neither in patients with nor without concomitant chemotherapy. The validation of a gene-signature that predicted patients most likely to benefit from vaccination could not be performed. This gene panel comprising immune related, Th1/IFNγ genes and chemokines for T cell homing, STAT1 and IRF1 regulated genes, was discovered to predict better DFS in a phase 2 placebo controlled study in NSCLC [45]. Recombinant protein, however, is not the most immunogenic vaccine concept, their processing by DC is not optimal [46] and this can also be deduced from the immunological responses that were reported earlier for this vaccine. First of all, spontaneous responses to MAGE3 are very rare and vaccine induced responses were measured only after an IVS of at least 10 days before the immune response was measured. In the previous trial only in 1 of 9 vaccinated patients with recombinant MAGE-3 protein and in 4 of 8 patients vaccinated with protein and AS02B adjuvant responded with a type 1 CD4+ T cell response. Furthermore, only 1 out of 9 HLA-A2 and 1 out of 5 HLA-A1 positive patients showed a CTL response after vaccination, respectively [47]. Thus, two trials are likely to have suffered from the low immunogenicity of the vaccine used, whereas in one it can be questioned if there were any tumor-specific responses. In the first trial the choice to go for a certain type of subgroup was based on a post-hoc analysis. Furthermore, also in NSCLC the immune contexture plays an important role with respect to the response to immunotherapy. For instance, NSCLC is known for its notoriously downregulation of HLA class I and this is associated with loss of the clinical effects of strong CD8 T cell infiltration. The same holds true for the expression of HLA-E which has a negative impact on infiltrating CD8 T cells and is overexpressed in 70% of the cases [48]. The importance of HLA class I expression for therapeutic vaccine outcome was also demonstrated in a metastatic melanoma patient who received an autologous melanoma vaccine. Three metastatic lesions strongly expressing HLA class I regressed whereas 3 other lesions had low to no HLA class I expression and progressed [49]. Also, the presence of a type 1 inflamed immune signature is important for responsiveness [50].

Overall it means that a full appreciation of cancer vaccines can only be obtained when cancer vaccines are trialed in settings that optimally support their purpose, that is to reinvigorate the T cell response against tumor antigens, and not asked to overcome the other immunological problems posed by tumors. It is most likely that vaccination of patients with cancer requires co-treatment with checkpoint antibodies since activated T cells will express co-inhibitory molecules [51]. In addition, upon IFNγ-exposure the tumor will adapt to resist the attack and start to express the ligands for these co-inhibitory molecules [9]. This needs to

be counteracted if one wants the vaccine-induced T cells to exert their function and control tumor growth [52,53].

2.2. All four levels of evidence for vaccine efficacy are observed in phase 1/2 TAA-vaccine trials

Bearing in mind the several reasons possibly explaining why cancer vaccines did not show a beneficial effect as single immunotherapeutic agent, the biological signs for success obtained in phase 2 trials should be carefully examined and placed into context of the several immune suppressive and escape mechanisms playing a role in patients with cancer, as they may obscure true vaccine activity.

2.2.1. HLA class I and II targeting tumor associated antigen vaccines

In five trials, different groups of patients were treated with vaccines targeting telomerase. Telomeres are shortened at each mitosis, limiting cell divisions, and tumors reset this clock by expressing telomerase that synthesizes new telomere units. The reverse transcriptase subunit of telomerase, hTERT, is often overexpressed and may function as a good tumor-associated antigen. In a phase 1/2 trial, 26 advanced mostly stage IV NSCLC patients, not receiving chemo or radiotherapy, were vaccinated with two telomerase peptides, GV1001 and GV1540. GV1001 is a 16aa telomerase peptide with promiscuous presentation in several different HLA class II molecules. In 13 of the 24 evaluable patients a GV1001 response developed. The immune responders displayed increased survival when compared to the non-responders **(Level 1)**. The detection of a GV1001 immune response was even after correction of potential confounders an independent prognostic factor for survival. Interestingly, 2 patients (stage 3a and stage 3b) were free of disease after 108 and 93 months and still have detectable T cell responses in blood [54,55]. In a phase 2 trial 23 inoperable stage III NSCLC patients received radiotherapy and weekly docetaxel followed by the GV1001 vaccine. In 13 of the 19 tested patients a long-term T cell response, measured by proliferation after one round of IVS, was found. Again, the immune responders displayed longer progression free survival (PFS) than non-responders [55]. A third trial in 46 patients with advanced NSCLC, having residual or progressive disease following front line therapy, received two injections with a binding-optimized HLA-A*0201-restricted TERT peptide and 4 injections with the native peptide. The detection of an immune response to the optimized and/or native peptide, as measured by ex-vivo IFNγ Elispot was associated with longer PFS and a significantly better OS. Moreover, among the immune responders there were three patients that had SD when they entered the trial but of whom the tumors started to shrink after vaccination, leading to a partial response (PR). In addition, 2 patients developed a SD while being progressive before vaccination [56]. Thus, in some patients with at low disease burden, cancer vaccination resulted in objective clinical responses **(Level 3)**. A case report on a patient with multiple metastatic lesions of ductal adenocarcinoma of the pancreas and treated 15 times with monocyte derived DC vaccine electroporated with hTERT mRNA [57], mentioned that this treatment resulted in a PR and long-term survival. A broad proliferative response to 9 of 15 tested hTERT peptides was measured. The response comprised IFNγ, TNFα and IL-2 producing CD4+ T cells while no reaction of CD8+ T cells was found. These responses developed slowly, several months after start of vaccination. In the fifth trial, three long hTERT-derived peptides (UV1), which were most frequently recognized by CD4+ T cells of long term cancer survivors, based on epitope spreading following vaccination with GV1001 [58], were used together with GM-CSF as vaccine in patients with prostate cancer receiving androgen deprivation treatment (ADT) as well as radiotherapy between month 4 and 6 of vaccination. De novo immune responses were detected in 18 of 21 tested patients, as measured after one round of IVS. The levels of prostate specific antigen (PSA) declined in 14 patients **(Level 2)** and 10 had no evidence of disease at the end of the trial. Progressive disease (PD) was defined as increase in serum PSA and/or appearance of new lesion. None of the patients with PD responded to the vaccine whereas the majority of patients with an SD displayed a response to 2–3 of the peptides. It was not clear whether the clinical response was due to the vaccine or due to ADT and radiotherapy [59]. Overall, vaccination against hTERT was associated with levels 1–3 of vaccine efficacy.

Shared tumor-associated antigens in melanoma were the first to be identified [10]. Vaccination of stage 4 metastatic melanoma patients with 3 HLA-A*0201 binding TAA-derived peptides combined either with GM-CSF or with IFNα2b or with the combination of both adjuvants revealed that these adjuvants did not improve the immunogenicity of the peptides. Of the 115 patients analyzed, only 35% made an immune response to at least one peptide, measured by ex-vivo IFNγ Elispot, indicating that this vaccine was not highly immunogenic in these patients. Of the 73 patients with clinical and immune data, 25 patients displayed a response at any of the two different time points studied and at least to one peptide. Immune responders had a significantly longer OS [25]. In another phase 2 trial, 61 patients with treatment refractory stage IV metastatic melanoma were vaccinated with 3 HLA class I binding peptides derived from the amino acid sequence of the tumor antigen survivin. Fifty-five patients were evaluable for clinical response and survival and 41 for immune reactivity [60]. Using ex-vivo IFNγ Elispot, a survivin-specific T cell response was detected at least once during the first 16 weeks of vaccination. The detection of a vaccine-induced type 1 T cell response was detected in 13 of 41 patients and more frequently observed in patients with less advanced disease and normal lactate dehydrogenase (LDH) levels, suggesting that less advanced disease is associated with less systemic immune suppression. Importantly, in 80% of the patients displaying CR, PR, or SD and only in 20% of the patients with PD a vaccine-induced immune response was detected, indicating an anti-tumor effect of these T cells. In general, vaccine-responders displayed a longer overall survival. In a phase 1/2 trial, 53 patients with advanced melanoma (stage III/IV) received a vaccine consisting of autologous DC loaded with a cocktail of melanoma antigen-derived HLA-A*0101 or HLA-A*0201 restricted native peptides from MAGE-1, MAGE-3, tyrosinase, MAGE-10, and analogues from MART1, gp100 and NY-ESO1 and 6 HLA class II peptides from MAGE-3, tyrosinase, gp100 and NY-ESO1. Later a 10-year pre-planned follow up was performed in [61]. Using different immune assays, each of the patients displayed type 1 T cell responses to almost all possible HLA class II peptides, sometimes to the HLA-A*0101 restricted peptides and almost to all of the HLA-A*0201 restricted peptides. Although in this trial no objective clinical responses were observed according to WHO criteria, some of the patients displayed slow regressions and eventually complete disappearance of individual metastases. Furthermore, after 13 years of follow-up, 19% of the patients with measurable disease are still alive, none of them except for one who received additional targeted therapy or immunotherapy. There were no correlations between the magnitude of the responses or the number of epitopes recognized, as measured after IVS, and clinical outcome, mostly because all patients responded to almost all epitopes in the vaccine. However, the intensity of the vaccine-injection site reaction, which may be a sign of a stronger immune response, was associated with longer OS **(Level 1)** [61]. This is reminiscent of other observations showing that flu-like symptoms and/or vaccine site reactions after vaccination were correlated with a stronger ex-vivo measured type 1 T cell response [62,63]. In addition, the emergence of eosinophilia after vaccination, possibly due to IL-2 and/or GM-CSF produced by the vaccine-activated T cells, was also significantly associated with long term survival in tumor bearing patients [61]. Thus, these melanoma vaccine trials provided evidence for vaccine efficacy at levels 1 and 3.

Twelve children with recurrent high-grade glioma were vaccinated with a cocktail of 3 HLA class I-restricted peptides, derived from the glioma-associated antigens survivin, IL-13R and EphA2, as well as a pan HLA-DR binding epitope from tetanus toxoid, all mixed in Montanide ISA 51 and then injected close to the powerful immune stimulator poly ICLC. Immune responses were found in 9 of the ten tested children as

measured by IFNγ Elispot after IVS. All children responded at least to EphA2 and 3 children also responded to the tetanus-derived helper epitope. The immune response waned in a few patients but in the other patients it was maintained for a long time. In one of the patients a level 3 evidence for vaccine efficacy was found. This patient had an anaplastic astrocytoma abundantly expressing EphA2, while the other glioma associated antigens were sporadically expressed. The patient developed a strong and persisting response to EphA2 and the helper peptide and this was associated with a very long-lasting PR [64].

Level 4 evidence for vaccine efficacy, that is a better clinical response in vaccinated patients versus an appropriate control group of non-vaccinated patients, was found in vaccinated patients with ovarian cancer. A vaccine consisting of autologous tumor cells, engineered to express GM-CSF/bi-shRNA furin DNA to block furin-mediated conversion of TGFβ pro-proteins into active immunosuppressive TGFβ1 and TGFβ2, was made. In a phase 2 trial, this vaccine was injected into women with stage III/IV ovarian cancer following CR on de-bulking surgery and chemotherapy. After the first randomization of 20 patients receiving the vaccine and 11 patients for control, preliminary data suggested clinical benefit and another 11 patients received only the vaccine. All 31 vaccinated patients developed a type 1 T cell response as measured by IFNγ Elispot against pre-processed autologous tumor cells. Vaccinated patients had a significantly longer time to recurrence than 11 non-vaccinated patients [65].

2.2.2. *Vaccination with defined HLA class I-restricted antigens*

HLA-A24 is the most common HLA class I allele in the Japanese population. Therefore, a whole series of trials have been performed with HLA-A24-restricted TAA-peptide vaccines. In a phase 1/2 trial, an HLA-A*02401 restricted peptide from KIF20A, which is significantly trans-activated in pancreatic cancer, was injected in 29 patients with metastatic pancreatic cancer who failed gemcitabine therapy. In 16 out of 23 tested patients the CD8+ T cell response to KIF20A increased and this was associated with injection site reactions. Level 3 vaccine efficacy was evident from the one CR and the objective shrinkage of some metastases in another 8 cases. The patient with a CR showed a strong and sustained (> 2 years) response to KIF20A as measured by HLA class I-multimers and IFNγ Elispot. Notably, in 3 cases the objective lesion shrinkage was not associated with a detectable T cell response, measured following two weeks of IVS [66], and it is not clear if this is a technical failure. In a follow-up phase 2 trial 68 chemotherapy naïve patients with advanced pancreatic cancer patients received 3 HLA-A*02401 restricted peptides from KIF20A, VEGFR1 and VEGFR2 in combination with gemcitabine irrespective of HLA type. In the end 38 patients were HLA-A*02401 positive. The PFS and OS did not differ between HLA-A*2401 positive and negative patients. Among the HLA-A*02401-positive subjects those who made an IFNγ-associated T cell response, measured after IVS, to KIF20A and/or VEGFR1 displayed a better OS. A similar observation was made for those patients with a strong injection site reaction [67]. A phase 2 study with OCV-C01 vaccine consisting of peptides from KIF20A, VEGFR1 and VEGFR2 with gemcitabine as adjuvant treatment for 30 surgically treated pancreatic cancer patients that were HLA-A*2402. 15 HLA-A*2402 negative patients received gemcitabine only. Possibly level 4 evidence for vaccine efficacy was found since the vaccinated patients had a better – but not significant - DFS than non-vaccinated patients. More than half of the patients displayed a CTL response to KIF20A, and this was associated with longer survival. Importantly, KIF20A expression was found in about 25% of the vaccinated patients, limiting the number of patients that could display a clinical response. Importantly, no recurrences were found in the group with a KIF20A+ tumor and all displayed a CTL response to KIF20A [68]. Two trials were performed in HLA-A*2401+ patients with advanced colorectal cancer failing standard therapy showing a number of patients with level 3 evidence of vaccine efficacy. First 18 patients were vaccinated with 5 different HLA-A*2401-restricted peptides from several onco-antigens and 2 peptides from VEGFR1 and VEGFR2. Level 3 evidence was manifested in 7 patients with 1 CR and 6 SD of 4–7 months. In addition, strong injection site reactions and an IFNγ-associated T cell response to three or more peptides, measured after IVS, was associated with longer survival [69]. The second study in 30 patients resulted in 3 PRs and in another 3 patients showing tumor shrinkage not fulfilling response evaluation criteria in solid tumors (RECIST). Nine patients showed an IFNγ-associated T cell response to all 7 peptides, measured after IVS. All nine patients were long term survivors and included 2 PR and 5 SD patients. The OS of patients responding to all 7 peptides was significantly longer than those responding to 6 peptides or less [70]. A phase II trial in 37 HLA*2402-positive patients with advanced head and neck cancer evaluated the injection of 3 peptides derived from 3 cancer testis antigens and observed level 3 evidence of vaccine efficacy in 15 patients, one exhibited a CR and 14 had SD. T-cell reactivity was found in 43–86% of the patients to each peptide. Patients who responded to all three peptides displayed superior PFS and OS then patients with responses to 0–1 peptides [71]. Level 3 evidence of cancer vaccine efficacy was also found in 3 out of 6 patients with advanced gastric cancer, who were vaccinated with an HLA-*2402 restricted peptide from lymphocyte antigen 6 complex locus K (LY6K). LY6K is an antigen associated with the malignant potential of cancer cells and is overexpressed in 85% of gastric cancers, albeit not by every cancer cell. A specific and robust T cell response was found in 4 patients after IVS but all patients responded. In one patient, classified as an SD, vaccination resulted in the initial shrinkage of 4 out of 5 evaluated tumors and this coincided with a decrease in serum CEA levels. Two other patients also showed SD [72]. These data clearly indicate that vaccination with LY6K peptide can mediate an antitumor effect but also show that immune escape is imminent when not all tumor cells express the targeted antigen. A vaccine comprising one HLA*0201 and one HLA-A*2402 restricted peptide derived from the carcinoembryonic antigen glypican-3 was injected in 32 patients with refractory ovarian clear cell carcinoma. The vaccine induced an ex-vivo T cell response in 15 of the 24 tested patients, measured by IFNγ Elispot. Expression of glypican-3 was found in 8 of 19 tested patients. The tumors of six patients showed reduced HLA class I expression. The expression of the protein, HLA class I and infiltration with TILs was not a predictive marker for survival. Two patients developed a PR [73], providing level 3 evidence that glypican-3 targeted vaccination may have impact on tumor growth. The first patient displayed multiple metastases before vaccination that rapidly progressed. A PR was achieved after 10 weeks with some lesions no longer visible but slow growth of a metastasis in a lymph node. This metastasis lacked glypican-3 expression and had a reduction in HLA class I as well as low number of tumor infiltrating lymphocytes (TIL). Concurrent with the PR, pretreatment tumor marker levels in serum dropped and remained flat until week 60. The second case, showed a drop in the serum tumor markers after the 7th vaccination and obtained a PR at week 37. Surprisingly, the primary tumor was glypican-3 negative but it is known that glypican-3 tumor expression is heterogeneous and depends on the location and timing of the biopsies [74]. This is not uncommon and has also been observed for other putative vaccine targets like XAGE-1b [75].

Overall, among all the patients vaccinated with HLA-A24-restricted CD8+ T cell epitopes there were five trials reporting level 1 evidence of clinical activity while level 3 evidence was found in 6 trials.

PR1 is an HLA-A*0201 restricted peptide that is recognized on myeloid leukemia cells by preferentially leukemia killing CD8+ T cells. In a phase 1/2 trial 66 HLA-A*0201 patients with either acute or chronic myeloid leukemia (AML, CML) or with myelodysplastic syndrome (MDS) were vaccinated at different dose levels of vaccine. Level 3 evidence of vaccine efficacy was found in 12 patients. PR1-specific CD8+ T cells were present in 85% of the patients at baseline. A vaccine-induce response, defined as a 2-fold increase in PR1-tetramer+ CD8+ T cells in the blood was observed in 53% of the patients. The vaccine-induced PR1-specific CD8+ T cells accumulated within the

central memory population. The TCR avidity of PR1-specific CD8+ T cells after vaccination was higher among the immune responders and interestingly, it was higher in immune responders with a clinical response than in immune responders lacking a clinical response. A more than 2-fold increase in PR1-specific T cells after vaccination was not related to dose or to the percentage of pre-existing PR1-specific T cells but was related to a lower disease burden at baseline, fitting with the other observations that clinical responses primarily were obtained in patients with low disease burden. Twelve patients showed a clinical response, 9 of which were immune responders. The other 3 did not display a vaccine increase in PR1-specific T cells but the avidity of the TCR of the pre-existing PR1-specific T cells changed and was higher than seen in immune responders without a clinical response or the other non-responders[76], suggesting that functional avidity maturation of tumor-specific T cells, known to be important for responses to viruses and cancer [77], forms a mechanism through which cancer vaccines can work.

Wilms' tumor gene 1 (WT1) is a potent transcriptional regulator, its expression correlates with cell proliferation and metastatic behavior of tumor cells. It is overexpressed in many different types of tumors [78]. Twenty-five patients with MDS were vaccinated with a WT1-peptide vaccine, comprising a CD4+ T cell epitope and a HLA-A*2402-restricted modified CD8+ T cell epitope. Eleven patients showed a CTL response as measured by HLA class I-multimers. No overt differences in the CTL response were found between patients with clinical benefit and those having progressive disease. Only five patients showed a WT-1 specific DTH response but the relation with clinical response was not reported, neither was the WT1-specific CD4+ T cell response reported [79]. This vaccine was also tested in 32 patients with advanced pancreatic cancer in combination with gemcitabine chemotherapy. In 18 of the patients a WT1-specific DTH response was observed. Eleven of the 16 patients with longer survival and none of the 7 patients with short survival showed a DTH response. Patients with a positive WT1-specific DTH response displayed superior OS. No differences were seen with respect to the number of WT1-specific CD8+ T cells. However, DTH+ patients displayed more naïve WT1-specific T cells at baseline and a significantly higher percentage of memory T cells than effector T cells after treatment than the poor responders [80], suggesting that the poor responders may have exhausted their WT1-specific T cell response while this population is still fit in DTH+ responders. An anchor-modified HLA-A*2402 restricted 9-mer WT1 peptide was injected in 21 patients with recurrent glioblastoma multiforme (GBM). Vaccination resulted in 2 PR and 10 SD. All patient tumors expressed WT1, but the patients with a PR had strong staining of tumor tissue, suggesting that the level of overexpression matters for clinical efficacy. The clinical responses could not be associated to the vaccine-induced T cell response since high frequencies of WT1-specific T cells were present before vaccination and did not increase after vaccination, even not in the clinical responder patients. Unfortunately, no data was presented on the activation of T cells [81] as a similar maturation of the T cell response seen after PR1 vaccination [76] may have occurred in these WT1 vaccinated clinical responders. Thirty patients in post-remission of AML but at very high risk of relapse were vaccinated with WT1 messenger RNA electroporated DCs [82]. Nine patients showed molecular remission, defined as the normalization of the WT1 mRNA tumor marker in the blood, 5 of which were sustained for a very long time. Four other patients showed disease stabilization for a minimum of 2 months. The survival of the vaccine-responders was significantly better than that of non-responders. Measurement of the circulating WT-1 specific T cell response was restricted to the measurement of an HLA-A*0201 restricted epitope but revealed an association between the increase in WT1-specific CD8+ T cells and clinical outcome. Notably, the presence WT1-specific CD8+ T cells in DTH-infiltrating T cells was correlated with long term clinical responses (at least 3 years). The latter observation confirms earlier studies in vaccinated melanoma patients, showing that the presence of TAA-specific T cells among skin-test infiltrating T cells predict clinical outcome [83]. Thus, WT1-specific vaccination shows levels 1a and 1b evidence in multiple trials. Only in one occasion objective clinical responses were correlated with immune data, providing level 3 evidence for WT1-targeted vaccine efficacy.

2.2.3. Personalized peptide vaccines based on pre-existing immunity

A series of trials have been performed with so-called personalized peptide vaccination (PPV). Here, vaccine-peptides are selected from a warehouse of HLA-class I restricted TAA based on the HLA type of the patient and the detection of pre-existing peptide-specific IgG reactivity against the TAA. Also in these trials levels 1 and 4 of evidence for vaccine efficacy is provided. In a phase 2 trial, 60 patients with advanced colorectal cancer failing at least one regimen of chemotherapy or targeted therapy were vaccinated with a maximum of four peptides [84]. In 63% of the 51 patients completing at least one series of 6 vaccinations, a CD8+ T cell response was detected by ex-vivo IFNγ Elispot. IgG responses to the selected peptides were increased in 94% of the patients. Patients with a concomitant increase in their CTL and IgG response (possibly reflective of CD4+ T cell reactivity) showed a better prognosis than the others. Both an increased T cell response and the number of peptides the patient responded to were predictive for favorable OS, once again suggesting that the magnitude and breadth of the response to cancer are important determinants. Similar observations were made in a single arm phase 2 trial where PPV-vaccinated patients with metastatic upper tract urothelial cancer. An increase in PPV-specific IgG reactivity was found in 19 of 37 patients and an IFNγ T cell response in 17 of 37 patients. Using a landmark time analysis, patients displaying both a humoral and cellular response to PVV had better OS than those patients with no, only IgG or only a T cell response [85]. In another phase 2 randomized trial, castration-resistant prostate cancer patients received dexamethasone alone or in combination with PPV. The vaccinated group of 37 patients displayed longer PFS, based on the level of serum prostate specific antigens, than the control group of 35 patients. Median OS was also longer. How the immune response related to outcome was not reported [86]. In a phase 2 randomized trial, vaccination of 39 patients with progressive bladder cancer after first-line platinum-based chemotherapy with a maximum of 4 peptides did not lead to improved PFS when compared to the control group, albeit that OS was improved. In addition, patients who developed a response to the vaccine displayed a longer PFS [87]. PPV was also tested in patients with previously treated advanced NSCLC. Patients received either docetaxel with PPV or docetaxel with placebo. No difference in PFS was observed when both groups were compared. Interestingly, within the vaccinated arm those patients displaying vaccine-induced increases in the peptide-specific IgG titer of at least 2-fold had a longer PFS and OS [88]. Another phase 2 randomized trial tested the addition of low-dose cyclophosphamide, with the intention to attack regulatory T cells, to PPV in patients with advanced biliary tract cancer [89]. No differences in the percentages of Tregs were observed between cyclophosphamide treated patients and the control group. Vaccine-induced T cell responses were observed in both groups and potentially were a bit higher in the combination treated patient group. While this combination group also showed a longer PFS, no clear relationship was found between the strength of the vaccine-induced immune response and survival. In summary, vaccine efficacy at the first level was found in 4 trials whereas level 4 evidence was provided in two trials of the 6 trials analyzed.

2.2.4. Vaccines targeting the overexpressed proteins HER2, MUC1 and CEA

The human epidermal growth factor receptor-2 (HER2) is a molecular driver in about a quarter of breast cancers. Antibody therapy to HER2 has dramatically improved the clinical outcome in breast cancer. When given in a neoadjuvant setting, 40–60% achieve a pathologic complete response (pCR) and this is associated with decreased recurrence rate and better OS [90,91]. CD4+ Th1 responses to HER2 are also detected in patients with HER2+ breast cancer but their numbers

decline with progressive disease. Low numbers of HER2 specific Th1 cells have been associated with an increased risk to recurrences after neoadjuvant therapy [92]. Similarly, a preexisting strong HER2-specific T cell response (measured by DTH or by IFNγ Elispot) is correlated with longer PFS in prostate cancer [93]. In breast cancer, elevated levels and broader reactivity of HER2-specific Th1 cells as measured by ex-vivo IFNγ Elispot correlated strongly with pathological CR following neoadjuvant treatment with HER2 antibody therapy, irrespective of general immune status of the patients [92]. Hence, vaccination against HER2 seemed a rational choice. Four patients with a non-pathological CR to neo-adjuvant therapy showed low numbers of HER2-specific Th1 cells before vaccination but a strong increase in overall levels and breadth of the HER2-specific T-cell response after the injection of autologous DC pulsed with 6 promiscuously HLA class II binding HER2 peptides [92]. The impact of the vaccine was tested in a larger trial, confirming its capacity to increase the levels and breadth of the HER2-specific Th1 response in most patients. However, the detection of these responses was much lower in the sentinel lymph nodes unless not only HER2 vaccination but also anti-estrogen therapy was given. Potential level 3 evidence could be seen as vaccination increased the pathological CR when the vaccine was given together with anti-estrogen therapy [94], but the effect anti-estrogen therapy on pathological CR was not tested. In a large trial with 298 clinically disease-free node-positive and high-risk node negative breast cancer patients, 153 patients received AE37 (HER2) + GM-CSF and 145 patients GM-CSF only. The vaccinated group showed ex-vivo detectable increased HER2-specific proliferation and increased numbers of IFNγ-producing HER2-specific T cells, for the subgroup of patients tested. No differences in the recurrent rate were seen comparing both groups. A preplanned subgroup analysis revealed that 78% of the vaccinated triple negative breast cancer patients (with low to intermediate HER2 expression) were still disease free versus 49% of these patients in the control group [95], providing level 4 evidence for vaccine efficacy.

Mucin-1 (MUC1) is expressed by many solid tumors. TG4010 is a vaccinia virus-based vaccine expressing full length MUC1 that was used to vaccinate 148 patients with MUC1-positive NSCLC [96]. Half of the patient group received cisplatin and gemcitabine chemotherapy whereas the other half received chemotherapy + TG4010. A longer PFS was seen in the vaccinated group. A pre-specified analysis of the CD8+ T cell response using HLA class I-multimer analysis did not reveal a strong response rate and was not different between the two arms [96]. No analysis of the CD4+ T cell response were performed because in a previous trial the response by CD4+ T cells, measured as a proliferative index of > 2, was deemed not informative while a response by CD8+ T cells, measured after a round of in vitro stimulation and found in 12 of the 21 patients with disease control, was associated with longer time to progression and OS [97]. Notably, the TG4010 induced MUC1-specific CD4+ T cell and CD8+ T cell response was found to be transient in two different trials [97,98]. In addition, a biomarker program had identified that the frequency of circulating CD16+CD56+CD69+ lymphocytes was higher in 37 vaccinated patients with a shorter time to progression and worse OS [96]. A phase2b/3 trial with 222 patients has been launched and the predictive value of this cellular biomarker was positively validated in the 2b part of trial [99]). Potentially, these activated lymphocytes have a negative effect on the immune system for instance by killing of DCs, activated CD4+ T cells and activated macrophages [100]. However, high frequencies were also associated with a higher incidence of adverse events in the vaccine group [96], suggesting that this may also underlie the difference in time to progression. Still, an increased progression free survival was found in the TG4010 group when compared to the placebo group, suggesting clinical benefit from MUC1 vaccination. Overall, these data suggest that the level 1 evidence obtained in the first trial could not be validated in the second trial. Despite the fact that potential level 4 evidence for vaccine efficacy was provided there was no strong link to vaccine-induced T cell reactivity.

Carcinoembryonic antigen (CEA) has also been considered as target antigen for therapeutic vaccines. In the past, we have shown that optimal response induction requires a balancing act to fine-tune the antitumor effect while lowering intestinal autoimmune pathology [101]. Twenty-seven patients with CEA expressing carcinomas were vaccinated with a DNA vaccine encoding an HLA-A*0201 restricted CEA epitope. This resulted in the detection of CEA-specific CD8+ T cells in 58% of the patients treated whom displayed no measurable disease at start of the trial, measured in an ex-vivo IFNγ Elispot assay [102]. Only a minority of patients with measurable disease showed a reaction to vaccination, indicative for disease burden associated immune suppression, and suggesting that is might be better to vaccinate in a minimal residual disease setting. Patients who reported diarrhea during the trial had a longer OS. Diarrhea was associated with a drop in the serum CEA levels (level 2). Most likely diarrhea was a reflection of an on-target autoimmune effect as the CEA peptide was shown to be presented on malignant and benign tissue, reminiscent of what has been shown after vaccination with melanoma associated antigens and vitiligo [103]. Also, chimeric antigen receptor T-cell therapy targeting CEA has been associated with respiratory toxicity due to transient CEA expression on lung epithelia caused by the precondition regimens [104] and has shown to induce severe colitis [105]. Potentially, reflections of on-target immunity to healthy tissues might also be seen as a level 3 of evidence, similar to vitiligo in the skin and severe ocular autoimmunity through destruction of normal melanocytes in patients with melanoma. This has been associated with a good efficacy of tumor immunotherapy [106].

The PANVAC vaccine targets both CEA and MUC1 and was used in a phase 2 trial to vaccinate 25 patients with metastatic breast cancer of any subtype in combination with docetaxel chemotherapy [107]. The 23 patients in the control arm received chemotherapy only. In the vaccine arm 56% of patients showed a CEA- and/or MUC1-specific immune response, measured after IVS, while this was the case for 40% in the control arm. There was a trend visible for improved PFS in the combination arm [107]. The data on MUC1 and CEA demonstrate that some of the TAA used in therapeutic vaccines may mediate antitumor effects but should be targeted with caution.

2.3. Therapeutic efficacy of cancer vaccines to treat virally-induced high grade lesions and cancers

About 20% of the cancers are induced by viruses, one well-known virus is human papillomavirus of which especially type 16 (HPV16) is highly oncogenic and causes tumors in the head and neck region as well as the anogenital region. Another oncogenic virus is the Merkel-cell polyomavirus. Both virus-induced cancers can respond to adoptive T cell therapies [108,109]. In addition, Merkel-cell carcinoma responds extremely well to PD-1 checkpoint blockade, showing an objective response rate of 56% in advanced Merkel-cell carcinoma [110]. This is much less the case for the HPV-induced carcinoma's [111]. The most likely reason for this is the presence of virus-specific CD4+ and CD8+ T cells in most of the patients with Merkel-cell carcinoma [112] and lack thereof in the majority of patients with a recurrent HPV-induced tumor [113–115].

To increase T-cell reactivity to HPV16 several types of vaccines have been developed which aim to harness the immune system against the viral oncoproteins E6 and E7, as they are critically involved in tumorigenesis. VGX-3100 is a DNA vaccine targeting oncoproteins of HPVs type 16 and 18. Immunization of patients with high-grade cervical lesions resulted in the induction of potent CD4+ Th1 responses and CD8+ CTL responses [116]. In a randomized phase 2b double blind, placebo-controlled trial, the vaccine induced regression in 50% of the 107 vaccinated patients with high-grade cervical lesions whereas this was observed in about 31% of the placebo group [117]. Post-hoc analyses showed that the regression of lesions was associated with an increase in the number of vaccine-induced T cells responding to E6 as measured by IFNγ Elispot [117] as well as CD137+CD8+ HPV-specific

T cells expressing perforin or granzyme in the blood and an increase in perforin+ T cells in the tissue [118]. The HPV DNA vaccine GX-188E also induced significant HPV-specific IFNγ-producing CD4+ and CD8+ polyfunctional T cell responses and the regression of high grade cervical lesions in 7 of 9 vaccinated patients [119]. ISA101 is an HPV16 synthetic long peptide (SLP) vaccine which was proven to be safe, highly immunogenic and capable of inducing type 1 CD4+ and CD8+ T cell responses in patients with HPV16-induced pre-malignant cervical lesions [62,120] and HPV16-induced end-stage cervical cancer [121,122]. Furthermore, it was shown to induce objective regressions of HPV16-induced high grade vulvar lesions in about 50% of the treated patients, in two independent trials, whereas spontaneous regression is only observed in 1.3% of the patients [123,124]. Notably, post-hoc analyses of the first trial showed a strong correlation between the breadth and magnitude of the ex-vivo vaccine-induced type 1 T cell response and clinical responsiveness [123,125] and this correlation was confirmed in the pre-defined analyses performed in a second trial [124]. Therapeutic ISA101 vaccination of patients with advanced or recurrent HPV16-positive cervical cancer installed HPV16-specific T cell reactivity in patients with a less suppressed immune status but the T cell response was much weaker than observed before in patients with HPV16-induced high grade vulvar disease. It also did not result in clinical responses [126]. Most likely, the lower T cell response was due to the apparent tumor-mediated leukocytosis observed in these patients as depletion of circulating CD14+ myeloid cells resulted in increased detection of T cell reactivity against recall antigens and the HPV16 oncoproteins [127]. In addition, chemotherapy-mediated normalization of the myeloid cell composition resulted in much stronger T cell responses to therapeutic vaccination when given to patients with advanced, recurrent or metastatic cervical cancer [127]. Moreover, it led to more cure in a mouse model for HPV16-induced cancers [127] and preliminary reported data reveal clinical benefit in those patients with the strongest immune response to the vaccine [128]. Upon activation of tumor-specific T cells they start to express co-inhibitory markers including PD-1 [51] suggesting that more benefit may be achieved when vaccination is combined with checkpoint blocking. In melanoma, combination of a TAA peptide vaccine was shown to be safe in combination with nivolumab [129]. In a phase 1/2 trial, patients with incurable HPV16-driven oropharyngeal cancers were treated with PD-1 checkpoint blockade and in order to boost the levels of HPV16-specific T cells, with the ISA101 HPV16 synthetic long peptide (SLP) vaccine. This doubled the objective response rate [130], when compared to earlier data [111].

A second example is vaccination against cytomegalo virus (CMV). Studies have shown that the CMV-derived phosphoprotein 65 (pp65) can be expressed in glioblastoma cells but not the surrounding healthy tissue, suggesting that this protein could function as a virus-derived tumor-specific target. In a small randomized and blinded clinical trial in newly diagnosed glioblastoma 12 patients were treated with autologous pp65 RNA-pulsed DCs with or without preconditioning of the vaccine site by injection of recall antigens. Preconditioning increased the accumulation of the injected DCs in the vaccine site-draining lymph nodes in a recall antigen-specific CD4+ T cell-dependent fashion. Not only did these patients display a better PFS and OS when compared to patients receiving only the DC vaccine but also the clinical response was associated with an increase in the number of pp65-specific IFNγ-producing T cells, with the two long term survivors showing the highest increase in pp65-specific T cells after vaccination [131]. In a more recent study, the immunogenicity of pp65-DC vaccination in patients with glioblastoma as well as the correlation between the strength of the pp65-specific immune response after vaccination with clinical outcome was confirmed. Patients whom displayed an OS > 40 months had a much more significant expansion in pp65-specific IFNγ producing T cells than those with an OS < 40 months [132].

In conclusion, there is strong evidence that the T cell response to viral antigens in human tumors plays an important role in controlling disease. The therapeutic vaccination trials in patients with pre-malignant disease provide levels 3 and 4 evidence of vaccine efficacy and preliminary data suggest that in combination with other immunotherapies (e.g. checkpoint blockade) clinical response rates to immunotherapy go up.

2.4. First signs of successful clinical translation of neoantigen vaccines

Good clinical responses to checkpoint blocking have also been associated with the presence of high numbers of mutations in tumors and the presence of T cells specifically recognizing these mutations [133]. Mutations in the DNA leading to a change in one or more amino acids of proteins (e.g. point mutations, insertions, deletions, frameshifts or breakpoints) may lead to a new class of peptides, called neoantigens, that are presented in MHC class I and II. They activate T cells with high affinity TCR because they have never been presented in normal tissue and thus bypass thymic tolerance. Spontaneous activation of neoantigen-specific CD4+ and CD8+ T cells have been documented in several types of tumors by several groups since 1994 [134] and hence neoantigens became a focus in the development of therapeutic vaccines [135]. The tools to identify MHC class I and class II-restricted neoantigens have undergone major technical advances allowing for their rapid identification [136]. In several mouse models, it was shown that neoantigens expressed by tumors not only functioned as targets for tumor-specific T cells responding to checkpoint therapy but also that vaccination with therapeutic long peptide vaccines or poly-epitope messenger RNA based on these mutant peptides induced tumor regression and rejection comparable to that of checkpoint blockade [137–140]. These results are similar to what was shown before with respect to the use of viral oncogene vaccines [30,141].

A deletion mutation affecting exons 2–7 of the EGFR gene (EGFRvIII) is found in a sizeable fraction of glioblastomas. A peptide containing the specific novel amino acid sequence created by this deletion mutation was conjugated to keyhole limpet hemocyanin, to increase its immunogenicity. The vaccine, called rindopepimut, has been tested in several phase 2 trials of patients with gross total resection of tumor and no evidence of progression after radiotherapy with concomitant temozolomide chemotherapy. In a first trial with 18 patients [142], 6 of 14 tested patients showed a rise in mutant-specific antibodies and this was associated with a better OS. Only 3 of the 17 tested patients showed a DTH response to the mutant peptide, indicating that not many patients were able to mount a T cell reaction to this mutant peptide. These 3 patients displayed an extremely good OS whereas no difference in OS was found when patients were grouped according to their DTH response to recall antigens. Importantly, 82% of the patients showed loss of EGFRvIII expression in recurrent tumors [142]. In the second trial, vaccination was performed during two different schedules of temozolomide chemotherapy. In one are 7 of 8 patients displayed a mutant-specific DTH response while in the other arm none of the patients responded. However, no differences in PFS or OS were seen. Notably, the increase in DTH reactivity was accompanied by a specific reduction in CD4+ T cells, an increase in CD4+ Tregs as well as mutant-peptide specific antibody titers [143], making one wonder if vaccination led to EGFRvIII-specific Th1 responses. Again in 11 of 12 recurrent tumors the expression of EGFRvIII was lost. Similar observations were made with respect to antibody titers and EGFRvIII expression in recurrent tumors in a third trial with 65 patients [144]. Because these trials showed an encouraging PFS and OS when compared to historical controls and despite the fact that EGFRvIII expression was rapidly lost, a randomized, double-blind, phase 3 trial was started. However, this study was terminated for futility as no difference in OS was seen during a pre-planned interim analysis [145]. Again, loss of the deletion mutant was seen in about 60% of patients in both groups but the Ab titers did not differ between patients with loss or persistent expression of the mutant EGFR, indicating that the humoral response is not a good immune correlate for clinical responsiveness and reinforces

the notion that the cellular immune response should be more closely monitored in order to value better the results obtained in these clinical studies. The reasons for failure of this trial are highly similar to the phase 3 trials that failed when TAA-targeting vaccines were used.

With the development of pipelines to identify neoantigens, personalized vaccine strategies have been developed. A first trial with neoantigen vaccination was performed by Carreno et al. [146]. Neoantigens were identified in tumors of patients with melanoma, confirmed to bind to HLA-A*0201 and loaded onto DC for vaccination. Neoantigen-specific T cells were detected after one round of IVS and isolated neoantigen-specific T cells were shown to recognize endogenously processed mutated proteins, showing their functionality. Another study utilized 13–20 SLP as vaccine to target up to 20 neoantigens per patient, admixed with poly ICLC in 8 patients with stage II/IVB melanoma after surgical resection with curative intent. Ex-vivo IFNγ Elispot analyses revealed immune responses to several pools of peptides, mostly CD4+ T cell mediated. Neoantigen-specific CD8+ T cells were detected after one round of IVS and > 30% of the cells were polyfunctional. Of all injected peptides, 60% were recognized by CD4+ and 15% by CD8+ T cells. In some, but not all, patients the vaccine-induced CD4+ and CD8+ T cells were able to recognize autologous tumor cells. Furthermore, CD4+ T cell were shown to respond to DC exposed to irradiated autologous tumor cells, showing natural presentation by DC of neoantigens. Interestingly, PD-1 blockade increased the breadth of the neoantigen T cell response [147]. Recently, a personalized RNA vaccine was tested in 13 patients with stage 3 and 4 melanomas after resection of their metastases and no radio-detectable lesions [148]. For each patient 10 mutations were selected and engineered into two synthetic RNA encoding 5 linear-connected 27mer peptides with the mutation in the middle. Patients were vaccinated percutaneously in the inguinal lymph node as this ensured efficient uptake of the RNA by DC. All patients completed treatment and T cell reactivity was detected against 60% of the predicted epitopes, with each patient responding to at least 3 epitopes. The majority of epitopes was exclusively targeted by CD4+ T cells, and 25% by both CD4+ and CD8+ T cells. All patients had recent history of recurrent disease and a high risk for relapse, but vaccination was associated with a strong reduction in the longitudinal cumulative recurrent metastatic events (Level 2). In addition, 3 of 5 patients with a metastasis at start of vaccination, showed objective clinical responses (1 CR, 1 PR, 1 MR). The effects seen are reminiscent of the outcomes seen in earlier trials with autologous tumor material. A DC vaccine with autologous tumor RNA was tested in 31 metastatic melanoma patients with follow-up to 10 years after the last patient was vaccinated. Based on DTH responses and in vitro T cell proliferation assays, 16 of 31 displayed reactivity to tumor loaded DC, 12 were negative and 3 inconclusive, indicating the induction of a tumor-specific response. Two patients showed disappearance of lesions, one even with CR for a couple of months, both later treated with checkpoint therapy and still alive. The presence of an immune response was associated with a significantly better OS and it was an independent predictor after correction for disease stage and performance. The 8 patients with > 20 months of survival were all immune responders [149]. Another trial used irradiated autologous melanoma cells conjugated to dinitrophenyl – in order to ensure tumor cell death - and mixed with BCG in order to enhance the vaccine's immunogenicity. Vaccination of 126 patients with stage 3b/c melanoma in the adjuvant setting revealed that patients with a strong DTH response to unmodified autologous tumor cells displayed a 5-year OS of 75% and DFS of 47%, whereas the no to weak DTH responders had a 5-year OS of 44% and DFS of 26% [150]. Interestingly, 35 vaccinated patients developed unresectable disease were treated with the CTLA4 checkpoint inhibitor ipilimumab. When compared to a similar group that had not been vaccinated before, vaccinated patients showed significantly more CR, PR and SD as well as longer OS. The antigens to which the immune system responded in these latter two trials are unknown but with our current knowledge are likely to involve neoantigens too. In addition, potential vaccine-induced responses to TAA expressed by the tumor may have fostered stronger reactivity to neoantigens as was shown by a trial in which a melanoma patient with low level of MAGE-specific CTL in blood after MAGE vaccination displayed tumor regression. Using TCR-Vβ cDNA libraries only a few of the vaccine-induced CTL were found in regressing metastases. However, they also found other TCR belonging to tumor-specific CTL enriched in regressing metastases and detectable in blood only after vaccination. These CTL recognized a neoantigen in the context of HLA-A2. Its presentation was increased in the presence of IFNγ suggesting that the attack of tumor cells by MAGE-specific CTL may have induced antigen-spreading of CTL recognizing truly tumor-specific antigens [151]. Thus, the first data in neoantigen vaccination trials indicate level 2 evidence for vaccine efficacy, however, it is likely that early autologous tumor cell based vaccine have triggered neoantigen-specific T cells. This makes it likely that also level 3 evidence for neoantigen vaccine efficacy exists.

2.5. Level 3 evidence for vaccines targeting immune suppressive mechanisms

A new development is the development of vaccines targeting molecules that suppress antitumor immunity. The transcription factor hypoxia-inducible factor-1α (HIF-1α) regulates the expression of genes involved in immunosuppression. Inhibition of HIF-1α increased the efficacy of tumor-specific T cells by increasing the production of their effector molecules and slowed down the growth of cancer cells in the 4T1 breast cancer model [152]. HIF-1α was also found to be a natural target for CD4+ T cells in patients with triple negative breast cancer. Three highly homologous peptides elicited type 1 immunity in mice and reduced mammary tumor growth in the C3(1)Tag basal-like/stem cell high murine model [153]. So far, no studies have been reported in a patient setting. Indoleamine 2,3 dioxygenase (IDO) is a potent inhibitor of T cells in patients with cancer and can be expressed by cancer cells and by suppressive myeloid cells. Interestingly, spontaneous T cell responses against IDO are detected in patients with cancer [154]. Level 3 evidence of vaccine efficacy was found in a study where 15 patients, with stable stage 3/4 NSCLC disease after standard chemotherapy, were vaccinated with an HLA-A*0201 restricted IDO peptide with imiquimod ointment as adjuvants applied 8 h before vaccination [155]. All patients developed an IFNγ-associated T cell response to the IDO peptide, as measured by Elispot after IVS. Potential on-target autoimmune effects related to IDO expression in the gastrointestinal tract were found. A number of patients remained in SD whereas one showed a PR of target lesions. No correlation was found between IDO expression in the tumor and clinical response to the vaccine, but the tumor of the patient with a PR had moderate IDO expression. Interestingly, the patient with PR and one long term clinical responder (SD for 2 years) showed long term stabilization of the kynurenine to tryptophan ratio, which is a measure for IDO activity. Two early progressive patients showed a strong expression of IDO in their tumor and a strong increase in this ratio. The IDO pathway is also linked to Treg biology via the induction of Tregs by IDO+ DC. In this trial, Tregs decreased in all patients during vaccine therapy [155]. More recently, also pre-existing PD-L1-specific cytotoxic T lymphocytes able to kill both PD-L1 expressing malignant lymphoma cells and normal immune cells, were described. In co-cultures the addition of PD-L1-specific CTLs increased the response of virus-specific CD8+ T cells in vitro [156–158], suggesting that may induce a similar effect in vivo. No trials have been performed.

2.6. Vaccines are not necessarily required to induce tumor-specific CD8+ T cells

Tumor-specific vaccines usually focus on the induction of tumor-specific CD8+ T cells. With the rising potential to rapidly identify neoantigens, attempts were made to use them in vaccines for boosting neoantigen-specific CD8+ T cells. Recently, the mutational landscape

was determined in 3 mouse tumor models and peptides with high likelihood to bind to MHC class I were used for vaccination. Unexpectedly, most of the peptides were recognized by CD4+ T cells. Furthermore, vaccination with such CD4+ T cell activating neoantigens induced potent tumor control and complete rejection of progressively growing tumors in mice. As expected [159,160], part of the mechanism included reshaping of the tumor microenvironment and antigen spreading by inducing CD8+ T cell responses to other epitopes [137,140]. The predominant recognition of neoantigens by CD4+ T cells after vaccination is also observed in humans [146,147]. Also in other mouse models where tumor-specific antigens are used, tumor control by CD4+ T cells is found. CD4 type 1 T-cells against a point mutation in isocitrate dehydrogenase type 1, a defining mutation in glioma, suppressed tumor outgrowth of subcutaneous mutant- isocitrate dehydrogenase type 1+ sarcomas [161]. MUC-1 specific CD4+ T cells prevented lung metastasis of MUC1+ tumors in mice [162]. HPV-specific IFNγ-producing CD4+ T cells controlled spontaneous cervical tumor outgrowth and progression in genetically engineered K14-HPV16 transgenic mice [163]. Notably, also the earlier mentioned therapeutic HPV vaccines are strong inducers of type 1 CD4+ T cell reactivity and in the trials with HPV16 SLP vaccine a clear relation was found between the strength of the ex-vivo CD4+ T-cell response and the clinical response of patients with vulvar lesions [124,125]. More anecdotal evidence is found in trials with tumor-associated antigens. A pancreatic cancer patient vaccinated with hTERT displayed a PR and long-term survival that was associated with a broad type 1 CD4+ T cell response while no hTERT-specific CD8+ T cells were found [57]. In depth analysis of long term NSCLC survivor in complete remission after hTERT vaccination also showed a broad hTERT-specific CD4+ T cell response and antigen-spreading to hTERT epitopes unrelated to the vaccine, including to one HLA-B7-restricted CD8+ T cell epitope. Such T cell activity was also observed in other clinical responders but not in non-responders [57,58]. Level 1 evidence of vaccine efficacy with the promiscuous HLA class II hTERT peptide was also reported [55]. Finally, a mixture of 12 MHC class I restricted epitopes and/or a mixture of 6 helper peptides derived from the melanoma antigens gp100, tyrosinase, MART-1, MAGE-A3 and MAGE A1, varying between 14–23 amino acids in length, have been used to vaccinate 175 patients with measurable stage IV melanoma. CTL responses, measured after IVS by IFNγ Elispot were found in 28–47% of the treatment groups whereas helper T cell responses, measured directly in a 5-day proliferation assay, were found in 40% of treated patients. T-helper reactivity was associated with production of IL-2, IL-12p70, IL-5, CXCL9 and CXCL10. Of the 148 eligible patients, 7 showed a PR and 27% had SD. The PR rate was higher in the groups receiving helper T cell epitopes. There was no association between the presence of a CTL response and a clinical response or OS. In the group of patients receiving the helper peptides, the proliferative response to the melanoma peptides but not to the recall antigen tetanus was strongly associated with the 1 year survival rate and OS, even after correction for clinical variables. Interestingly, the overall response rate was the highest in patients responding to both the CTL and the helper T cell vaccine [164]. Although the authors did not formally exclude that the longer peptides used – a format known to be of higher immunogenicity [165] – also induced CTL responses, the data suggest CD4+ T cell responses are relevant for the control of tumors. This notion is sustained by the earlier observation that the adoptive transfer of autologous NY-ESO1-specific CD4+ T cells induced regression of refractory metastatic melanoma [166] as well as by the observation that TIL of clinically responding stage IV melanoma patients comprise neoantigen-specific CD4+ T cells [167,168] and the observation that the infusion of neoepitope-specific CD4+ T cells mediated tumor regression in patient with cholangiocarcinoma [169].

3. The influence of host immune factors on vaccination and survival

3.1. The impact of circulating myeloid cells and granulocytes on vaccine efficacy

The NSCLC patients vaccinated with GV1001 not only were examined for their response to vaccination but also analyzed with respect to the presence and potential impact of immune suppressive myeloid cells. The PBMC of the patients were analyzed for the presence of two types of MDSC, the CD14+, HLA-DRlow monocytic MDSC (mMDSC) and the Lin-, CD33+CD11b+, HLA-DR-MDSC. A group of healthy donors (not matched for age or sex) were taken as controls. Among the 22 patients, 16 displayed a T cell response. The Lin-, CD33+CD11b+, HLA-DRneg MDSC were higher in those vaccinated patients lacking an immune response but as a group the percentage of these MDSC was not higher than in the healthy controls. The mMDSC were higher in the patient group and had an impact on OS within the group of immune responders. A low percentage of mMDSC was associated with longer PFS (60 vs 7.8 months) and an extended OS (73 vs 21 months) [170]. This was not the only trial where an impact of MDSC on therapy and survival was observed. In melanoma, patients treated at the point of a low lymphocyte to monocyte ratio display a worse response to chemotherapy [171]. In addition, melanoma patients with high levels of mMDSC experienced worse survival and display lower reactivity to melanoma associated antigens [172] and display less of a response to checkpoint therapy [173]. The detection of T cell reactivity against tumor antigens in combination with the levels of mMDSC also strongly correlated to survival in melanoma [172–175]. MDSC also defined survival in breast cancer. Patients who mounted an HER2-specific T cell response and had a lower frequency of Lin-, CD33+CD11b+, HLA-DR-MDSC displayed a higher 5-year survival rate. The patients with high levels of these MDSC lacked a CD8+ T cell response to HER2 [175].

Manipulation of the levels of different myeloid cells may have a positive impact on vaccine outcome and survival. Patients with progressive cervical cancer display higher levels of circulating CD14+ myeloid cells, the depletion of which results in the detection of stronger T cell reactivity to recall antigens and tumor antigens while chemotherapy-induced normalization of their levels is associated with a stronger response to therapeutic tumor vaccination [127]. A combination of TAA-mRNA transfected DC vaccination and docetaxel chemotherapy resulted in a decline in mMDSC. This decline in mMDSC was associated with a longer disease specific survival in prostate cancer patients [176]. Analyses of the PBMC of 74 metastatic melanoma patients treated with DC vaccination, revealed that a low expression of phophatidylethanolamine binding protein 1 (PEBP1) after but not prior to vaccination, was associated with a poor overall survival after vaccination. This was confirmed in a second cohort of 95 patients. PEBP1 expression correlated with genes in T cell responses but inversely with genes of myeloid cells and STAT3 associated inflammation as well as the myeloid to lymphoid cell ratio. Patients who did well displayed a rise in PEBP1 after vaccination [177]. The data of these last 3 studies suggest that a change in the balance between immune promoting versus immune suppressive myeloid cells increases the tumor-specific T cell response and improves patient outcome.

So far MDSC were hard to define phenotypically and functional assays were poorly specific but we have started to define the function of each phenotypically defined putative MDSC type in humans using in vitro sorted MDSC. These analyses revealed strong suppressive function for the mMDSC, variable suppression by the CD33+ MDSC and no suppression by the putative CD33- MDSC. Attempts to reduce the MDSC with gemcitabine, based on mouse models showing decreases in splenic MDSC and better tumor immunity, failed in patients when focusing on the frequencies of CD11b+HLA-DRlow/neg MDSC [178], mMDSC or CD33+ MDSC while the levels of the non-suppressive CD33neg myeloid cells dropped steeply [31,32]. Using an easy to reproduce gating

strategy, we showed that the levels of mMDSC were highly predictive for survival in ovarian cancer but so were the levels of DC. The ratio between these cells had a prediction specificity and sensitivity of 87.5% for better overall survival [32]. In the TC-1 mouse model it was shown that the presence of activated macrophage like DCs is important for the outcome of immune-mediated chemotherapy effect [30,179], and therapeutic tumor vaccination [180]. In melanoma patients treated with PD-1 blocking antibodies, the levels of CD14 + CD16-HLA-DRhi (classical monocytes) is associated with longer survival and better response to checkpoint therapy [181]. These observations are supported by the observation that in addition to the number of infiltrating CD8 + T cells, also the number of activated DC and ratio of M1 to M2 macrophages not only determines overall survival but also predicts the response to adoptive T cell therapy in melanoma [182].

Neutrophils, which comprise the population of granulocytic MDSCs may have a systemic immune suppressive effect in cancer patients. In the RNActive CV9201 study, targeting 5 TAA in NSCLC patients, the neutrophil to lymphocyte ratio was inversely correlated with progression free and overall survival. Furthermore, two mutually exclusive expression signatures in the peripheral blood characterized patients as either having a T and NK cell signature or having a myeloid cells and inflammation signature. Patients with the latter signature had shorter PFS and OS [183]. A high neutrophil to lymphocyte ratio was associated with a less broad vaccine-induced T cell response after PPV vaccination of patients with advanced colorectal cancer [69], indicating that neutrophils may also impact on the efficacy of the vaccine to stimulate tumor-specific T cell reactivity.

Altogether, these studies make a strong case for using the levels of different types of circulating myeloid cells (immune suppressive and stimulatory), in addition to the myeloid to lymphoid ratio and the detection of T cell reactivity for clinical decision support. This notion is sustained by a recent study in which a peripheral immune score was developed. The score was based on peripheral immune subsets reflecting immune function in a positive way, including central memory CD4 + and CD8 + T cells, and the ratio of these two subtypes over ICOS + Tregs. It also evaluated the levels of potentially negatively acting immune subsets, indicated by CD4 + or CD8 + T cells expressing more than two of the CTLA-4, PD-1 and TIM3 checkpoints, the percentages of ICOS + Treg, CD11c + DC expressing PD-L1, CD33 + HLA-DRlow/neg MDSC expressing PD-L1 and that of CD3-CD56 + NK cells lacking expression of Tim3 [184]. The immune cells were assigned to bins based on tertiles to ensure that patients with similar frequency of a given subset were assigned to the same bin. Then a score was applied being zero to the highest bin with an expected negative result or to the lowest bin if that subset should have a positive response. Conversely, 2 points were given to the other extreme bins. When pretreatment PBMC of 48 breast cancer patients, whom received either docetaxel or docetaxel with a PANVAC vaccine, were analyzed with a scoring method based on classic phenotypes, no association with PFS was found. However, if the peripheral immune score was applied then a correlation with PFS could be observed for the vaccine treated group but not in the control arm [184]. This suggests that the peripheral immune score could help to identify the patients who benefit the most from vaccination. The predictive value of this approach then was confirmed in a group of prostate cancer patients treated with either a bone-seeking radionuclide alone or in combination with PROSTVAC vaccine. Again, only in the vaccine arm the peripheral immune score was predictive [184], confirming an earlier report [185].

3.2. The impact of regulatory T cells and NK cells in trials

A few trials reported that not only MDSC but also regulatory T cells may mediate an impact on vaccine efficacy and/or on survival. Among the patients responding with a GV1001-specific T cell response after vaccination, those with low frequencies of CD4 + CD25 + CD127low/neg Foxp3 + Tregs had significantly increased PFS and OS. In addition, the quality of the T cell response was better as indicated by the higher IFNγ/IL5 and IFNγ/IL13 ratio [170]. Similarly, breast cancer patients mounting a CD8 + T cell response to Her2 and having relatively low frequencies of $CD4^+Foxp3^+CD127lowCD25^+$ Tregs displayed a better survival [175]. A subset of CD4 + T cells was shown to suppress tumor immunity by the action of membrane bound TGFβ in mice [186]. One study reported that vaccination with the MAGE-A3 may lead to the induction of such Tregs in patients with metastatic melanoma [187]. Interestingly, disappearance of these CD4 + TGFβ + Tregs was observed in melanoma patients immunologically responding to vaccination – as measured by DTH test - while in the immunologically non-responding group the frequencies of CD4 + TGFβ + Tregs rose to four times the levels observed in the responders. The stage IV melanoma patients who showed an immune response to the vaccine had longer progression free and overall survival [188], suggesting that the vaccine expanded Tregs have a negative impact on vaccine efficacy and survival. Indeed, T cells that are genetically engineered to resist TGFβ-signaling were much more active against mouse melanoma [189]. Vaccine-induced Tregs were also observed in patients with high grade lesions of the vulva after HPV16 SLP vaccination. These patients showed a weaker effector T cell response to the vaccine and were less likely to show objective clinical responses [125]. However, the observations were not confirmed in a second study [124].

To interfere with Tregs, inoperable but stable metastatic colorectal cancer received cyclophosphamide (50 mg, twice a day on days 1–7 and 15–21) and were vaccinated with modified virus Ankara expressing the tumor antigen 5T4. In 12 of the 27 vaccinated patients the percentage of CD4 + Foxp3 + Tregs dropped with about 40% or more. This decrease was associated with better PFS [190]. In another study, metastatic castration-resistant prostate cancer patients received cyclophosphamide at a 50 mg dose, every day for 7 weeks in combination with PPV vaccination or PPV alone. The decrease in percentage Tregs from baseline frequencies was higher in the cyclophosphamide arm but so was an increase in CD33 + CD14-MDSC [191], suggesting that unintentionally one suppressive mechanism was replaced by another when cyclophosphamide is given.

Finally, the frequency of circulating CD16 + CD56 + CD69 + lymphocytes (NK cells) was higher in 37 vaccinated NSCLC patients with a shorter time to progression and worse OS in a phase 2b trial [96]. The predictive value of this biomarker was positively validated in a phase 2b/3 trial with 222 NSCLC patients and was only operational in patients receiving the vaccine as it had no association with survival in non-vaccinated patients [99]. Potentially, these activated lymphocytes have a negative effect on the immune system for instance by killing of DCs, activated CD4 T cells and activated macrophages [100].

4. Conclusions

All sorts of cancer vaccines have been tested in the clinic and with the exception of a few, most trials did not reveal strong correlations between vaccine-induced immunity and signs of clinical impact. This is mostly due the fact that the number of true clinical responders in these trials is rather low to allow for statistical comparisons, limiting the analyses to showing that the clinical responders at least did mount a vaccine-induced type 1 T cell response. There are several reasons to explain the limited efficacy of vaccines, including: a) the choice for less immunogenic vaccine platforms reflected by a low percentage of patients responding, the need for in vitro stimulation to detect the responding T cells and the transient nature of the induced response [25,27,47,55,66,67,69,96] ; b) the choice to target suboptimal antigens, which are heterogeneous expressed resulting in antigen loss variants and tumor escape [72,145,192] or not dominant enough [193,194]; and c) the lack of a supportive systemic and tumor environment in cancer patients, all reducing the potential to obtain sufficient numbers of vaccine-induced tumor-reactive T cells that can enter the tumor bed and exercise their effects on tumor growth. If a cancer

vaccine is applied in settings of low disease burden [61,65,68,102,148] or pre-cancer [116–119,123,124] when immune suppression is expected to be less, or in a setting that systemic immune suppression is mitigated [127], the vaccine-induced T cell responses are stronger, broader and of an Th1 and/or CTL type. This coincided with level 4 evidence for vaccine efficacy in ovarian cancer and in pancreatic cancer [65,68]. Vaccination of patients with premalignant disease revealed a good correlation between tumor-specific Th1/CTL responses and clinical outcome. On the grounds that a type 1 immune contexture of cancer is associated with better response to therapy [2], extrapolation of the immune correlation found in patients with premalignant disease towards patients with cancer is plausible.

Most cancer vaccine trials report level 1 evidence of vaccine efficacy using survival benefit for immune responders as a surrogate marker for the detection of an effective antitumor response. While extension of life is the ultimate goal of immunotherapy, one may wonder how strong this type of evidence is. In one trial patients were vaccinated with HLA-A24 restricted peptides. There was no difference in survival between vaccinated patients bearing the HLA-A24 restriction element and those not having this HLA type, yet the immune responders within the HLA-A24-positive group displayed better survival than those failing to mount a T cell response [67]. In another randomized trial, no differences were seen between vaccinated patients or controls, but patients who developed a humoral response displayed a better clinical outcome [88]. A similar observation was made in the context of DTH responses and survival [143]. Furthermore, some studies report that patients with a response to 7 different peptides have a longer survival than those with a response to 6 peptides [70] whereas in other studies a similar difference in survival is found for patients responding to 3 peptides versus 0–1 peptides [71,84]. A simple explanation for these observations would be that the presence or more broader response to the vaccine rather reflects a better immune status of the cancer patients than true control of tumor growth. This notion is sustained by the regular observation that higher levels of circulating immune suppressive cells are associated with a lower response to vaccines and with worse survival. However, one can't exclude that a DTH response reflects more functional cells or that a broader response prevents escape, thus still reflects valuable data. Therefore, studies presenting level 1 evidence for vaccine efficacy in the context of assays that control for immune status and the levels of immune suppressive cells increase the merit of their data. Type 2 evidence, which is more directly related to the behavior of tumors, is not often used, Changes in the serum levels of the tumor markers PSA, CEA, CA125 or CA19-9 [59,73,102] can be measured but many tumors lack appropriate serum tumor markers that truly reflect disease burden. Furthermore, the number and timing of metastatic events [148] as used for melanoma is not likely to be useful for other types of tumors.

Level 3 evidence was defined as signs of on-target autoimmunity or regression of individual metastases, objective clinical responses after vaccination. Two trials in which an overexpressed self-protein was targeted, reported signs of potential on-target autoimmunity [102,155]. More importantly, there were at least 18 trials showing regression of premalignant lesions or tumors after vaccination with a series of different antigens over a spectrum of different tumors. In general, the patients with such a clinical response showed a robust or more active type 1 T cell response [56,57,61,66,69,72,76,82,123–125,148–150] which in the case of premalignant disease showed a strong correlation with clinical outcome [123–125]. The regressions of tumors in cancer patients, albeit a few per trial, form an attest to the capacity of cancer vaccines to induce a functional immune response, which under the right – probably less immune suppressive – conditions even can control tumor growth. Most likely that is the case why only a few trials have presented level 4 evidence for vaccine efficacy. Vaccination of patients with premalignant lesions resulted in a strong tumor-specific Th1/CTL response and a higher regression rate than in the non-vaccinated control group [117]. Moreover, vaccination in a minimal residual disease setting of ovarian cancer using an engineered autologous tumor vaccine, resulted in tumor-specific type 1 T cell immunity and prevented against the development of recurrences when compared to non-vaccinated controls [64]. Similarly, a non-significant longer PFS was found in a trial where vaccination of pancreatic cancer patients was performed after surgery. However, in this trial HLA-A24+ were vaccinated and received gemcitabine, while the HLA-A24-negative patients received only chemotherapy. Thus, this result should be taken with some caution [68]. Cancer vaccination potentially is associated with level 4 evidence in two other trials. The PFS as measured by serum PSA levels was better in castration resistant prostate cancer patients receiving PPV with dexamethasone chemotherapy than in those receiving chemotherapy only. But no immune response was measured [85]. In addition, PPV vaccination of patients with progressive bladder cancer resulted in a better OS but not a better PFS than the control group [87].

At this point it is fair to conclude that there are quite a number of trials in which cancer vaccines induce the required type 1 T cell response and regression of lesions or metastases. The immunotherapy of cancer field has realized earlier that currently many vaccine platforms have been optimized to do their job [24], that is the induction of tumor-reactive CD4+ and CD8+ T cells to levels allowing ex-vivo detection in the blood. For the use of cancer vaccines in the treatment of patients with cancer, new studies will require investigators to address the reasons for successful regressions rather than focusing on signatures associated with OS in the absence of such regressions. Understanding what is similar among the level 3–4 responders and where they differ from non-responders will teach the way to apply the vaccine with the right co-treatments for the right patient. I expect that many overlaps will be found with parameters defining the success of other immunotherapeutic treatment options.

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Declaration of interest

SHvdB is one of the inventors on the patent for the use of synthetic long peptides as vaccine but holds no financial interest. SHvdB serves as a paid member of the strategy board of ISA Pharmaceuticals (NL) that exploits this patent, as well as performs contractual work for ISA Pharmaceuticals. SHvdB also has served as adhoc scientific advisor for IO Biotech (DK) that develops a vaccine against proteins overexpressed in immune suppressive cells. No other potential conflict of interest relevant to this article was reported.

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