APPROACHES TO ADVANCE CANCER VACCINES TO CLINICAL UTILITY

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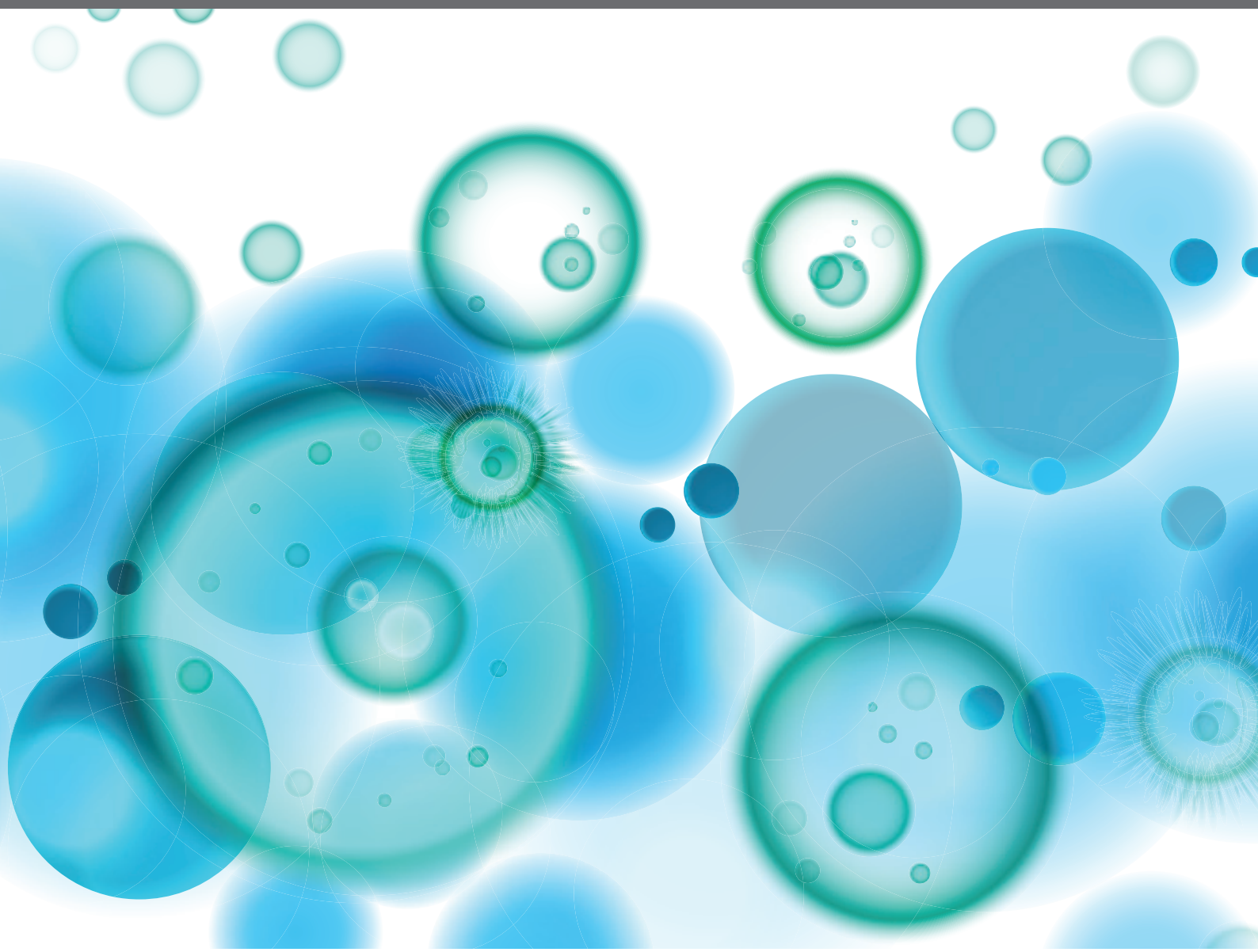

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APPROACHES TO ADVANCE CANCER VACCINES TO CLINICAL UTILITY

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Although cancer vaccines have yielded promising results both in vitro and in animal models, their translation into clinical application has not been very successful so far. Through the success of immune checkpoint inhibitors, the tumor immunotherapy field revived and led to important new insights. A better understanding of the functional capacity of different dendritic cell (DC) subsets and the immunogenicity of tumor antigens, more particularly of neoantigens, have important implications for the improvement of cancer vaccines. These insights can guide the development of novel strategies, to enhance the clinical utility of cancer vaccines. The aim of this Research Topic is therefore to provide a comprehensive overview of current issues regarding cancer vaccine development with an emphasis on novel approaches toward enhancing their efficacy.

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EDITORIAL
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Editorial: Approaches to Advance Cancer Vaccines to Clinical Utility

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Keywords: cancer vaccines, personalized, adjuvant, antigen-presenting cell, *in situ* vaccination, immunosuppression, biomarkers

Editorial on the Research Topic

Approaches to Advance Cancer Vaccines to Clinical Utility

Although cancer vaccines have yielded promising results both *in vitro* and in animal models, their translation into clinical application has not been very successful so far, even though encouraging results from small early phase trials are reported. Junco et al. describes the 10-year follow up of Heberprovac, a GnRH1 peptide vaccine linked to a tetanic toxoid epitope in prostate cancer patients. Kjeldsen et al. reports on the 6-year follow up of an indoleamine-2,3-dioxygenase (IDO) peptide vaccine in non-small cell lung cancer. Both vaccines target endogenous proteins, are tolerated well long-term, and are safe and show durable responses. Delivering durable benefits is a unique feature of immune therapy, hence the emergence as "Breakthrough of the Year" 2013 (1). Through the success of immune checkpoint inhibitors, the tumor immunotherapy field revived and led to important new insights. A better understanding of the functional capacity of different dendritic cell (DC) subsets and the immunogenicity of tumor antigens, more particularly of neoantigens, have important implications for the improvement of cancer vaccines. These insights can guide the development of novel strategies, to enhance the clinical utility of cancer vaccines. The aim of this Research Topic was therefore to provide a comprehensive overview of current issues regarding cancer vaccine development with an emphasis on novel approaches toward enhancing their efficacy.

Current cancer treatments are becoming more and more **personalized** based on the patient's specific tumor characteristics instead of a one-size-fits-all approach (2). This concept is also true for cancer immunotherapies. Mastelic-Gavillet et al. describes personalized dendritic cell (DC)-based vaccination and mentions the importance of targeting private tumor antigens, such as **neoantigens**. Related to this, Klausen et al. discuss the use of alternative neoantigens resulting from JAK2 and CALR mutations in hematological malignancies. They also depict the use of regulatory proteins, PD-L1 and PD-L2, as target antigens. This latter is conceptually similar to the IDO vaccine trial described by Kjeldsen et al. as the immune target does not need to identify the tumor, but focuses on the suppressive environment. In the trial of Junco et al., the chosen target is a driver of tumor growth. Vermaelen discusses the recent efforts taken to improve the selection of tumor antigens to use as targets in cancer vaccines and their visibility. Xiang et al. identifies the most optimal peptide for vaccination from three antigens expressed by gynecological tumors.

An important issue to consider when aiming to increase the efficacy of cancer vaccines is the use of the right **adjuvant** (3). Besides using DC's as nature's adjuvant, several other approaches are available. In their paper, Xiang et al. describe that polystyrene nanoparticles can induce T cell responses to tumor antigen peptides although not through conventional inflammation. Vermaelen gives an overview of the adjuvant formulations that have been developed to unlock clinically relevant immune responses against cancer antigens, which comprise both immune stimulation and suppressing the suppressors. However, a reality check of the vaccine formulations tested clinically in lung cancer shows that clinical

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successes are limited and that traditional approaches from the infectious diseases' vaccine field cannot be translated to cancer treatment as such. Ho et al. also report recent insights in clinically relevant vaccine adjuvants that impact DC cross-presentation efficiency. Furthermore, they emphasize that the mode of action of adjuvants in general, and on antigen cross-presentation in DCs in particular, is important for the design of novel adjuvants as part of vaccines able to induce strong cellular immunity. Kartikasari et al. describe the epigenetic effects of vaccine adjuvants on immune cells and cancer cells and propose epigenetic interventions that could improve cancer vaccines.

Another crucial component for the induction of a successful anti-tumor response is the use or targeting of the right **antigen-presenting cell**. DCs are the most professional antigen-presenting cells but, even between the different DC subsets significant functional differences have been reported (4). The review by Clappaert et al. provides a nice overview of the different myeloid cell types that are present in tumors, including DCs, and how they can be harnessed for cancer therapy. Since efficient cross-presentation of tumor antigens is warranted, the current evidence points toward the **cross-presenting DC** subset ($CD141^+$ DC in humans, $CD8\alpha^+/CD103^+$ DC in mice) as the most promising target, which is discussed by Mastelic-Gavillet et al. and Ho et al. In this respect, Botelho et al. show specific binding and uptake of a fusion protein of Xcl1 and OVA synthetic long peptide (SLP) by $Xcr1^+$ DCs. The potent adjuvant effect on the induced T cell response was associated with sustained tumor control. Thus, developing Xcl1-SLP-Fc fusion proteins as an off-the-shelf vaccine targeting cross-presenting DCs might be an economical and easier alternative to *ex vivo* DC vaccines. Viral vectors constitute another approach to modify DCs *in situ*, as discussed by Goyvaerts and Breckpot. Their attractiveness lies in the fact that they can be targeted and then simultaneously deliver the encoded tumor antigen to antigen-presenting cells as well as behaving as Th1-polarizing adjuvant via the viral vector backbone. However, the antiviral immunogenicity also carries their weakness for which solutions are discussed.

DC targeting can also be achieved via so-called ***in situ* vaccination** approaches, to induce local release of tumor antigens from the tumor itself (5). Yasmin-Karim et al. report that stereotactic body radiation therapy (SBRT) synergizes with intratumoral injection of agonistic anti-CD40, resulting in regression of non-treated contralateral tumors and formation of long-term immunologic memory in a pancreatic mouse model. Locy et al. discuss how oncolytic viruses, radiotherapy, physical therapies, growth factors, and cytokines can stimulate anti-tumor immune responses through the induction of immunogenic cell death, the attraction of different immune cell populations and by alleviating immune suppression. Next challenges for *in situ* vaccination include the accessibility of the tumor and the need to develop approaches to circumvent local immunosuppression.

FUTURE PERSPECTIVES

Although it has come a long way, there is still a lot of room for cancer vaccine optimization. First, the best vaccination approach might differ for "hot tumors (immunogenic)" vs. "cold tumors (non-immunogenic)." Vermaelen describes the importance to focus on **lymphocyte entrance and the local suppression** in the tumor mediated by receptors/ligands (checkpoints), cells (Treg, MDSC), and metabolism (IDO, adenosine, lack of arginine, etc). Strategies to handle tumor associated myeloid cells are more extensively elaborated by Clappaert et al.

Second, **biomarkers** can guide physicians in their treatment decision to obtain a faster selection of the most effective treatment. Highly reliable molecular and/or cellular biomarkers for vaccine efficacy are still to be identified. Mastelic-Gavillet et al. summarizes that in non-small cell lung cancer $BDCA1^+$ DC/$BDCA3^+$ DC ratio in peripheral blood correlated with survival, as did $CD56^{dim}$ cytotoxic NK cells in glioblastoma. The expression of chemokine receptor CXCR4 on $CD8^+$ T cells and CD32 on monocytes correlated with immunological responders. However, these still require further validation. Epigenetic mapping could be a promising next type of biomarker, but is still in its infancy according to Kartikasari et al.

Finally, the indication for which the vaccine developed is of major importance. Due to the highly immunosuppressive nature of the tumor microenvironment, it is clear that cancer vaccination strategies will have to be integrated in **combination therapies** to tackle tumor-induced immunosuppression (6). Current standard of care therapies can have immune modulating properties or serve as adjuvant. Some are described by Locy et al., as mentioned above. Klausen et al. mentions upregulation of cancer testis antigens by hypomethylating agents given to patients with high-risk myelodysplastic syndrome. Practically, the influence of different standards of care in each indication need to be taken into account to foster clinical implementation, in particular when vaccination would not be applied as a first line treatment. Equally important, is looking at the development of new therapies in that indication that might become the next standard of care and existing therapies for other indications that can serve as good adjuvants as mentioned by Ho et al. The review paper of van Willigen et al. delineates the position of DC therapy in the current and future cancer treatment landscape for glioblastoma, melanoma, prostate cancer, and renal cell carcinoma.

Personalization, as indicated in this Research Topic, either through the *in situ* or *ex vivo* use of the right type of autologous cell and/or by choosing the best specific target for each tumor or its microenvironment currently holds a lot of promise. Optimized clinical trials will now have to reveal whether this brings cancer vaccine efficacy to the next level.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. AV and ST wrote the manuscript. CB performed critical revision.

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ORIGINAL RESEARCH
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Safety and Therapeutic Profile of a GnRH-Based Vaccine Candidate Directed to Prostate Cancer. A 10-Year Follow-Up of Patients Vaccinated With Heberprovac

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Heberprovac is a GnRH based vaccine candidate containing 2.4 mg of the GnRHm1-TT peptide as the main active principle; 245 μg of the very small size proteoliposomes adjuvant (VSSP); and 350 μL of Montanide ISA 51 VG oil adjuvant. The aim of this study was to assess the safety and tolerance of the Heberprovac in advanced prostate cancer patients as well as its capacity to induce anti-GnRH antibodies, the subsequent effects on serum levels of testosterone and PSA and the patient overall survival. The study included eight patients with histologically-proven advanced prostate cancer with indication for hormonal therapy, who received seven intramuscular immunizations with Heberprovac within 18 weeks. Anti-GnRH antibody titers, testosterone and PSA levels, as well as clinical parameters were recorded and evaluated. The vaccine was well tolerated. Significant reductions in serum levels of testosterone and PSA were seen after four immunizations. Castrate levels of testosterone were observed in all patients at the end of the immunization schedule, which remained at the lowest level for at least 20 months. In a 10-year follow-up three out of six patients who completed the entire trial survived. In contrast only one out eight patients survived in the same period in a matched randomly selected group receiving standard anti-hormonal treatment.

Heberprovac vaccination showed a good security profile, as well as immunological, biochemical and, most importantly, clinical benefit. The vaccinated group displayed survival advantage compared with the reference group that received standard treatment. These results warrant further clinical trials with Heberprovac involving a larger cohort.

Keywords: advanced prostate cancer, GnRH/LHRH vaccine, hormone ablation, hormone sensitive cancer, overall survival

INTRODUCTION

The early landmark studies of Huggins and Hodges established the hormonal dependence of prostate cancer and provided the basis for the use of androgen deprivation in its treatment (1).

Reduction of plasma testosterone to castrate levels, either through surgical castration (orchiectomy), or of oral or injectable estrogens, became the standard therapy for disseminated prostate cancer in the following 40 years (2–6). In the early 1980s, LHRH analogs were added as an alternative to achieve reversible pharmacologic castration (7–10).

By the mid 1990's, an immunological approach (LHRH vaccines) had been designed and tested in men to achieve androgen deprivation to treat prostate cancer (11, 12) and in post-menopausal women to test gonadotropin inhibition (13). The efficacy of the neutralizing action of LHRH/GnRH through the involvement of hormone-specific antibodies has been demonstrated in a wide range of animal species, including humans. Such studies have involved either passive immunization by infusion of anti-LHRH antibodies (14) or vaccination with the LHRH peptide coupled to tetanus or Diphtheria toxoid (DT) molecules as carriers (11–14), or LHRH in multiple antigen peptide (MAP) constructs (15). These approaches are impractical for widespread commercial application since passive immunity is inefficient and expensive (16) and the use of peptide–toxoid conjugates and MAP constructs produce variable results (17). On top of that, the GnRH-tetanic toxoid conjugates since their big size can induce anti-haptenic immunosuppression and such process became difficult to reproduce at industrial scale (18).

In order to overcome these limitations, the Heberprovac vaccine candidate was designed, which contains the modified pEHWSYPLRPG GnRH sequence, chemically coupled to the 830–844 T helper epitope of the tetanic toxoid (TT) in the same synthetic process. Such approach breaks immune tolerance to hormone, by eliciting anti-LHRH neutralizing antibodies that induce immunological castration (19). The administration of seven Heberprovac immunizations, followed by radiotherapy in six advanced prostate cancer patients, resulted in 100% immunogenicity, testosterone drop to castration levels, and PSA normalization. These clinical results had never been reported for a GnRH-based vaccine.

MATERIALS AND METHODS

Ethics and Methodological Aspects

The current clinical trial complied with the principles of the Declaration of Helsinki on clinical investigation in humans. It was approved by the Scientific and Ethics Committee of the Marie Curie Oncology Hospital, in Camaguey, Cuba, as well as by the National Regulatory Authority of Cuba (CECMED). The patient's informed consent was recorded before the study was started. An intermediate endpoint was established to identify the high-risk cases and poorly responding patients, who then received the usual disease treatment as recommended by the medical guidelines. The intermediate evaluation was setup to ensure protection of patients with low immunization response. The adverse events were evaluated by The Common Terminology Criteria for Adverse Events, Version 3.0[7] http://ctep.cancer.gov/protocoldevelopment/electronic_applications/docs/ctcaev3.pdf.

Trial Design

It was a single arm, open, prospective study in which a randomized external group of patients with locally advanced and metastatic prostate cancer was used. The main goal of the trial was to evaluate the product safety according to the local and systemic adverse events (AE) and signs of efficacy. The sample size (N) was calculated in 6–8 patients for the immunized and for the external group receiving the standard therapy. During the study, safety and tolerance of the vaccine candidate were monitored by rigorous control of the adverse events, and calculation of the occurrence frequency. The survival of vaccinated patients was compared with a cohort of patients bearing advanced prostate cancer, selected with the same criteria, received the standard anti-hormonal treatment.

Patients and Eligibility

From January to March 2007, eight men diagnosed with advanced (stage 3–4) prostate cancer (TNM classification, 1992) were recruited at the Uro-Oncology Department of the Marie Curie Oncology Hospital in Camaguey, Cuba, based on clinical, biochemical and anatomical-pathological criteria. Previously, all patients signed an informed consent. The prostate biopsy was performed using trans-rectal ultrasound with a biopsy device (ALOKA 2004, Japan). The eligibility criteria also included leukocytes $>3.0 \times 10^9$/L, lymphocytes $>1 \times 10^9$/L, thrombocytes $>100 \times 10^9$/L, and hematocrit >30%. The exclusion criteria for the treatment included previous immunological treatment of up to 2 months before the beginning of the immunization schedule, as well as significant levels of anti-GnRH antibodies, and decompensated chronic diseases (asthma, epilepsy, autoimmune diseases, immunodeficiency, anemia, uncontrolled urinary sepsis and renal, hepatic and cardiovascular diseases) **Diagram 1**.

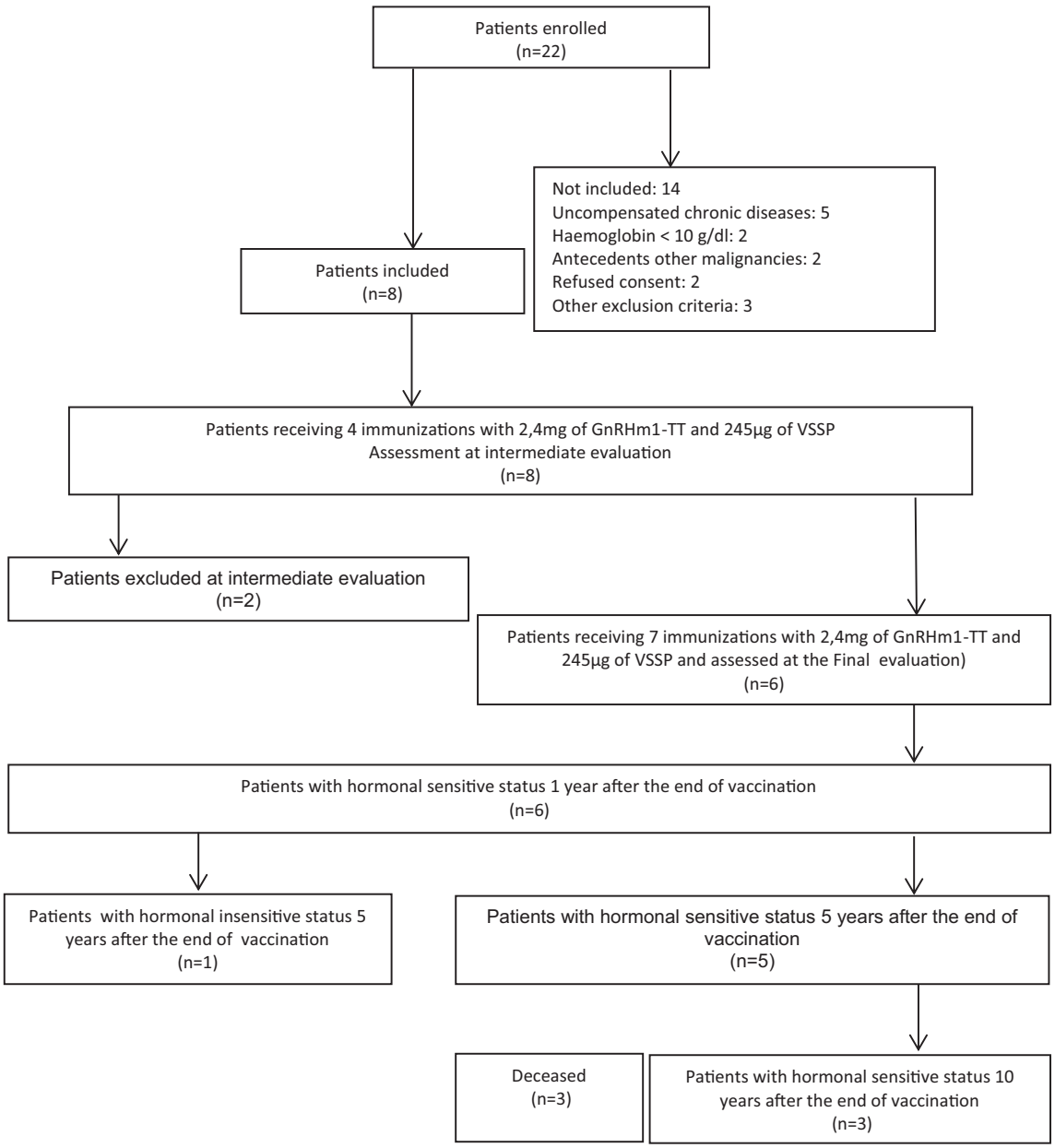

DIAGRAM 1 | Outcome of patients included in the Phase I clinical trial with Heberprovac. CONSORT diagram.

Vaccine Composition and Treatment Schedule

The vaccine consist of a mixture of three components: the 27 amino acid GnRHm1-TT peptide synthetized and supplied by The Center of Genetic Engineering and Biotechnology (CIGB), Cuba, in 2.4 mg 2R vials; Montanide ISA 51 VG adjuvant from Seppic, France; and VSSP, a Neisseria meningitidis derived adjuvant produced and supplied by the Center of Molecular Immunology (CIM), Cuba, in 0.8 mg/0.5 mL vials. Before immunization, the peptide was resuspended in VSSP adjuvant and mixed (50:50 v/v) with the Montanide ISA 51VG oil adjuvant, in order to form a water-in-oil emulsion that was added to a total volume of 700 µL, and injected intramuscularly to patients. All patients received seven doses of a vaccine containing 2.4 mg of the peptide, 245 µg of VSSP, and 500 µL of Montanide ISA-51. The first four doses were administered fortnightly, and the remaining three were applied monthly. A month after vaccination ended, a total of 60 Gy radiotherapy (RT) was assessed using a Co-60 radioisotope (**Figure 1**). The patients' response to vaccination was evaluated at recruitment, after the fourth and seventh immunizations, and after receiving RT.

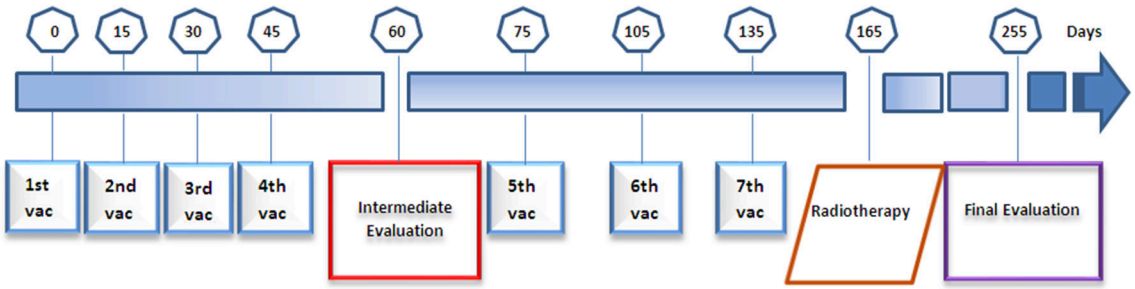

FIGURE 1 | Schematic overview of the immunization schedule, radiotherapy, and evaluation interventions practiced to the patients participating in the Heberprovac Phase I clinical trial.

Clinical and Complementary Assessment

The patients underwent general physical examination, digital rectal exam (DRE), and laboratory imaging and analysis. The imagenological examination included transrectal and trans-abdominal ultrasound and bone gammagraphy to determine possible metastases. Blood samples were drawn for routine checkups at recruitment and 15 days after the fourth and seventh immunizations, and after the patients received RT for general clinical laboratory parameters, as well as for anti GnRH antibody titers, using an ELISA kit. For the biochemical and endocrine evaluation, serum PSA was determined by ultra-micro-analysis system (UMELISA, CIE, Cuba) and the testosterone levels were quantified through a radioimmune assay (RIA, CISBIO, France). Since the main goal of the trial was to measure the product safety, the local and systemic adverse events were carefully assessed. Systemic toxicity was evaluated for 72 h after each vaccination. It included measurements of temperature, blood pressure, respiratory frequency 30 min after each injection, and later, every hour during 4 h. The patients completed the physical examination in 72 h, using the standard supervision applied to in-patients, through anti-GnRH quantification plus serum PSA and testosterone determinations.

Long Term Follow-Up of Patients

Follow up was made every 3–4 months for 10 years since the end of the immunization. The parameters evaluated in each medical consultation were the same as for the previous evaluation of patients during the clinical trial development: DRE, anti-GnRH antibodies, serum testosterone and PSA. Imaging methods: Trans-rectal ultrasound (Aloka, Japan) was used at the diagnosis and at the final evaluation for prostate biopsy. In order to look for nodules and metastases, we carried out Tc 99 Gammagraphy scan. After the completion of treatment the patients were followed up for a further period of 10 years. Survival of patients that completed the vaccination schedule was compared with a parallel sample of patients ($n = 8$) with similar disease status, who received standard anti-hormonal treatment.

Statistics

The data were double entered and validated using Microsoft Access, and then imported into SPSS 13.0, for analysis. The frequency distribution and central tendency and dispersion were estimated by mean standard deviation, median, interquartilic range (QR), and the maximum and minimum values (range) for qualitative and quantitative variables.

For each type of adverse event, the frequency distribution (IC 95%) was estimated with the classical and Bayesian statistics. For survival, statistical analysis was carried out using Log Rank test.

RESULTS

Study Population

Between March and July 2007, eight men with confirmed diagnosis of advanced prostate cancer (stages III/IV) were included in the safety study with the vaccine candidate Heberprovac. At the same time, 8 patients with advanced prostate cancer were randomly selected in the uro-oncology service, who began treatment with the standard therapy for prostate cancer and were used as external control group (EG). **Tables 1A**, **B**. The age of patients ranged from 63 to 78 years old (71.3 years on average). All patients had high Gleason score confirmed by the histological study. The patients were evaluated at recruitment, after the fourth and last (7th) immunizations, the later after they received the RT (**Figure 1**). The treatment schedule was completed in 6 patients, who were followed up for recurrence during 10 years (2007–2017) **Diagram 1**.

Adverse Events

The vaccine was well tolerated despite the presence of side effects and adverse reactions (see below) that coincided with the protocol safety hypothesis. No vaccine-related events exceeded grade II. The intermediate evaluation was made to check safety. Two patients (04 and 06) were removed from the study for presenting signs of clinical and biochemical progression of the disease (interruption criteria).

The observed local and systemic adverse events are summarized in the **Table 2**. All patients reported local pain at the vaccination site. Three of them developed a slight swelling around the injection site. Other events reported were local redness and swelling, skin atrophy, induration, and erythema. Systemic adverse events included fever, muscle pain and flu-like symptoms in all the six patients that finished the treatment. Late adverse events were mainly associated with the hormone deprivation caused by the vaccine, and included libido

TABLE 1A | TNM classification and Gleason score of patients included in the Phase I clinical trial with Heberprovac.

Prostate cancer patients vaccinated with Heberprovac		
Patient no.	**TNM classification**	**Gleason score**
MC01	**T3M0N0: Stage III**. Prostatic tumor with extracapsular extension to both prostate lobules. No meta, no ganglionar nodules	Prostatic ADC Gleason 9 in all the studied fragments. Predominant pattern 4
MC02	**T3bM0N0: Stage III.** Prostatic tumor with extracapsular extension to both prostate lobules. No meta, no ganglionar nodules	Prostatic ADC Gleason 9 in left lobule. Gleason 8. Predominant pattern 4. Right Lobule hyperplastic
MC03	**T3bN0M1b Stage IV.** Prostatic tumor with extracapsular extension to both prostate lobules. Bone metastases. No ganglionar extension	Prostatic ADC Gleason 8 in 100 % of the simple. Right lobule Hyperplasia
MC04	**T4aN0M1b Stage IV.** Prostatic tumor with extracapsular extension to both prostate lobules that infiltrate vesical neck, rectus; with bone meta. No ganglionar infiltration	Prostatic ADC. Gleason 10 in all the studied fragment of the right and left lobules
MC05	**T3aN0M0**. **Stage III.** Prostatic tumor with extracapsular extension to one prostate lobule. No meta, no ganglionar nodules	Prostatic ADC of all studied fragment of right lobule. Gleason 8. Left lobule, ADC, Gleason 8 in 100% of the samples
MC06	**T3bN0M1b Stage IV.** Prostatic tumor with extracapsular extension to both prostate lobules. Bone metastases. No ganglionar extension	Undifferentiated Carcinoma of muscle tissue. Gleason 10
MC07	**T3aN0M0 Stage III.** Prostatic tumor with extracapsular extension to one prostate lobule. No meta, no ganglionar nodules	Prostatic ADC of right lobule. Gleason 8 in all the samples. Left lobule Hyperplastic
MC08	**T3aN0M0 T Stage III.** Prostatic tumor with extracapsular extension to one prostate lobule. No meta, no ganglionar nodules.	Prostatic ADC of left lobule. Gleason 10 in 100% of samples. Right lobule hyperplasic. Gleason 6

TNM correspond to patient classification according to the American Joint Committee on Cancer (20).

TABLE 1B | TNM classification and Gleason score of patients non-included in the clinical trial that were used as control external group.

Non included Prostate cancer patients (External group)		
Patient no.	**TNM classification**	**Gleason score**
EG03	**T4 N1M0: Stage IV**. Prostatic tumor with extracapsular extension to both prostate lobules. No meta, ganglionar nodules	Prostatic ADC Gleason 8 in all (4) studied fragments. Predominant pattern 4
EG05	**T4 N0M1: Stage IV** prostatic tumor with extracapsular extension to both prostate lobules. Bone metastases, no nodules	Prostatic ADC Gleason 9 in both lobules. Predominant pattern 5
EG06	**T3aN0M0. Stage III.** Prostate tumor with perineural and perivascular extension to both prostate lobules. No Bone metastases. No ganglionar extension	Prostatic ADC Gleason 7 in 4 out 5 samples studied. Predominant pattern 4
EG09	**T4b N1M1b Stage IV.** prostatic tumor with extracapsular extension to both prostate lobules that infiltrate bladder. Bone metastases and ganglionar infiltration	Prostatic ADC. Gleason 10 in all the studied fragments of the right and left lobules
EG11	**T3bN0M0**. **Stage III.** Prostatic tumor with extracapsular extension to both prostate lobules. No metastases, no ganglionar infliltration	Prostatic ADC of the prostate. Right lobe, Gleason 8. Left lobe, Gleason 9 in all the samples
EG12	**T3bN1M1b Stage IV.** Prostatic tumor with extracapsular extension to both prostate lobules. Bone metastases. No ganglionar extension	Undifferentiated Carcinoma of prostate with muscle tissue infiltration. Gleason 10
EG14	**T3aN0M0 Stage III.** Prostatic tumor with extracapsular extension to one prostate lobule. No meta, no ganglionar nodules	Prostatic ADC of right lobule. Gleason 8 in all the samples. Predominant pattern 4
EG17	**T3aN0M0 T Stage III.** Prostatic tumor with extracapsular extension to one prostate lobule. No metastases	Prostatic ADC of right lobule. Gleason 9. Predominant pattern 5

TNM correspond to patient classification according to the American Joint Committee on Cancer (20).

decrease, sexual dysfunction, breast tenderness and weakness. Remarkably, not a single case of Gynecomastia was observed for the vaccinated group. However, in the case of the control group, it is important to point out that 75% of patients reported hot flushes between 15 and 20 days after the injection of Zoladex, as well as an increase in urinary symptoms after the first two administrations of the GnRH analog. Similarly, symptoms depending of hormonal ablation as asthenia, sexual erectile dysfunction and decreased libido were observed in the 60–100% of patients, respectively (**Table 2**).

TABLE 2 | Most reported adverse events in Heberprovac vaccinated and control group prostate cancer patients.

Variables registered by patients		Vaccinated	%	Control Group (%)	
Local	Pain in the injection site	5	62.5	1	12.5
	Edema	5	62.5	1	12.5
	Skin atrophy	4	50.0	2	25.0
	Increase of volume	3	37.5	1	12.5
	Erythema	2	25.0	0	0.00
	Induration	4	50.0	0	0.00
	Crusty lesion	4	50.0	0	0.00
	Residual macula	1	12.5	0	0.00
	Scarring reaction	1	12.5	0	0.00
Systemic	Fever	6	75.0	1	12.5
	Anemia	1	12.5	3	37.5
	Asthenia	3	37.5	5	62.5
	Bradycardia	1	12.5	2	25.0
	Headache	1	12.5	2	25.0
	Depression	1	12.5	3	37.5
	Decreased libido	6	75.0	8	100.0
	Diarrhea	1	12.5	1	12.5
	Sexual erectile dysfunction	4	50.0	7	87.5
	Hypertension	3	37.5	2	25.0
	Hot flushes	0	12.5	6	75.0
	Gynecomastia	0	0.00	5	62.25

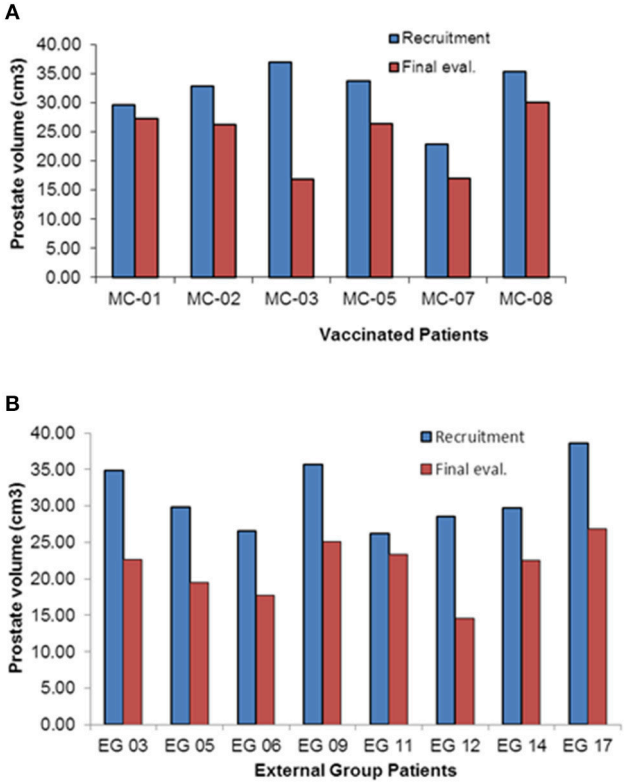

FIGURE 2 | Prostate volume evaluation by trans-rectal ultrasound of the prostate cancer patients included in the clinical Heberprovac clinical trial and the External Group of prostate cancer patients with similar stage. **(A)** Individual measurement of prostate volume of patients before the treatment and after finishing the full immunization schedule and RT. **(B)** External Group prostate measurement using transrectal ultrasound before and after complete standard hormonal therapy and RT.

Clinical and Imaging Evaluations

The evaluation of prostate size according to the DRE at recruitment for the trial showed that 7/8 patients possessed T3 prostate size, while one patient (MC04) displayed T4 prostate; the largest prostate size according to TNM classification (20). These data were confirmed using trans-rectal ultrasound.

Of the eight patients initially included in the trial, six completed the immunization schedule, and in two cases (patients MC04 and MC06) the treatment was interrupted and the patients had to abandon the trial after the intermediate evaluation, due to progression of the disease manifested as elevated PSA and creatinine, urinary obstruction, hydronephrosis, and renal failure that forced them to discontinue immunization.

The completion of treatment with the 7th Heberprovac immunization plus RT, resulted in a significant reduction of the prostate size in the six patients that concluded the full schedule and in the 100% of patients of the control group, considering the prostate size by DRE and trans-rectal ultrasonography.

Transrectal ultrasound data of prostate volume for each patient is summarized in **Figure 2**. For immunized patients, the most important prostate reduction was observed in the patient MC 03, with 55% prostate reduction. Patients MC 07, MC 05, and MC 02 underwent between 20 and 25% prostate volume reduction, whereas patient MC 08 had around 18% reduction. Patient MC 01 just suffered a 5% of prostate reduction at the time **Figure 2A**. The overall prostate volume reduction observed was 23.4%, in comparison to the moment of recruitment ($P < 0.01$). On the other hand, patients who received standard therapy also had an important benefit in relation to the reduction of the size of the prostate. In this way patients EG 03, EG 05, EG 06, and EG17 had a decrease of 30% or more of the prostate size. The patient EG 12 was the one that showed a greater reduction of prostatic size among all with 49%. The remaining 2 patients showed a decrease of 10 and 29% of the prostatic volume in relation with the beginning (**Figure 2B**).

Anti-GnRH Immune Response and Surrogate Biochemical Markers

Heberprovac is a vaccine candidate designed to generate anti-GnRH antibodies. Such humoral immune response was evaluated at the mid and end stages of the trial and compared with the values at the moment of patient recruitment.

Table 3 shows Testosterone and PSA correspondence with the anti GnRH antibody titers. All patients generated anti-GnRH antibodies after the fourth immunization. Two patients (MC

TABLE 3 | Anti GnRH antibodies, Testosterone and PSA levels at recruitment, intermedia and final evaluation of prostate cancer patients immunized with Heberprovac.

Patient no.	Anti GnRH antibodies (Dilution titers)		Testosterone levels (nmol/L)			PSA levels (ng/ml)		
	After 4th immun.	After 7th immun.	At recruitment	After 4th immun.	After 7th immun.	At recruitment	After 4th immun.	After 7th immun.
MC-01	3,200	12,800	4.4	0.13	0.249	22.9	12.49	0.43
MC-02	3,200	12,800	2.31	2.75	0.079	32.3	15.86	0.52
MC-03	6,400	12,800	4.55	2.04	0.041	46	25.50	0.83
MC-04	1,600	*	3.91	3.15	*	34.9	45.17*	*
MC-05	6,400	25,600	2.79	3.82	0.99	50	31.08	3.99
MC-06	1,600	*	4.94	4.68	*	16	22.95*	*
MC-07	3,200	12,800	3.39	6.46	0.10	3.80	2.09	0.36
MC-08	800	6,400	4.02	1.85	0.02	6.90	6.91	0.78

Means that the patient interrupted the treatment and were not evaluated at this time.

03 and MC 05) developed 1:6,400 anti-GnRH antibody titers; three patients (MC01, MC 02, and MC 07) reached 1:3,200; two patients (MC 04 and MC 06) developed 1:1,600 titers; and one patient (MC 08), developed 1:800 anti-GnRH antibody titers. After completion of the reminder three immunizations, the anti-GnRH immune responses continued increasing and reached 1:25,600 in patient MC 05. Four patients (MC 01, MC 02, MC 03, MC 07) generated 1:12,800 antibody titers. The lowest anti-GnRH antibody response corresponded to patient MC 08, who developed 1:6,400 anti-GnRH titers. As mentioned previously, patients MC 04 and MC 06 showed disease progression, and did not complete the treatment; hence, they were excluded from the final evaluation.

Such anti-GnRH immune responses corresponded with a significant drop in testosterone, found in 3/8 patients (MC 01, MC 03, and MC 08) just 15 days after the fourth immunization. Upon completion of the immunization schedule and the conclusion of the radiotherapy, 100% of the patients that met the criteria of continuity in the trial, underwent testosterone castration under 1 nmol/l ($p < 0.001$) (**Table 3**).

The patient's PSA kinetics was evaluated in parallel during the entire immunization schedule. Such measurements experienced a change from a mean of 26.6 ng/ml at recruitment, to 20.2 ng/ml after the fourth immunization ($p > 0.05$). The completion of the immunization schedule however, yielded complete PSA normalization in the six patients that concluded the protocol ($p < 0.001$) (**Table 3**). It is important to note that the PSA decline started when the anti-GnRH antibodies reached titers similar to or higher than 1:3,000. **Figure 3** represents the inverse relation between anti-GnRH antibody titers and the PSA levels, the higher the anti GnRH titers, the lower the PSA values.

Also, the anti-GnRH antibody isotypes generated with the vaccine candidate Heberprovac were determined. After finishing the fourth immunization, the highest antibody response in all the patients was of IgM subtype, followed by IgG1 and IgA, in that order (**Figure 4A**). After the end of the immunization schedule and once the patients had received the radiotherapy, the IgG1 isotype increased significantly and exceeded the IgM values. The IgM anti-GnRH immune-response however, kept a more regular distribution among all the patients that finished the trial. Besides, the IgG2, IgG4, and IgE in the serum samples represented <10% of the total immunoglobulins detected (**Figure 4B**).

Long Term Clinical, Biochemical, and Immunological Follow-Up of Patients During 10 Years

The primary endpoint of this phase I clinical trial of the vaccine candidate Heberprovac was to evaluate the acute and long term safety of the product which are described in 3.2 and **Table 2**.

Progression Free Survival Time (PFS) and Overall Survival (OS)

The secondary endpoint of this study was to test the capacity of Heberprovac to induce anti-GnRH antibodies, to reduce testosterone and PSA serum levels and, most importantly, to determine the patient overall survival. The **Figure 5** shows a correlation between anti-GnRH antibody titers, testosterone and PSA levels of the six patients receiving seven doses of Heberprovac and radiotherapy after a 10 year follow up. The highest anti-GnRH antibody titers in serum were reached immediately after the end of the vaccination schedule, ∼5 months after the beginning of the trial, with a mean value of 1:14,000.

Accordingly, testosterone values dropped to castration levels, and PSA normalization was observed in all patients at the time of final evaluation. The patient follow up showed that a year after the start of vaccination, the anti-GnRH antibody titers dropped to about half (average 1:6,000) of those seen by the end of the vaccination schedule. The anti-GnRH titers continued to decrease over time, but values remained above background for about 3 years (**Figure 5**). In accordance with the anti-GnRH seroconversion, during this period the testosterone concentration in serum remained at castration levels, and the PSA levels continued normal. Patients MC 03 and MC 05 showed testosterone and PSA relapsing, which was controlled with additional standard hormonal therapy. However, patient MC 03, responded only temporarily to the additional second line of hormonal ablation, and died 3 and a half years after finishing the treatment.

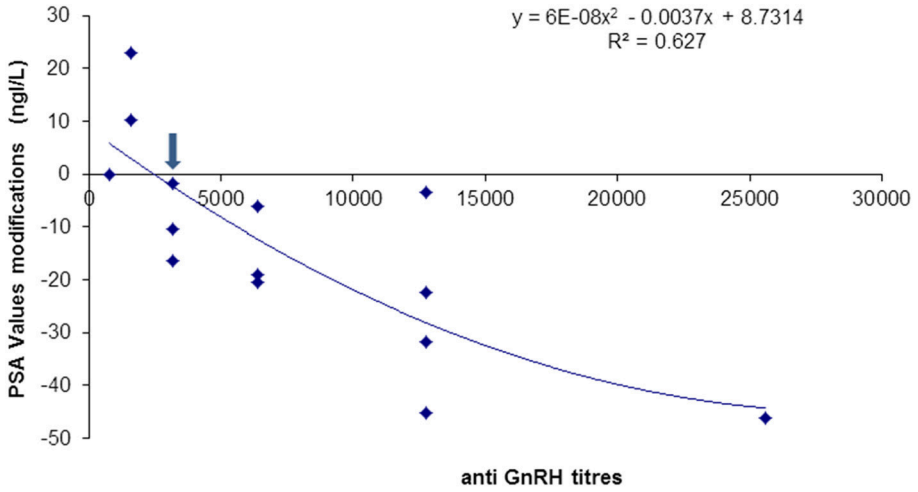

FIGURE 3 | Anti-GnRH antibody titers and PSA values modifications in patients immunized with Heberprovac. Anti-GnRH antibody titers of 1:3,000 or higher (arrow), correlated with a decrease in the PSA values in all patients. A statistical correlation using a quadratic regression was significant ($R^2 = 0.627$).

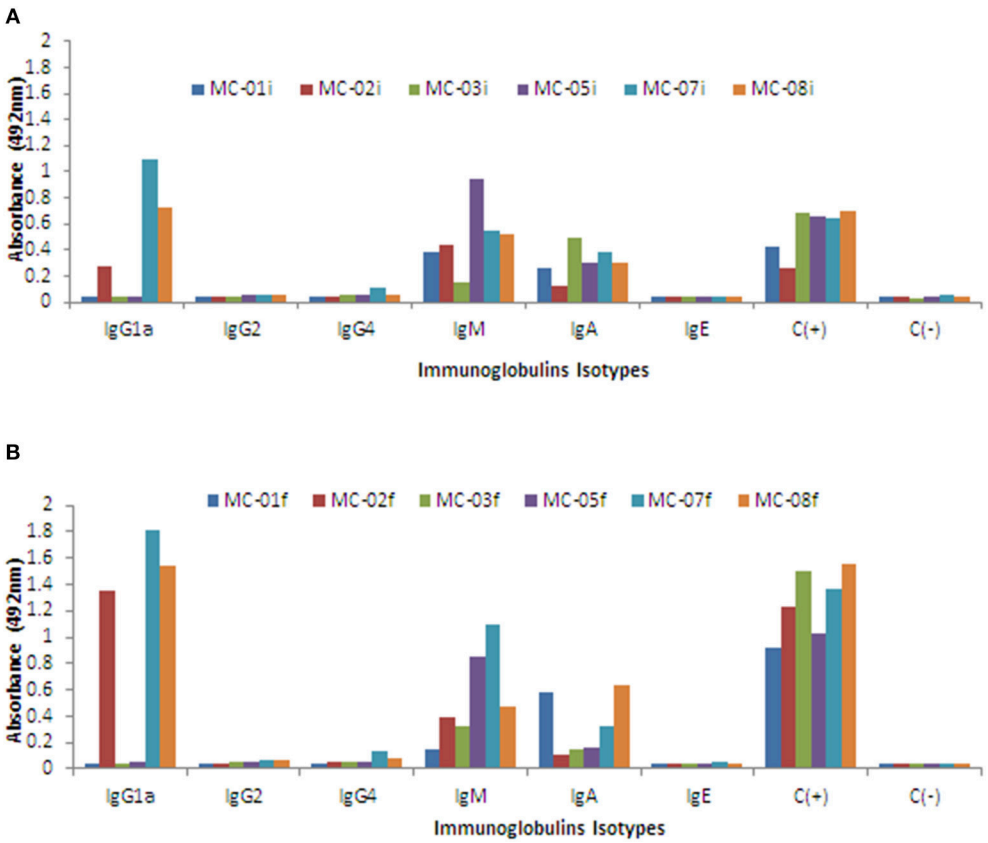

FIGURE 4 | Schematic representation of anti GnRH antibody levels by isotypes tested during the intermediate and final evaluation of prostate cancer patients immunized with Heberprovac. **(A)** Individual values of anti-GnRH seroconversion by isotypes after the administration of 4 doses of Heberprovac**.** The most significant anti-GnRH antibody seroconversion were of IgM, IgG1, and IgA isotypes. **(B)** Individual anti GnRH seroconversion by isotypes of prostate cancer patents that completed all seven immunizations and received RT. The higher anti-GnRH antibody titers were found for IgG1, IgM, and IgA isotypes, respectively. Statistical significance was calculated using an ANOVA test followed by the Dunn comparison test. The i and f that appear in the legend of **(A,B)** refer to the intermediate and final evaluations, respectively.

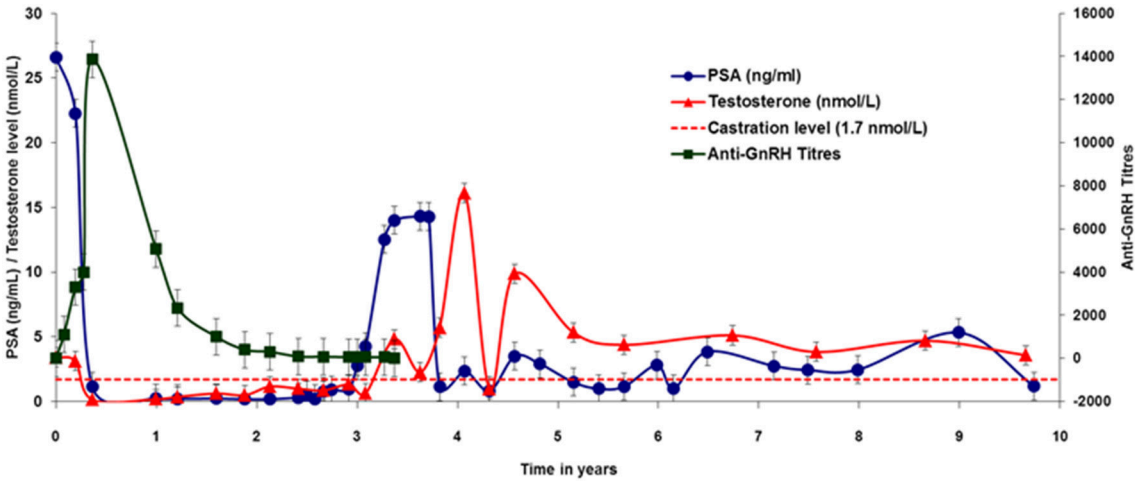

FIGURE 5 | Ten-year follow up of 6 patients that completed the trial schedule with Heberprovac and received RT. The colored lines and each point represent the mean of the anti GnRH antibody titres (black), Testosterone values (nmol/L), (red), and PSA levels (ng/mL), (blue) at different moments of the trial. Maximal antibody titers corresponded with the Testosterone decrease to castration levels and PSA normalization between 5 and 6 months after the beginning of the trial. Note that, after an initial peak, the antibody titers dropped to about 50% 1 year after the treatment was completed, and were nearly cero at the end of the second year. However, testosterone continued at castration levels and PSA stayed normalized until the third year after treatment. Peaks of testosterone and PSA were observed between years 3 and 5 and corresponded to patients relapsed. Prism Graph Pad v6.1 was used for graph.

Also, from 3.5 to 5 years post-immunization, an increase in the testosterone levels was observed in patients MC 01, MC 07, and MC 08 (**Figure 5**). But it just raised the PSA values in patient MC 05, who responded very fast to the use of GnRH analog Zoladex

The overall survival data of this study are summarized in the **Figure 6**. For the immunized group, patient MC 07, who maintained prostatic disease clinically and biochemically controlled, developed a primary lung cancer and died several months later. By the ninth year after the treatment, patient MC 08, who had never manifested PSA relapse or required any additional treatment for prostate cancer, died of pneumonia at age 82. Ten years after the end of the treatment with Heberprovac, 3 out of 6 patients that completed the treatment schedule are alive and have a clinically and biochemically controlled disease (**Diagram 1**). However, in the case of the control group that received standard anti-hormonal treatment, only 1 out 8 patients (12.5%) survive and keep hormone sensitiveness (**Figure 6**). The first patient of control group died after the third year (EG 09) as result of bone metastases and anemia. Patients EG 05 and EG 12 fell in a state of castration resistance and died at 5 and 7 years after the disease diagnosed, respectively. Patients EG 03 and EG 06 died from non-related prostate disease after 8 and 9 years of the treatment began, respectively. Finally, patient EG 11 suffered brain metastases and patient EG 14 was affected by bone metastases and kidney infiltration that generated renal insufficiency. Both patients succumbed 10 years after finished the treatment.

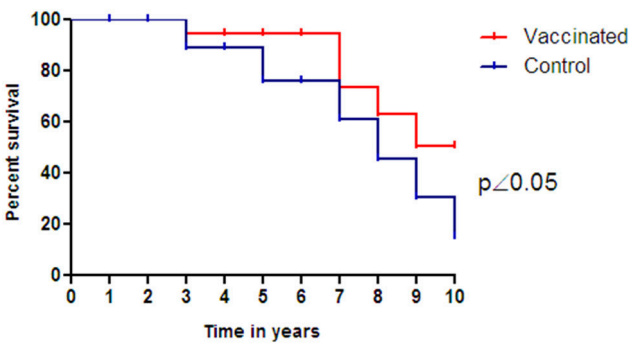

FIGURE 6 | Kaplan-Meier curve for survival of prostate cancer patients receiving GnRH vaccination (Heberprovac) ($n = 6$) and patients that received standard anti-hormonal treatment during the same time period ($n = 8$). On completion of the treatment they were followed up for 10 years, after which 3 out 6 patients completing Heberprovac vaccination and 1 out 8 receiving the anti-hormonal standard treatment are alive. The statistical analysis was carried out using Log Rank test and demonstrated survival benefits for the vaccinated arm ($p < 0.05$).

DISCUSSION

The combined use of adjuvant hormone and radiation therapies to treat high-risk prostate cancer patients has improved significantly results, with about 80% of patients disease-free (and no PSA failure) for 5 years (21).

The Gonadotropin-releasing hormone (GnRH) is critical for the normal functioning of the reproductive system. The administration of either polyvalent or monoclonal anti-GnRH antibodies in males, leads to decreased testicular size, cessation of spermatogenesis, and a severe reduction of testosterone levels, as does immunization with the GnRH-carrier conjugates (17, 22).

A number of studies have shown that the GnRH vaccines have promising application for managing hormone-dependent cancers (prostate and breast cancer) (23–25). However, the

clinical application of these synthetic vaccines requires the availability of a powerful adjuvant to enhance antibody responses that effectively block hormone-receptor binding, for instance using GnRH analogs conjugated to bacterial toxoids, such as diphtheria (DT) or tetanic toxoid (TT) (26).

This paper describes a novel GnRH vaccine candidate (Heberprovac), which overcomes the limitations reported for other vaccine candidates in terms of anti-GnRH antibody responses and their efficacy. The fact that 100% of patients developed significant anti-GnRH antibody titers, and in turn all of them normalized or decreased PSA below 4 ng/mL during the final evaluation, represents an important achievement in relation to all the previous vaccine candidates based on GnRH (18, 27). Indeed, this is the first time that such efficient antibody responses have been reported using a GnRH-based vaccine.

The improved results provided by Heberprovac, could be partially considered as a consequence of amino acid change of L-Glycin by L-proline at the sixth position of the native GnRH that breaks the natural "U" conformation of the GnRH peptide. This change, along with the incorporation of the 830–844 TT epitope, leads to the formation of a longer and more rigid molecule that impairs hormone-receptor interaction and supports a better antigen processing and presentation thanks to its high promiscuity to existing haptenic molecules of different origins (12, 19, 28).

In addition, Heberprovac combines the GnRHm1-TT peptide formulated with the adjuvant Montanide ISA 51 (oil adjuvant) and VSSP, that belongs to the new generation of adjuvants based on pathogen-related molecules identified as danger signals recognized by the innate immune system (29). VSSP is proved to have the ability to activate mouse and human dendritic cells, *in vitro* and *in vivo*, with the corresponding IL-12p40/p70, TNF-α, and IL-6 production (30, 31).

Since Heberprovac effectiveness will depend mainly on the anti-hormonal effects caused by anti-GnRH antibodies capable of inducing immunocastration, the antibody titers, isotype maturity, and antibody affinity should correlate with such vaccine effects.

As expected, most anti-GnRH antibodies elicited after the first immunizations were of IgM isotype. At the end of immunization schedule, the antibodies switched to IgG1 and IgG2 subtype patterns in most patients. Several reports have shown that adjuvation of peptide vaccines with Montanide ISA 51 VG induces powerful antibody responses with a mixed Th1/Th2 profile, thanks to their capacity to expand lymphocyte subpopulations, particularly IFNγ that produces CD4 and CD8 T cells [production (30, 31)].

Regardless of the anti-GnRH antibody isotype proportion that prevailed in each patient, the testosterone values dropped significantly in all the cases at the end of the immunization schedule and radiotherapy. Interestingly, when the anti-GnRH antibody production reached titers $\geq$1:3,000, the PSA levels dropped to normal values in all the patients. This correlation could represent a prognostic indicator of patient responses to immunization with Heberprovac. However, further studies including a larger number of patients are required.

The high anti-GnRH immune response and the drastic reduction of testosterone levels in patients with advanced prostate cancer induced by Heberprovac in the current study, has not been reported before for similar candidates in clinical trials (11–13, 18, 28). Nevertheless, the most striking result of this study is, undoubtedly, the higher rate of survival after a 10-year follow up (see below). Remarkably, the immunological and endocrine parameters correlated with normalization of PSA serum levels in 100% of patients, elimination of urinary obstruction symptoms, and normalization of prostatic signs, according to the data obtained with the DRE and transrectal ultrasonography of the 6 patients who completed the clinical study. Interestingly, a year after the end of the trial, the breast tenderness observed during the first months disappeared, seemingly in relation to the discrete increase in the testosterone levels. A decrease in sexual libido was maintained while testosterone in serum remained at castration levels, and it was more evident in older patients (MC 02, MC 05, and MC 07). However, two of these patients had prior episodes of sexual erectile dysfunction. The remaining patients, including the MC 03 patient, who died of metastatic lesions 3 and a half years following treatment completion, showed a partial recovery of their sexual libido when the testosterone levels exceeded 5 nmol/L (data not shown). It was remarkable that, throughout the study, none of the patients suffered gynecomastia or hot flushes. However, the control group that received the standard antihormonal treatment, although it did not manifest any of the symptoms associated with the inflammatory response generated by the vaccines, showed a profuse symptomatology of testosterone suppression as the decrease in sexual libido, hot flushes, erectile sexual dysfunction and muscle weakness in the 60–100% of the patients, indistinctly. The occurrence of these adverse events, observed in the control group and commonly reported during hormonal therapy (32–35), were not observed with Heberprovac immunization. This is likely due to the gradual testosterone decrease induced by the vaccine in contrast to the rapid castration induced by analogs and antagonists of GnRH (36–40).

The long-term evaluation of patients immunized with Heberprovac, demonstrated a 50% survival in a 10 years follow up. In contrast, the parallel control group of patients receiving standard therapy for advanced prostate cancer demonstrated a significantly lower survival rate (12.5%) in the same period ($p < 0.05$) (**Figure 6**). We believe that the slow and progressive form of hormonal ablation produced by Heberprovac vaccination could be a determining factor in a longer delay in the transition from prostatic tumors to castration resistance (CRPC) and hence in the superior survival of Heberprovac vaccinated patients. Other aspects such as the value that the use of adjuvants such as VSSP could have in the generation of an immune spreading against the prostate tumor should also be explored.

Concerning long-term disease control in the vaccinated patients, only one patient (MC 03) died before 5 years of treatment. This case was a patient with metastatic prostate cancer at recruitment, and persistent symptoms of bone pain who, nevertheless, showed a vigorous immune response after vaccination that corresponded with a decrease in testosterone to castration levels, PSA normalization, and prostate size reduction,

as shown by DRE and trans-rectal ultrasound. Besides this case, only one patient (MC 05) experienced a biochemical recurrence in the fourth year of the clinical trial and required hormonal treatment. Patients MC 01 and MC 08 showed a testosterone recovery of 10 and 15 nmol/L, respectively, however, they maintained normal levels of PSA, and did not require any additional treatment until 6.5 and 7 years.

Patients MC 07 and MC 02, both over 80 years old, died seven and 9 years after the start of the clinical trial, respectively, by causes unrelated to prostate cancer and its treatment. In both cases the patients exhibited complete disease control at the time of death, and never required additional hormone manipulation or another type of therapeutic strategy.

Altogether, these results are suggestive of a positive impact of vaccination with Heberprovac in overall patient survival compared with those receiving the standard treatment. Response to the vaccine correlated with the antibody titers raised against GnRH as well as with PSA reduction and castration levels of serum testosterone. Nevertheless, the value of such parameters as biomarkers of response need to be further confirmed in a future clinical trial with a larger cohort of prostate cancer patients.

AUTHOR CONTRIBUTIONS

JJ, RR, FF, IB, MDC, LC, CV, EB, EP, RBa, RBr, GG, AHG, AnC, AdC, ARa, AC-A, LH, and FS: conception and design of the study and writing and revision of the manuscript. LG, LP-F, ARo, AHG, OR, ML, MMed, LdQ, AA, CM, MMen, MMa, GM, AG, PR, RM, YF, MC, HT, DB, KC, PS, MQ, VM, MA, NA, CC, SA, IV, LA, ErR, ElR, PCe, PCa, MCS, IF, and LF: clinical trial assessment.

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Durable Clinical Responses and Long-Term Follow-Up of Stage III–IV Non-Small-Cell Lung Cancer (NSCLC) Patients Treated With IDO Peptide Vaccine in a Phase I Study—A Brief Research Report

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Background: Long-term follow-up on a clinical trial of 15 stage III-IV NSCLC patients treated with an Indoleamine 2,3-Dioxygenase (IDO) peptide vaccine (NCT01219348).

Methods: Fifteen HLA-A2-positive patients with stable stage III-IV NSCLC after standard chemotherapy were treated with subcutaneous vaccinations (100 μg IDO5 peptide, sequence ALLEIASCL, formulated in 900 μl Montanide) biweekly for 2.5 months and thereafter monthly until progression or up to 5 years. Here we report long-term clinical follow-up, toxicity and immunity.

Results: Three of 15 patients are still alive corresponding to a 6-year overall survival of 20 %. Two patients continued monthly vaccinations for 5 years (56 vaccines). One of the two patients developed a partial response (PR) of target lesions in the liver 15 months after the first vaccine and has remained in PR ever since. The other patient had a solitary distant metastasis in a lymph node in retroperitoneum at baseline which normalized during treatment. All following evaluation scans during the treatment have been tumor free. The vaccine was well tolerated for all 5 years with no long-term toxicities registered. The third long-term surviving patient discontinued vaccinations after 11 months due to disease progression. Flow cytometry analyses of PBMCs from the two long-term responders demonstrated stable CD8+ and CD4+ T-cell populations during treatment. In addition, presence of IDO-specific T-cells was detected by IFN-γ Elispot in both patients at several time points during treatment.

Conclusion: IDO peptide vaccination was well tolerated for administration up to 5years. Two of 15 patients are long-term responders with ongoing clinical response 6 years after 1st vaccination.

Keywords: cancer, immunotherapy, NSCLC, IDO, peptide vaccine

INTRODUCTION

Lung cancer is the leading cause of cancer death in both men and women worldwide, with non-small-cell lung cancer (NSCLC) accounting for 85–90% (1). At the time of diagnosis most patients have stage III–IV inoperable disease with a poor prognosis and a 5-year overall survival of <5%.

Previously, first-line standard treatment for the majority of patients with metastatic NSCLC, when no targetable alteration is revealed, was platinum-based chemotherapy, but only 15–30% of the patients responded (2).

Cancer immunotherapy, a treatment that boosts the body's natural defense to fight cancer has greatly evolved the last decade, and is now the standard of choice in many solid tumors. Nivolumab and Pembrolizumab, both PD-1 blocking antibodies and Atezolizumab a PD-L1 blocking antibody are approved by FDA and EMA for second line treatment for NSCLC and Pembrolizumab as first line treatment for patients with tumors expressing PD-L1 (3–5). All three antibodies work by relieving the suppression of the anti-tumor immunity, thereby boosting the immune system to kill cancer cells. Multiple immune regulatory targets are being investigated these days, among others indoleamine 2,3-dioxygenase (IDO).

IDO is an intracellular enzyme that catalyzes the rate-limiting step in degradation of Tryptophan (T) leading to local depletion and an increase in Kynurenine (K) metabolites (6). An upregulation of IDO in tumor cells leads to depletion of T which suppresses T-cell function and survival (7). Because T and K concentration can be measured from patients' serum, IDO activity can be monitored by computing K/T ratio (8). Consequently, cancer patients, including lung cancer, exhibit higher K/T ratios compared to healthy donors suggesting elevated IDO activity in cancer patients, thus proposing IDO as a valuable target in cancer.

IDO-specific T-cells have been shown to influence adaptive immune reactions in both cancer patients and healthy donors. Further, we have shown that these IDO-specific T-cells are cytotoxic effector cells capable of recognizing and killing both cancer cells and immunosuppressive dendritic cells *in vitro*. These findings justified clinical testing of an IDO derived peptide vaccine with the aim of boosting the IDO specific cytotoxic T-cells (9). A phase I vaccination study was performed at our institution from 2010 to 2012 including 15 HLA-A2+ stage III/IV NSCLC patients, demonstrating significant improved overall survival when compared with the group of excluded patients because of HLA-A2 negativity (10). Here, we present the long-term clinical and immunological outcomes of the treatment.

MATERIALS AND METHODS

Patients

Fifteen HLA-A2 positive patients with biopsy verified stage III–IV NSCLC in stable disease after standard chemotherapy were treated with subcutaneous vaccinations (100 μg IDO5 peptide, sequence ALLEIASCL, formulated in 900 μl of the adjuvant Montanide) (11). This study was carried out in accordance with the recommendations of GCP with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the National Board of Health and the local Ethics Committee at the Capital Region of Denmark. The initial study (NCT01219348) results have previously been reported (10). Patients were enrolled from June 2010 to May 2012 and treated every second week for 2.5 months and thereafter monthly

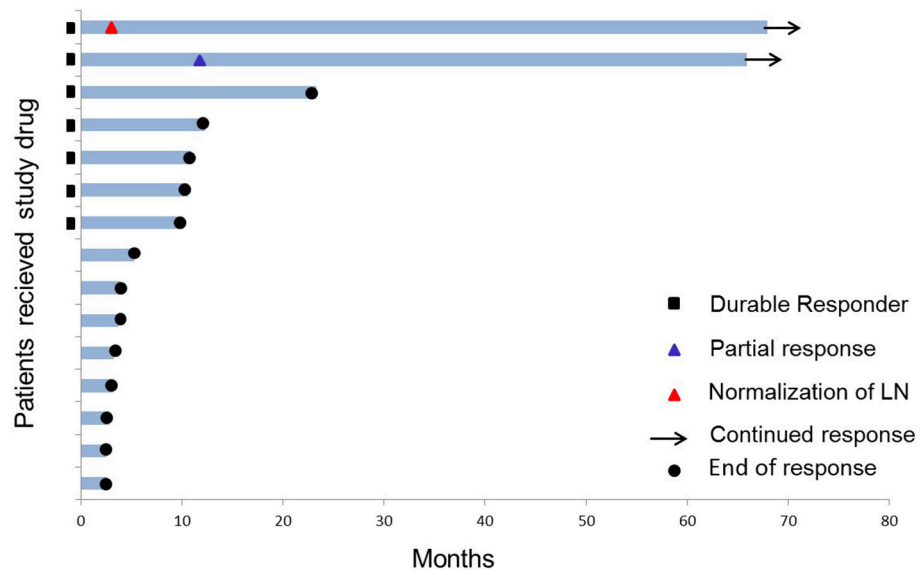

FIGURE 1 | Swimmer plot of the 15 stage III–IV HLA-A2+ NSCLC patients who received study drug (IDO peptide vaccine). Two of 15 patients are long-term responders (as of 6 years after the 1st vaccine). Durable response is defined as >8.5 months clinical treatment benefit.

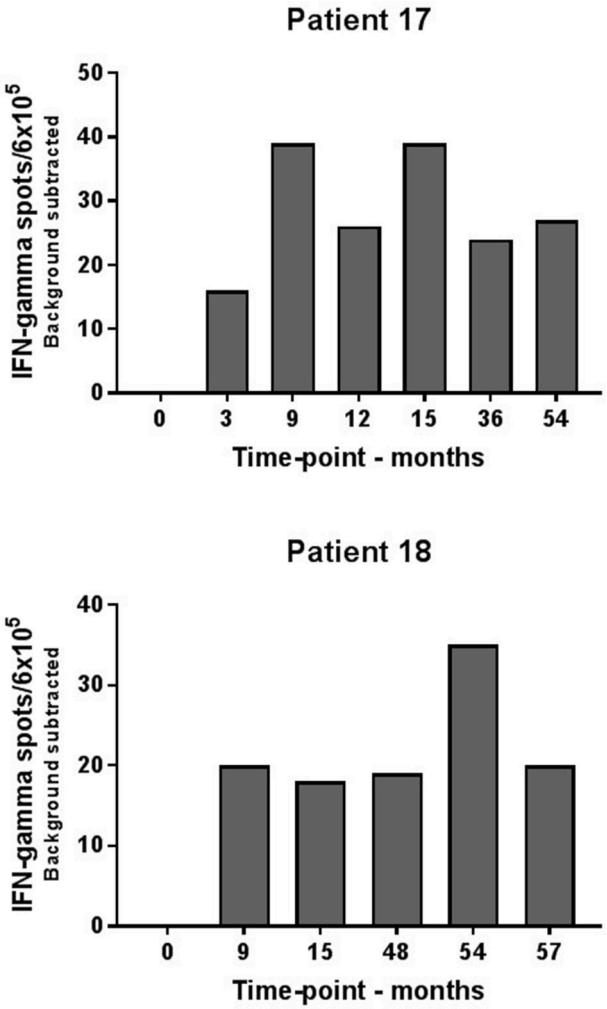

FIGURE 2 | Elispot analysis of IDO-specific T-cells in consecutive blood samples from long-term responders. IDO specific T-cells were demonstrated in patient #17 and #18 at several time points during IDO vaccination course.

until progression or up to 5 years. Two of the 15 patients have completed 5 years of vaccination, enabling evaluation of potential long-term toxicity according to CTCAE version 4.0. Furthermore, long-term clinical benefit was evaluated by CT or PET-CT scans according to Response Evaluation Criteria in Solid Tumors 1.1 (RECIST 1.1) at baseline and every third month for a completion of 5 years follow-up.

Patient Material

Peripheral blood mononuclear cells (PBMC) were obtained from peripheral blood by Lymphoprep technique by gradient centrifugation every third month during vaccination from the two long-term responders. Isolated cells were frozen immediately with 90% humanized AB-serum and 10% dimethyl sulfoxide and stored at −180°C.

Elispot

To assess whether IDO vaccination resulted in measurable T-cell responses in the two long-term patients, we performed indirect IFN- ELISPOT as previously described. Briefly, PBMCs were stimulated once in *ex vivo* medium +5% HS, 120 U/L interleukin-2 and 15 umol/L IDO5 peptide prior to analysis to extend the sensitivity of the assay. After 7 days in culture, cells were counted and analyzed in IFN-y ELISPOT. Nitrocellular bottomed 96-well plates (MultiScreen MAIP N45; Millipore) were coated with IFN-y capture mAb (Mabtech) overnight. Wells were washed, blocked by *X-vivo* medium and the effector cells were added in duplicates at different concentrations with or without 5 umol/L of the IDO5 peptide. Plates were incubated overnight and medium was discharged and wells washed prior to addition of biotinylated secondary Ab (Mabtech). Plates were incubated at room temperature (RT) for 2 h, washed and avidin-enzyme conjugate was added to each well. Plates were incubated at RT for 1 h and the enzyme substrate NBT/BCIP (Invitrogen Life Technologies) was added to each well and incubated at RT for 5–10 min. Upon the emergence of dark purple spots, the reaction was terminated by washing with tap water. The spots were counted using the ImmunoSpot Series 2.0 Analyzer (CTL Analyzers).

Flow Cytometry

PBMC samples were thawed in 37°C RPMI medium 1640 + GlutaMAX (Life Technologies) and thereafter washed in RPMI and stained in PBS containing 0.5% bovine serum albumin. For phenotyping of CD3+ T-cells, the following antibodies were used: CD45RA-FITC, CD62L-PE, CCR7-PE-CY7, CD3-APC, CD8-BV421, CD4-HV510 (BD Biosciences), CD27-PerCP (Nordic Biosite). Natural Killer cells, B-cells, and γ/δ cells were stained with the following antibodies: CD16-FITC, CD56-PE, CD19-PE-CY7, CD3-APC (BD Biosciences), and γ/δ -BV421 (Nordic Biosite). Myeloid derived suppressor cells were stained with: CD33-FITC, HLA-DR-PerCP, lineage = CD3-, CD19-, and CD56-PE-Cy7, CD11b-APC (BD Biosciences), CD14-BV421 (Nordic Biosite). Regulatory T-cells were stained with CD45RA-FITC, CCR4-PerCP-Cy5.5, CD127-PE-Cy7, CD4-APC, CD25-BV421 (BD Biosciences), FoxP3-PE (eBiosciences). Dead cell marker APC-Cy7 near IR (Invitrogen) fluorescent reactive dye was used to exclude dead cells. For intracellular staining of transcription factor FoxP3, we used Transcription Factor Staining Buffer set (eBioscience) according to guidelines issued by the manufacturer.

RESULTS

Long-Term Clinical Follow-Up

Three of the 15 patients are still alive (as of May 2018) corresponding to a 6-year overall survival of 20% (**Figure 1**). One patient was excluded from the trial due to progression after 11 months; the two other patients continued to be on monthly vaccination for 5 years with no other anti-cancer therapy given. They each received a total of 56 vaccines. Both patients had IDO expressing tumors (30–50%) by immunohistochemistry (10).

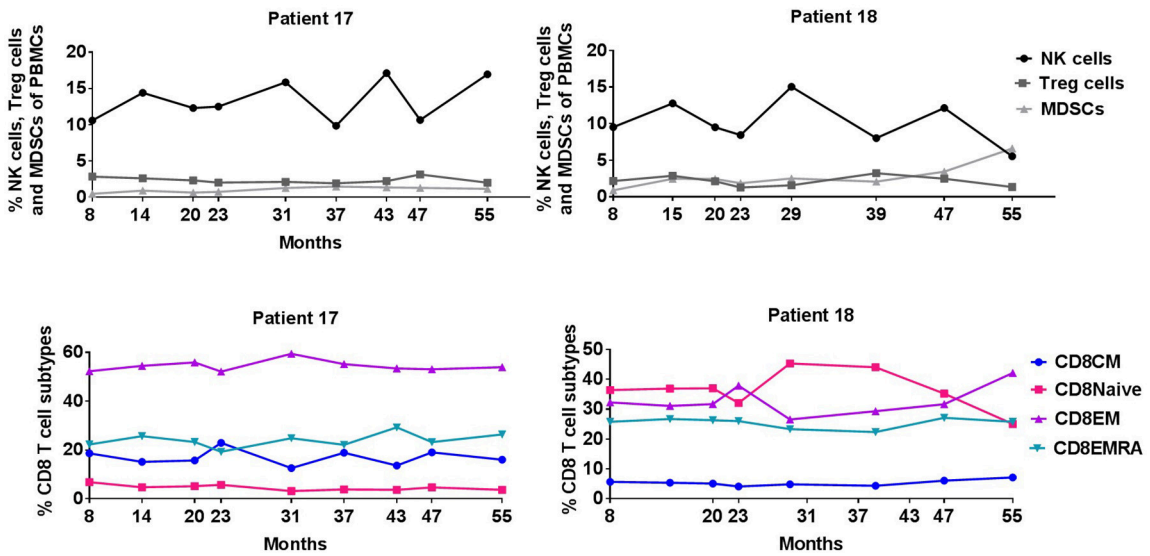

FIGURE 3 | Percentage of NK cells, Treg cells, MDSCs, and CD8+ T cell subpopulations during IDO vaccination course.

One of the two long-term responders (#18) was diagnosed with stage IV adenocarcinoma in 2009 (localized in lung and liver) and was initially treated with 1st line Carboplatin and Pemetrexed, 2nd line Erlotinib followed by 3rd line Docetaxel before inclusion in the trial in 2012. The patient achieved a partial response (PR) of target lesions in the liver 15 months after the first vaccine was administered and has been in ongoing stable PR for 6 years.

The other long-term responder (#17) was diagnosed with stage III adenocarcinoma in 2009; initially treated with an upper right lobectomy and subsequently 1. line Cisplatin and Vinorelbine. Further dissemination lead to a left adrenalectomy in 2010 due to a metastasis, followed 1 year later by 2. line Cisplatin and Pemetrexed for retroperitoneal lymph node recurrence before inclusion in the IDO vaccination trial in 2012. The patient had a solitary metastasis in a retroperitoneal gland (1.3 cm) at baseline which was normalized at 2nd evaluation during IDO vaccination. Absence of recurrent disease have been confirmed by CT ongoing for 6 years.

The third long-term survivor had stage IV disease and was treated with 4 lines of therapy before trial inclusion. The patient progressed after 11 months on IDO vaccination (14 vaccines administered) and was referred to standard of care where additional four lines of therapy have been given.

Long-Term Toxicity

The vaccine was well-tolerated in both long-term responders receiving the vaccine for 5 years and no CTCAE grade 3–4 adverse events were observed. Both patients are in good performance status (PS 0) and only experienced grade 1 or 2 local reactions at the injection site; i.e., redness, itching, and subcutaneous granuloma. All three local reactions are known AEs to the adjuvant Montanide.

Long-Term Immunity

Consecutive ELISPOT analyses for evaluation of peripheral blood immune reactivity to the IDO peptide were established for the two long-term responders during their 5 years of treatment. Immune-monitoring demonstrated detectable vaccination-induced IDO specific T-cell responses at several time-points during vaccination on the two patients as opposed to baseline samples (**Figure 2**).

Consecutive flow cytometry analyses of PBMCs during continuing vaccination (available from 8 to 56 months) were also performed on the two long-term responders (**Figure 3**). Peripheral blood percentages of CD8+ and CD4+ T-cells did not change significantly during vaccination as well as subpopulations of naïve, effector memory (EM), central memory and EMRA T-cells. Additional FACS analyses of natural killer (NK) cells, CD4+ regulatory T cells (Tregs), and myeloid derived suppressor cells (MDSCs) were also stable during vaccination for 5 years.

DISCUSSION

As published in 2013, vaccination in a phase I trial with an epitope derived from IDO in 15 patients with disease stabilization after standard chemotherapy demonstrated long-lasting PR+SD of at least 8.5 months in 47% of the patients (10). Historically, median PFS in patients with stage IV NSCLC treated with at least one line of chemotherapy is ∼6–7 months (12). This long-term follow-up 6 years after IDO vaccine initiation shows a 20% 6-year overall survival as compared to historical data with a 5-year OS <5%. The improved OS obviously needs confirmation in a larger randomized clinical study. Still, two of 15 patients have ongoing clinical response 6 years after vaccination initiation and have not received additional anti-neoplastic treatment following the vaccination period.

Importantly, the two patients with ongoing clinical response have received 56 vaccines in total over 5 years, with only local and manageable side effects and no grade 3–4 toxicity reassuring the vaccine to be safe for administration for a long period.

Many vaccine trials in NSCLC have shown a vaccination induced immune response; usually an increase of target specific cytotoxic T-cells as observed in our trial. Unfortunately, this has not translated into significant survival advantages in phase III trials to date testing antigenic target vaccines, whole cell vaccines and vector based vaccines. In terms of toxicity, all tested vaccines have shown less toxicity compared to immune checkpoint inhibitors and chemotherapies (13–17). The demonstration of enhanced immune response without concomitant survival benefit suggests that vaccine therapy might benefit from combination with other therapeutic modalities such as checkpoint inhibitors, chemotherapy or radiation therapy.

Although the immune checkpoint inhibitors have shown tremendous potential, response rates remain relatively low in lung cancer. Two PD-1 inhibitors (Nivolumab and Pembrolizumab) and one PD-L1 inhibitor (atezolizumab) have been approved by FDA and EMA for 2nd line treatment in NSCLC and Pembrolizumab for first line treatment in patients whose tumors have high expression of PD-L1 (>50%). Durvalumab, a PD-1 inhibitor, is approved by FDA for stage III NSCLC patients post chemoradiotherapy (18). Tumor-associated macrophages (TAM) and MDSCs play important roles in tumor immune evasion and their presence in the tumor limit the accumulation of T-cells. An understanding of IDO-reactive T-cells may lead to a treatment strategy improving effectiveness of checkpoint inhibition by activation of IDO specific T-cells reacting toward both tumor- and regulatory cells at the tumor site, thereby leading to local inflammation and diminished immune inhibition.

We hypothesize that vaccine induced activated IDO-reactive T-cells would attract T-cells into the tumor, resulting in inflammation, inducing PD-L1 upregulation on cancer cells as well as immune cells and thereby generating targets more susceptible to anti-PD-1/PD-L1 immunotherapy.

We therefore suggest that combination of a PD-1 blocking antibody and the IDO derived peptide vaccine potentially could increase clinical benefit in patients with NSCLC. To this end, a clinical phase I/II trial is running at our institution with the combination of an IDO and PD-L1 derived peptide vaccine in combination with Nivolumab for patients with metastatic melanoma. Pre-clinical toxicity data show no additional toxicity with the combination compared to Nivolumab alone (NCT03047928).

Epacadostat an IDO inhibitor plus Pembrolizumab have been tested in patients with NSCLC resulting in response rates up to 40–50% and with no additional toxicities in a phase I/II study (19). Currently a phase III trial (ECHO-305/NCT03322540) is running. However, Epacadostat and Pembrolizumab failed to improve progression free survival compared to Pembrolizumab alone in a phase III trial in patients with metastatic melanoma (ECHO-301/KEYNOTE-252 trial). Extensive biomarker analyses are being conducted to contribute to the understanding of the failure.

Presently, a randomized phase II clinical trial is being initiated in patients with NSCLC combining PD-1 blocking antibody and this IDO derived peptide vaccine (Keynote-764).

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

JK performed the experiments, interpreted data, and wrote the paper. IS and MA conceived the project, designed research, interpreted data, and wrote the paper. LE-N, TI, and AM interpreted data and edited the paper.

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REVIEW
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Personalized Dendritic Cell Vaccines—Recent Breakthroughs and Encouraging Clinical Results

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With the advent of combined immunotherapies, personalized dendritic cell (DC)-based vaccination could integrate the current standard of care for the treatment of a large variety of tumors. Due to their proficiency at antigen presentation, DC are key coordinators of the innate and adaptive immune system, and have critical roles in the induction of antitumor immunity. However, despite proven immunogenicity and favorable safety profiles, DC-based immunotherapies have not succeeded at inducing significant objective clinical responses. Emerging data suggest that the combination of DC-based vaccination with other cancer therapies may fully unleash the potential of DC-based cancer vaccines and improve patient survival. In this review, we discuss the recent efforts to develop innovative personalized DC-based vaccines and their use in combined therapies, with a particular focus on ovarian cancer and the promising results of mutanome-based personalized immunotherapies.

Keywords: dendritic cells, vaccines, cancer, immunotherapy, neo-antigens

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INTRODUCTION

Dendritic cells (DC) are the most potent professional antigen-presenting cells (APC) and play critical roles in regulating the innate and adaptive immune responses (1). In their immature state, DC patrol the tissue microenvironment and become activated in the presence of foreign pathogens. This activation occurs following stimulation by exogenous danger signals via pattern recognition receptors (PRR) such as Toll-like receptors (TLR) (2, 3) and leads to DC migration to the draining lymph node and the presentation of the processed epitopes to T cells (4). During the T cell activation, DC engage the T-cell receptor (TCR), secrete specific cytokines and stimulate the immune responses toward TH1, TH2, or Tregs depending on the cytokine environment. Due to their proficiency at antigen cross-presentation (i.e., the presentation to both $CD4^+$ and $CD8^+$ T cells), DC have been used as vaccine platforms to induce anti-tumor cytotoxic T lymphocyte (CTL) CD8 immune responses (5–8).

Various types of DC-based vaccines have been evaluated in clinical trials. The most commonly used preparation involves the reinfusion of *ex-vivo* derived DC pulsed with tumor-associated antigens (TAAs) or tumor cell lysates and stimulated with a defined maturation cocktail. In the earlier trials, the gold standard maturation cocktail included the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in combination with prostaglandin E2 (PGE2) (8–10). However, despite the important roles of PGE2 in promoting DC migration (11) and in enhancing T cell proliferation (12), it has also been shown that PGE2 may induce differentiation of regulatory T cells (13),

increase the expression of the pro-tolerogenic enzyme indoleamine 2,3-dioxygenase (IDO) (14), and may limit IL-12p70 production (15). As these PGE2-related activity may curtail the anti-tumoral immune response, alternative methods of *ex vivo* maturation of DC have been explored such as the triggering of co-stimulatory pathways (e.g., CD40-CD40L) (16) and the activation of the TLR using agonists such as poly IC (TLR3) (17), resiquimod (TLR7/8) (8) and 3-O-deacylated monophosphoryl lipid A (MPLA) (18), a modified TLR4 agonist with less toxicity than LPS. Moreover, DC subsets have been directly targeted *in vivo* by administration of TAAs directly to DC or by intra-tumoral administration of immunomodulatory molecules to activate local DC.

Although, DC-based vaccinations looked promising after Sipuleucel-T (Provenge®) approval in 2010, a DC-based immunotherapy for the treatment of advanced prostate cancer (19), unfortunately, the vaccination against established malignancies has generally shown limited clinical benefit. There are a number of potential factors that can impact the efficiency of DC-based vaccines. For instance, there is a reduction TAAs expression by tumor cells leading to immunosuppression and the immune evasion of cancer cells. Tumor cell elimination may also be blunted by the immune suppressive barriers overexpression, such as checkpoint receptor signaling (CTLA-4, PD-1/PD-L1) and immunomodulatory cellular subsets [Tregs and myeloid-derived suppressor cells (MDSCs)] (20, 21). Moreover, there are evidences of defects in both the number and functions of DC subsets, which facilitate tumor progression and immune evasion (22–29). Overall, the transition of DC from an *in vitro* cell culture to an *in vivo* immunosuppressive environment may alter the effectiveness of DC-based immunotherapy.

Therefore, ongoing trials using DC-based vaccines are evaluating the use of combined immunotherapies to favor DC activation and promote T cell functions, and overcome tumor immune evasion. The Indian government agency (CDSCO-Central Drugs Standard Control Organization) recently approved in 2017 an autologous monocyte-derived and tumor lysate-pulsed mature DC-based vaccine (APCEDEN®) for treatment of four cancer indications (prostate, ovarian, colo-rectal and non-small cell lung carcinoma) (30). The multicentric phase II clinical trial by Bapsy et al. (31) demonstrated that this formulation was safe and well-tolerated in patients with refractory solid tumors. Moreover, the efficacy profile of APCEDEN® therapy demonstrated a survival benefit of >100 days (30).

HUMAN BLOOD DENDRITIC CELLS

DC originate from the common myeloid bone marrow progenitor cells and can be found in both, lymphoid and non-lymphoid tissues in an immature state (1). DC are heterogeneous and consist of multiple specialized subtypes, which are defined based on their phenotypic and functional characteristics, including morphology and immunological features (expression of surface markers, cytokines, chemokines, and transcription factors). The homology of human DC and mouse DC populations have been extensively studied using transcriptional profiling (32–36). In humans, all DC express high levels of MHC class II molecules (HLA-DR), and lack lineage-specific surface markers for T cells (CD3), B cells (CD19/20), and natural killer cells (CD56). The DC subtypes found in the blood are myeloid DC (mDC) (also termed $CD11c^+$ conventional DC, cDC), which can be further divided into $CD141^+$ mDC, $CD1c^+$ mDC, and $CD123^+$ plasmacytoid DC (pDC) (37). The $CD1c^+$ mDC account for the majority of the mDC population in the human blood representing approximately 1% of all mononuclear cells, with the $CD141^+$ mDC representing only 0.1%. Compared with $CD141^+$ mDC, the $CD1c^+$ mDC have an inferior capacity to cross-present antigen to $CD8^+$ T cells (35, 38). Human $CD141^+$ DC are homologous to the mouse cross-presenting $CD8\alpha^+/CD103^+$ DC, and are characterized by the exclusive expression of XCR1 and Clec9A (33, 39–43). The pDC are specialized producers of type I interferons in response to viruses (44) and can, on one end, induce Tregs expansion and tolerance (45, 46), while effectively cross-present antigens to CTL (47–49). Using mass cytometry (i.e., CyTOF), Guilliams et al. identified that the combination of the two markers (CADM1 and CD172a) could be used as flow cytometry markers to identify the conventional subsets of mDC across tissues and species (human, macaque and mouse) (50). Thus, $CD141^+$ DC can be defined as $CADM1^{hi}CD172a^{lo}$, while the $CD1c^+$ mDC correspond to $CADM1^{lo}CD172a^{hi}$ cells. Notably, the conventional identification of mDC or pDC (37) has lately been challenged by a study, which, using single-cell transcriptome profiling, demonstrated that human blood DC could be further stratified into six distinct populations (51). This increasing knowledge about DC subsets will certainly be exploited for the design of novel strategies to improve the clinical efficacy of cancer vaccines.

The isolation of DC subset is another for the generation of DC-based vaccine has also improved over the years. Initially, DC subsets were isolated directly *ex vivo* from the peripheral blood to produce DC-based vaccines for immunization of B cell-lymphoma patients against their TAAs (52). As DC have a low frequency in peripheral blood, low numbers of DC were isolated using this method. Nowadays, most clinical studies employ monocyte-derived DC (MoDC) in the generation of DC-based vaccine because of the relative ease at obtaining sufficient number of cells from peripheral blood and their functionality (53, 54). MoDC are a subset of DC exhibiting common features with cDC (55), including the ability to migrate, to potently stimulate $CD4^+$ and $CD8^+$ T cells, to produce key cytokines (IL-1, IL-6, TNF-α, IL-12, and IL-23) (56), and to express cell surface markers such as CD11c and MHC II (55). Autologous MoDC can be obtained by culturing human peripheral blood monocytes ($CD14^+$) in the presence of GM-CSF and IL-4 (57) with the resulting vaccines eliciting tumor-specific T cell responses and some clinical efficacy (56).

With recent technological advances in isolation of specific immune cell populations, second generation DC vaccine have

Abbreviations: DC, Dendritic cell; APCs, Antigen-presenting cells; CTL, Cytotoxic T lymphocyte; TAAs, tumor-associated antigens; MoDC, Monocyte-derived DC; OS, Overall survival; TILS, tumor infiltrating lymphocytes.

focused on the collection of blood-derived primary DC subsets. As previously mentioned, naturally circulating DC have a low frequency in peripheral blood (<1% of leukocytes). Nonetheless, there exist significant transcriptional and functional differences between the blood-derived DC in comparison with the *in vitro* generated MoDC suggesting that blood-derived DC may be superior for therapeutic vaccination (32, 58). Early phase I results suggest that vaccination with peripheral blood-derived pDC or mDC is safe and well-tolerated amongst patients with advanced-stage melanoma (59), prostate carcinoma (60) or acute myeloid leukemia (61). One such trial is based on a novel type of blood-derived DC vaccine is being assessed within the collaborative European project entitled "Professional cross-priming for ovarian and prostate cancer" (PROCROP). For this trial, a $CD141^+$ subset of blood-derived mDC, which has superior capacities at cross-presenting TAAs to $CD8^+$ T cells (39, 42, 62), is being evaluated as a personalized DC vaccine.

Altogether, clinical trials have yet to prove that blood-derived DC vaccines are more efficacious than *in vitro* generated MoDC (63). For instance, the development of second generations of DC-based vaccines may also face multiple technical challenges such as the limited availability of cells that can be purified, the large amount of blood or leukapheresis to be collected, and the negative effects of chemotherapy that may reduce the number of DC in the peripheral blood (64).

DENDRITIC CELL DYSFUNCTION IN CANCER

Optimal DC function is necessary for the initiation of protective anti-tumor immunity. Yet, it is known that immunosuppressive factors expressed by the tumors cells, including IDO (65, 66), Arginase I (67), IL-10 (68, 69), TGF-β (23, 70), PGE2 (71, 72), and VEGF (73–77), can impair the differentiation, maturation, and function of the host DC (78–80), which may become tolerogenic and favor the stimulation of regulatory T cells (81, 82). For instance, high level of intratumoral pDC is associated with poor disease outcome across several tumor types (83, 84). The impairment of DC differentiation (80, 85), and the resulting inadequate antigen-presenting functionality of DC, contributes to T cell anergy or exhaustion is well documented in cancer. In a breast and pancreatic cancer study, tumor-derived granulocyte-stimulating factor induced alterations in the development of $CD141^+$ DC, which were associated with impaired $CD8^+$ T cell responses and correlated with poor clinical outcomes (86). An additional mechanism contributing to the impaired antigen processing ability of intra-tumoral DC is the accumulation of pathological amount of lipid by the DC due to up-regulated expression of scavenger receptor A (SR-A) (87). These lipid-laden DC have reduced capacity to stimulate allogeneic T cells (87).

It was previously demonstrated that DC derived from patients with advanced cancer are weak stimulators of T cells compared to healthy volunteers (88). In some tumors, as cancer progresses, tumor-infiltrating DC accumulate and switch from immunostimulatory to regulatory phenotypes (23), and correlates with the increased expression of negative costimulatory molecules such as TIM3 (89), PD-L1 and PD-1 (90) as well as the production of L-Arginase (91). In fact, this is a predominant mechanism of DC dysfunction in ovarian carcinoma, with $PD\text{-}1^+$ $PD\text{-}L1^+$ $CD277^+$ DC accumulating in the tumor over the course of the disease (90, 92). The increased expression of PD-1 was shown to affect the function of DC by inhibiting NF-κB activation, and was associated with decreased T cell activity and reduced tumor-infiltrating T cells in advanced cancer (93). CD277 was shown to be universally expressed in ovarian cancer-infiltrating DC and may affect the expansion of TCR-stimulated T cells.

Therefore, the immunosuppressive DC, controlled by the tumor microenvironment, plays an important role in supporting tumor progression, and probably limiting the success of DC-based vaccine in cancer patients. There is increased awareness on the influence of age-related changes on the development of tumors and on treatment prognosis. Aging has already a profound effect on DC function, affecting numbers and functions of pDC (94), and inducing substantial changes in gene expression profile of $CD1c^+$ DC as illustrated by significant down-regulation of antigen presenting and energy generating genes (95). Thus, to overcome systemic immune dysfunction and augment DC-induced responses *in vivo*, many investigators are combining DC-based vaccines with tumor-damaging agents or considering the use of DC-based vaccines to treat earlier in the course of the disease (96). Notably, combining CD40 agonists with TLR3 activation was shown to be sufficient to reverse the immunosuppressive phenotype of tumor-infiltrating DC into APCs capable of priming anti-tumor T cell responses (97).

ACTIVE INGREDIENTS OF DC-BASED CANCER VACCINES

Tumor Antigens

TAAs are a crucial component of DC vaccines as they represent the targets for CTL-generated anti-tumor immune response. Non-mutated self-antigens resulting from over-expression of tissue- or lineage-specific genes induced by transformation induce low T cell reactivity due to central tolerance mechanisms. Conversely, mutated neo-antigens are generated by somatic mutations due to the tumors' inherent genetic instability rendering them tumor-specific and private, with the advantage of being recognizable for T cells and not impacted by central tolerance.

Defined Antigens

The most widely used cancer vaccines tested so far were based on defined, shared TAAs (e.g., MART-1, gp100, CEA, PSA, p53, NY-ESO-1, MAGE-A3), which are HLA restricted (98–103). Both, individual and the combination of several defined antigens were tested, but only achieved limited clinical efficacy (104–106). A potential disadvantage of immunotherapy targeting one or few defined TAAs is the possibility of rapid development of tumor escape variants that lose the expression of these epitopes (107). Using multiple (defined or undefined) antigens as vaccine targets may be crucial for achieving significant clinical benefit and may overcome the challenge of tumor escape via antigen-loss.

Neo-Antigen-Targeted Approaches

The high mutational rate of tumor cells results in the expression of neo-antigens that are tumor specific. The identification of patient specific TAAs, including both shared tumor antigens and neo-antigens, is now possible using next-generation sequencing (NGS) and bioinformatics tools (e.g., NetMHC) (108) complemented or not by direct isolation of HLA-bound peptides (immunopeptidome) and mass spectrometry (MS) analysis (109). The personalized cancer vaccine can be manufactured based on neo-antigens that have been identified and used to manufacture peptides or RNA for the pulsing of DC. Nonetheless, two major challenges arise from this approach: the time between tumor resection and first vaccine injection, which can reach several months, and the cost of the neo-antigen identification process.

Three recent Phase I clinical trials confirmed promising potential of personalized cancer vaccines based on neo-antigens (110–112), with the study by Carreno et al. utilizing DC-based vaccine (110). Whole-exome sequencing was carried out to identify somatic mutations in tumors from three patients with melanoma and short peptides coding for seven neo-antigens were pulsed onto autologous DC. Despite the small sample size, the study proved that neo-antigen cancer vaccines could elicit neo-antigen specific T cell response with some patients showing stabilized or non-recurrent disease (110).

Whole Tumor Preparations

In indications where surgery can be performed as part of the treatment, the resected tumor tissue can be used as a source of patient-specific TAA by preparing a tumor cell lysate. Alfaro et al. used freeze-thaw lysis from biopsies to generate glioma-specific lysate (113). The treatment induced IL-12 production in each patient and circulating tumor cells markedly dropped in 6 of 19 cases with five patients experiencing disease stabilization (114). The immunogenicity of tumor cell lysate can be enhanced using alternative lysate preparation methods such as freeze-thaw, UV irradiation or oxidation treatment (115–120). Our group showed that tumor cells oxidation using hypochlorous acid (HOCl) combined with freeze-thaw cycles results in primary necrosis of tumor cells, and increases immunogenicity of the resulting tumor lysate (121). The main advantages of using autologous tumor lysate as a source of TAAs are the absence of HLA restriction and the reduced time and cost of manufacturing in comparison to the neo-antigen prediction strategies.

RECENT ACCOMPLISHMENTS IN PERSONALIZED DC-BASED IMMUNOTHERAPY

Current Treatment Strategies for Advanced Ovarian Cancer

A DC-based vaccine generated by differentiation of autologous Mo-DC pulsed with HOCl oxidized autologous tumor cell lysate (OC-DC vaccine) was tested in platinum-treated, immunotherapy-naïve, recurrent ovarian cancer patients in a single-center, multi-cohort, non-randomized phase I trial (122). During the study, a total of 392 vaccine doses were administered intra-nodally under ultrasound guidance without serious adverse events. The results of the first of three cohorts was reported by Tanyi et al. (122). In this study, the DC-based vaccine was administered either alone, in combination with bevacizumab or in combination with bevacizumab and low-dose intravenous cyclophosphamide until disease progression or vaccine exhaustion. This OC-DC vaccine induced T cell responses (increased in IFN-γ production) to autologous tumor antigens, which were detected in 11 of 22 evaluable patients on week 12. Moreover, this antitumor immune response was associated with significantly prolonged survival with increased neo-antigen specific T cells responses, both previously recognized and non-recognized neo-epitopes.

Overall from the 25 patients treated two (2) patients showed partial response and 13 patients experienced stable disease, which persisted for a median of 14 months from enrolment. Of note, vaccine responders experienced significantly longer progression-free survival (PFS) compared to non-responders patients. The 2-year overall survival (OS) rates of the responder patients was 100%, whereas the 2-year OS of non-responders was 25%. The best results were obtained with the triple combination of vaccine plus bevacizumab and cyclophosphamide. This study demonstrated that the use of OC-DC vaccine was safe and elicited a marked antitumor immunity, including tumor-specific neo-antigens. Altogether, personalized DC vaccines using whole tumor lysate can drive responses to private antigens and, in combination with other immunotherapy treatments, can greatly improve clinical outcome.

Promising Phase 3 Studies in Progress

An exhaustive list of DC-based studies is available in **Table 1**. Notably, a phase 3 trial is currently testing DC vaccine loaded with autologous tumor lysate (DCVax-L) in patients with newly diagnosed glioblastoma following surgery as add-on to the standard of care combining radiation and chemotherapy (NCT00045968; Northwest Therapeutics). Patients are receiving temozolomide plus DCVax-L ($n = 232$) or temozolomide and placebo ($n = 99$). DCVax-L is administered intra-dermally six (6) times the first year and twice per year thereafter. Following recurrence, all patients are allowed to receive DCVax-L. The first reported results showed that the median OS was 23.1 months from surgery as compared with the 15-17 months achieved with SOC only in past studies (123). Only 2.1% of patients had a grade 3 or 4 adverse event related to the vaccination treatment. Due to its safety profile, this DC vaccine has the potential to be administered in a wide range of indications and applied in a wide range of combinations.

Another phase 3 study is currently evaluating the efficacy adjuvant vaccination using RNA-loaded autologous DC vaccine to treat patients with uveal melanoma (NCT01983748). This study will compare standard of care treatment with vaccination (8 intravenous of vaccine over 2 years).

Finally, a phase 3 study is currently evaluating active immunization in adjuvant therapy of patients with stage 3 melanoma with natural ($BDCA3^+$) dendritic cells (nDC) pulsed with peptides (NCT02993315). Patients will receive nDC vaccine by three (3) intranodal injection per cycle for a maximum of three

TABLE 1 | Table of current ongoing clinical trials using personalized DC-based vaccines.

		NCT number	Indication	Interventions	Phase	Enrollment	Start date	Estimated primary completion date
Tumor lysate	1	NCT00703105	Ovarian cancer	Ontak (anti-CD25)|DC vaccine + ontak	Phase 2	36	2008	2018
	2	NCT01204684	Glioma|Astrocytoma|Astro-oligodendroglioma|Glioblastoma	Autologous tumor lysate-pulsed DC + 0.2% resiquimod|DC vaccination + polyICLC	Phase 2	60	2010	2018
	3	NCT01635283	Newly diagnosed or recurrent low-grade glioma	Tumor lysate-pulsed autologous DC vaccine	Phase 2	18	2012	2019
	4	NCT01946373	Malignant melanoma	Cyclophosphamide|Fludarabine|T cells|Interleukin-2|DC vaccine	Phase 1	10	2013	2018
	5	NCT01973322	Malignant melanoma stage III|Stage IV	Arm 1: autologous DC loaded with autologous tu lysate (DC vaccine) + RT|Arm 2: DC vaccine + IFN-α|Arm 3: both arm 1 and 2 + RT|Arm 4: DC vaccine	Phase 2	24	2013	2019
	6	NCT01957956	Newly diagnosed glioblastoma	Tumor lysate-pulsed autologous dendritic cell vaccine + temozolomide	Early phase 1	21	2013	2016
	7	NCT01808820	Malignant glioma|Glioblastoma	Dendritic cell vaccine|Tumor lysate|Imiquimod|Leukapheresis	Phase 1	20	2013	2019
	8	NCT02496520	Advanced solid tumors, sarcoma|Central nervous system tumor	Dendritic cells|Surgery as needed|Chemotherapy as needed|Radiation: radiation therapy as needed	Phase 1|2	10	2014	2018
	9	NCT01803152	Sarcoma|Soft tissue sarcoma|Bone sarcoma	Biological: dendritic cells vaccine|Lysate of tumor|Gemcitabine|Imiquimod|Leukapheresis	Phase 1	56	2014	2019
	10	NCT02718391	Malignant melanoma	DC pulsed with autologous tumor lysate	Phase 2	120	2015	2019
	11	NCT02301611	Malignant melanoma	Autologous Tumor Lysate (TL) + Yeast Cell Wall Particles (YCWP) + Dendritic Cells (DC) (TLPLDC Vaccine)|Placebo	Phase 2	120	2015	2019
	12	NCT02503150	Metastatic colorectal cancer	Antigen pulsed dendritic cells + chemotherapy|Chemotherapy	Phase 3	480	2015	2019
	13	NCT02678741	Metastatic melanoma	TLPLDC vaccine in addition to standard of care checkpoint inhibitor of choice	Phase 1|Phase 2	45	2016	2019
	14	NCT03395587	Newly diagnosed glioblastoma	Autologous DC pulsed with autologous tumor lysate	Phase 2	136	2018	2022
	15	NCT03360708	Recurrent glioblastoma	Cytokine-induced killer cells|Tumor lysate-pulsed autologous DC vaccine	Early phase 1	20	2018	2022
	16	NCT03014804	Recurrent glioblastoma	Autologous dendritic cells pulsed with tumor lysate|Nivolumab	Phase 2	30	2018	2020
RNA	17	NCT01983748	Uveal melanoma	Autologous DC loaded with autologous tumor RNA	Phase 3	200	2014	2022
Peptide	18	NCT02775292	Adult solid neoplasm|Childhood solid neoplasm|Metastatic neoplasm	Aldesleukin|Cyclophosphamide|Fludarabine phosphate|Nivolumab|NY-ESO-1 reactive TCR retroviral vector transduced autologous PBL|NY-ESO-1(157-165) peptide-pulsed autologous DC vaccine	Phase 1	12	2017	2019
Tumor neoantigen	19	NCT01885702	Colorectal cancer	Neoantigen-loaded DC vaccination	Phase 1|2	25	2010	2016
	20	NCT03300843	Melanoma|Gastrointestinal|Breast|Ovarian|Pancreatic cancer	DC vaccine loaded with neoantigen coding peptide	Phase 2	86	2018	2027

(3) cycles or placebo injections to determine if adjuvant nDC vaccination improves 2-year RFS rate.

PREDICTIVE MARKERS FOR THE CLINICAL EFFICACY OF DC-BASED VACCINES

Another path to the improvement of DC-based vaccine efficiency is based on the identification of surrogate biomarkers of the triggered immune response against the tumor that would strongly and uniformly correlate to vaccine efficacy. Studies have identified different potential biomarkers of clinical responses to DC-based vaccination. For instance, in melanoma, two (2) candidate genes were identified with a predictive value for a positive outcome to a DC-based immunotherapy (124). The chemokine receptor CXCR4 and the receptor for the FC portion of IgD (CD32) were over-expressed in the lymphocytes cell membranes and in the monocyte populations in immunological responder patients as compared to non-responder patients (124). Higher CXCR4 protein expression was found in $CD8^+$ T cells pre- and post- whereas higher CD32 protein expression in monocyte populations was identified in responder patients at pre-treatment time points (124). In a recent phase II study in patients with glioblastoma, DC vaccination induced a significant and persistent activation of $CD56^{dim}$ cytotoxic NK cells, whose increased response was strongly associated with prolonged survival, while $CD8^+$ T cells had only a poor contribution to anti-tumor responses (125). In NSCLC patients, the survival time was closely associated with the $BDCA1^+$ DC/$BDCA3^+$ DC ratio in peripheral blood after DC immunotherapy (126).

Tumor-infiltrating lymphocytes (TIL) are examined extensively in various cancer types, including epithelial ovarian cancer, with their presence found to be an important prognostic factor (127–134). Additionally, in ovarian cancer, infiltrating Tregs in the tumor microenvironment correlate with poor prognosis (135–137). In the context of DC-vaccination, in glioma, the TIL content was identified as a predictor of clinical response (138). An increased overlay in the TCR repertoire of TIL and circulating T cells correlated with improved responses to DC-based vaccination and overall survival (138). Hence, the TIL content may be used as a selection tool to identify patients who could potentially benefit from DC vaccination therapy.

In terms of monitoring anti-tumor vaccine trials, a study by Kirkwood et al. found that functional assessment of T cells such as interferon-γ production is preferable as opposed to frequency or phenotype of effector T-cells (139). In a multicenter study (ECOG E1696), where melanoma patients were treated with a peptide vaccine, there was a significant difference in OS by immune response status. Immune responders, patients whose T cells exhibited interferon-γ response (against to one or more of the three antigens measured by ELISPOT) lived longer than the nonimmune responders (median OS, 21.3 vs. 10.8 months; $P = 0.033$).

In conclusion, highly reliable molecular or cellular biomarkers of the clinical efficacy of personalized DC-based vaccines are still missing. Prospective longitudinal studies will help identify predictive prognostic and treatment-efficacy biomarkers using "Omics" data (140) and systems biology analysis. Therefore, there is an urgent need for clinical studies beyond phase II to demonstrate that DC-based vaccines can induce durable objective responses and improve long-term survival in cancer patients, and maybe identify strong correlate for all malignancies.

CONCLUSIONS

The development and success of DC-based immunotherapies has been hampered by several factors; (1) the immunosuppressive tumor microenvironment, particularly in advanced stage of the disease (2) the limited capacity of systemically administered DC to localize to the tumor-draining lymph nodes, (3) the low avidity of TAAs-specific T cells, and (4) the lack of reliable prognosis biomarkers. The rapidly increasing knowledge about DC subsets and the tumor-induced suppressive microenvironment must be exploited to design novel and improved cancer vaccines. The future of DC vaccines will certainly rely on combination therapies. As discussed in this review, recent studies have shown the great potential of such strategies, especially when using personalized DC vaccines. Overcoming the cancer immunosuppressive environment will reveal the real therapeutic potential of such DC vaccine.

AUTHOR CONTRIBUTIONS

BM-G and KB wrote the manuscript. All authors, BM-G, KB, CB, POG, and LEK contributed to manuscript revision, read and approved the submitted version.

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MINI REVIEW
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Novel Strategies for Peptide-Based Vaccines in Hematological Malignancies

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Peptides vaccination is an interesting approach to activate T-cells toward desired antigens in hematological malignancies. In addition to classical tumor associated antigens, such as cancer testis antigens, new potential targets for peptide vaccination comprise neo-antigens including JAK2 and CALR mutations, and antigens from immune regulatory proteins in the tumor microenvironment such as programmed death 1 ligands (PD-L1 and PD-L2). Immunosuppressive defenses of tumors are an important challenge to overcome and the T cell suppressive ligands PD-L1 and PD-L2 are often present in tumor microenvironments. Thus, PD-L1 and PD-L2 are interesting targets for peptide vaccines in diseases where the tumor microenvironment is known to play an essential role such as multiple myeloma and follicular lymphoma. In myelodysplastic syndromes the drug azacitidine re-exposes tumor associated antigens, why vaccination with related peptides would be an interesting addition. In myeloproliferative neoplasms the JAK2 and CALR mutations has proven to be immunogenic neo-antigens and thus possible targets for peptide vaccination. In this mini review we summarize the basis for these novel approaches, which has led to the initiation of clinical trials with various peptide vaccines in myelodysplastic syndromes, myeloproliferative neoplasms, multiple myeloma, and follicular lymphoma.

Keywords: peptide vaccination, follicular lymphoma, multiple myeloma, myeloproliferative neoplasms, myelodysplastic syndrome, PD-1, cancer testis antigen, neo-antigens

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INTRODUCTION

Cancer vaccine therapy is based on the principle of activating an immune response toward cancer cells. The concept dates back to the Nineteenth century when William Coley attempted to raise an immune response against cancer by exposing patients to bacterial extracts (1). In the view of modern research standards Coley's results are questionable, but since then the field has evolved immensely and modern therapeutic cancer vaccines induce potent anti-tumor immune responses. The field of therapeutic cancer vaccines involves a variety of methods including cellular vaccines, RNA/DNA based vaccines, viral vaccines, and peptide/protein vaccines described in detail by

Gou et al. (2) Peptide vaccines hold the advantage of short production times and easy administration and will be the focus of this review. This method is based on peptides from selected tumor proteins that are injected into patients along with an immune activating adjuvant. After injection, the peptides are processed by antigen presenting cells and presented to T cells in the draining lymph node, as illustrated in **Figures 1B,C**. T cells recognizing the presented epitopes are primed to recognize cells expressing the target proteins, as these are presenting the epitopes on the cell surface. The vaccine field is fueled by the continuous discovery of targetable epitopes.

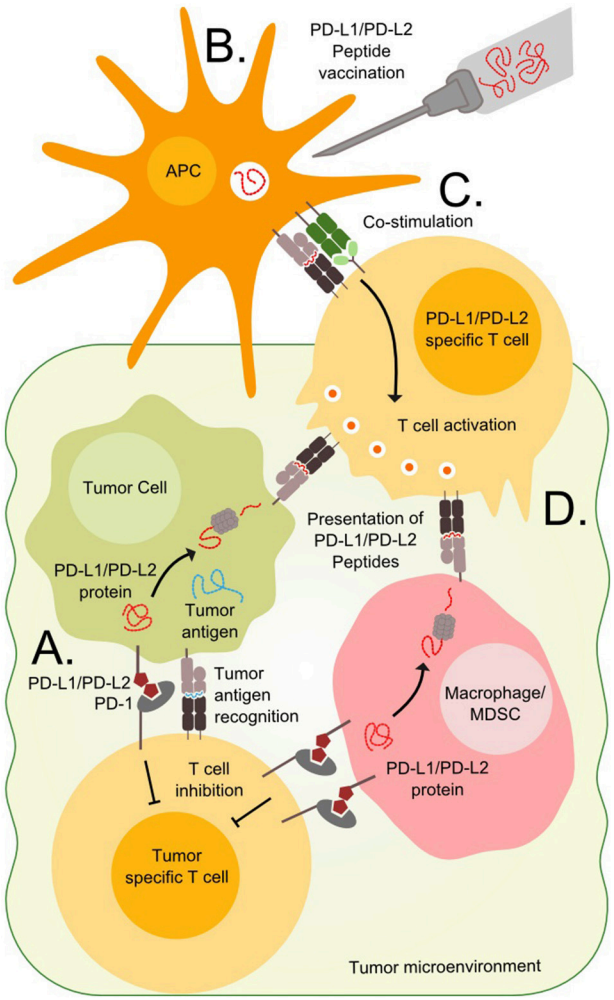

FIGURE 1 | Targeting PD-L1 and PD-L2 expressing cells. **(A)** T cells in the tumor microenvironment often express PD-1 and are vulnerable to stimulation from the ligands PD-L1 or PD-L2 expressed on tumor cells or tumor infiltrating cells such as macrophages or Myeloid-derived suppressor cells (MDSC). **(B)** Immunogenic peptides derived from the PD-L1 and PD-L2 can be injected in the patients where they are endocytosed and processed by antigen presenting cells (APC). **(C)** The APCs present the peptides to T cells in the draining lymph node along with co-stimulatory signals, which are necessary for priming and optimal cytotoxicity. **(D)** Tumor cells, macrophages and MDSCs expressing PD-L1 and PD-L2 also present epitopes derived from these proteins on surface MHC molecules and are vulnerable to primed PD-L1 and PD-L2 specific T cells.

Such epitopes are either neo-antigens, which are formed by somatic mutations that generate a novel mutant antigen, or non-mutated antigens that are overexpressed by the neoplastic cells. Unfortunately, therapeutic cancer vaccination has yet to show significant clinical impact. Limitations to this approach involves a variety of immune escape mechanisms including defected antigen presentation identified in many tumors and T cells unable to find or penetrate the tumors, which might be a minor issue in hematological malignancies as these by nature are less immune restricted than solid tumors (3). Another major limitation is the immunosuppressive mechanisms employed by tumor cells and regulatory cells in the tumor microenvironment (**Figure 1A**) (2). Immune checkpoints such as the PD-1/PD-L1 pathway inhibit activated T cells and thereby prevent an effective antitumor response. Monoclonal antibodies blocking these pathways known as checkpoint inhibitors allow the activated T cells to function regardless of the suppressive signals from the surroundings. Checkpoint inhibitors have proven effective in both solid and hematological cancers (4). However, not all tumors respond to checkpoint inhibitors and they are associated with serious side effects. Targeting the checkpoints through therapeutic vaccination offers a novel way to directly target regulatory pathways in the tumor microenvironment and potentially modify tolerance to tumor antigens. Like the checkpoint inhibitors the vaccine approach might relieve the immune suppression and potentiate anti-tumor T cell responses, but in addition, the vaccine may recruit activated T cells to the tumor site and promote epitope spreading when the target cells are killed. Addressing the immune regulatory mechanisms is essential to improve the outcomes of peptide vaccination.

In this mini review we summarize novel strategies to overcome immune suppression and enhance tumor recognition, which have led to clinical trials in myelodysplastic syndrome, myeloproliferative neoplasms, multiple myeloma, and follicular lymphoma.

TARGETING IMMUNE CHECKPOINTS IN MULTIPLE MYELOMA

Multiple myeloma (MM) is a neoplastic disease of plasma cells with hallmarks including hypercalcemia, renal insufficiency, anemia, and bone lesions. In the recent years several new treatment options have become available, which has improved the median survival. However, the disease is still incurable. All cases of MM are preceded by the precursor state monoclonal gammopathy of undetermined significance (MGUS) and some patients progress via an intermediate state termed smoldering multiple myeloma (SMM) (5). Since the majority of genetic mutations are already present in the precursor states, changes in the microenvironment are believed to impact the risk of progression (6). The microenvironment in MM is severely immunosuppressive (7), and decreased humoral and cellular immune responses to viral and neoplastic epitopes in patients with MGUS and SMM are risk factors for progression to MM (8). Progression from MGUS to MM

is also correlated to the expression level of the immune checkpoint molecule programmed death ligand 1 (PD-L1) on MM cells (8). PD-L1 interacts with the molecule PD-1 on T cells and serves as a powerful negative regulatory signal, which plays a major role in the normal physiologic maintenance of immune self-tolerance, reviewed in Keir et al (9). In symptomatic MM, T cells and natural killer (NK) cells in the tumor microenvironment display increased amounts of PD-1, and MM-cells, osteoclasts and dendritic cells demonstrate elevated levels of PD-L1 (10–16). One study showed that PD-L1 is variably expressed on clonal plasma cells in newly diagnosed MM patients (17). The PD-1/PD-L1 pathway not only promotes the progression of myeloma indirectly by immune evasion; bone marrow stromal cells induce myeloma cells to express PD-L1, which results in increased tumor cell proliferation and reduced susceptibility to anti-myeloma chemotherapy (18). Extramedullary plasmacytomas from patients with late stage MM are characterized by increased expression of PD-L1 (19). Furthermore, the level of PD-1 on T cells is inversely correlated with overall survival (20). Additionally, patients display increased levels of PD-L1 on myeloma cells at relapse or when refractory to treatment, and is associated with an aggressive disease phenotype (21). Increased numbers of T cells with upregulated PD-1 and an exhausted immune phenotype is identified in patients that relapse after high-dose chemotherapy followed by allogeneic hematopoietic stem cell transplantation (HDT-ASCT), indicating that the PD-1/PD-L1 axis could be an important determinant of early relapse after HDT-ASCT (22).

We have characterized T cells in cancer patients that are able to recognize peptides derived from PD-L1 protein, and demonstrated that specific T cells isolated and expanded from these patients are able to recognize and kill PD-L1 expressing cells (23, 24). PD-L1 specific T cells target both tumor cells as well as PD-L1 expressing cells in the microenvironment (**Figure 1D**) (25, 26). Furthermore, stimulation of T cell cultures with PD-L1 peptide was *in vitro* shown to boost the antineoplastic effect of a dendritic cell (DC)-vaccine (27). This effect is likely based on the ability of PD-L1 specific T cells to kill regulatory PD-L1 positive cells in the cell culture, consequently leading to an attenuated immune regulation.

Based on these observations, we have initiated a phase I study testing safety and efficacy of PD-L1 peptide vaccination as a monotherapy consolidation after HDT-ASCT in patients with MM. Furthermore, we are initiating a vaccination study with PD-L1 peptide for patients with SMM. Of note, monotherapy with the anti PD-1 monoclonal antibody (mAb) nivolumab did not show effect in MM (28). Several combination studies of PD-1 specific mAbs have been halted by the Food and Drug Administration (FDA) due to increased mortality in the experimental arms. The halt has recently been lifted on several studies, but the difficulties using anti-PD-1 mAbs for MM underline the need for development of alternative approaches to target the PD-1/PD-L1 pathway in MM.

TARGETING IMMUNE CHECKPOINTS IN FOLLICULAR LYMPHOMA

Follicular lymphoma (FL) is an incurable disease characterized by waxing and waning courses of the disease and is often monitored without the need for active treatment. Over time the disease expands and there is a substantial risk of transformation to more aggressive lymphomas. The mainstay treatment is chemotherapy and anti-CD20 mAbs. Since FL is an indolent disease, it is believed to be ideal for vaccination therapy, which has been explored in FL, in the form of anti-idiotype cancer vaccines. So far this approach has failed to show clinical benefit when tested against placebo or chemotherapy in phase III trials (29–31). There are many possible reasons for the lack of success in these trials, but the immunosuppressive microenvironment in FL is a probable explanation. A gene expression study in FL revealed that the gene signature from regulatory immune cells was an independent adverse prognostic factor (32). Another study looked at the gene expression of specific immunosuppressive proteins in the microenvironment and found 24 out of 54 to be upregulated in FL compared to healthy tissue (33). PD-L1 and programmed death ligand 2 (PD-L2) were among the upregulated genes, which also was confirmed by immunohistochemistry. Both PD-L1 and PD-L2 play a role in immune suppression and contribute to the reduced cytotoxic potential of effector T cells (34). In FL PD-L1 expression has also been identified on tumor-infiltrating macrophages (35).

The clinical relevance of the PD-1 pathway was investigated in a phase I checkpoint inhibition trial, where heavily treated FL patients were treated with the PD-1 blocking mAb Nivolumab as monotherapy. 4 out of 10 had an objective response and one achieved complete response (CR) (28), indicating that the PD-1/Ligand pathway could be important for successful vaccination therapy. As mentioned above, cytotoxic PD-L1 specific T cells can be expanded in cultures by stimulation with PD-L1 derived peptides. Likewise, immunogenic PD-L2 epitopes have been identified, and spontaneous immune responses against these epitopes have been observed in cancer patients (36). Additionally, PD-L2 specific T cells are cytotoxic to PD-L2 expressing tumor cells. Based on these findings and additional unpublished data, we are conducting a phase I vaccination trial with PD-L1 and PD-L2 derived peptides in relapsed FL as maintenance after chemotherapy (NCT03381768). This vaccine is primarily targeting the PD-L1 and PD-L2 positive tumor infiltrating macrophages known to stimulate tumor vascularization and moreover have been correlated with disease transformation and poor prognosis (37, 38). Furthermore, the macrophages seem to have a lymphoma propagating role by secretion of IL15 (39). Thus, by targeting PD-L1 and PD-L2 expressing tumor- and regulatory cells in FL, we hope to shift the immunological balance toward tumor elimination.

TARGETING CANCER TESTIS ANTIGENS IN MYELODYSPLASTIC SYNDROME

Myelodysplastic syndrome (MDS) is a malignant disorder characterized by clonal expansion of mutated myeloid precursor

cells, resulting in an accumulation of blasts in the bone marrow and cytopenia due to ineffective hematopoiesis. MDS responds poorly to chemotherapy, and the only curative treatment is allogeneic HSCT (allo-HSCT), which most often is not feasible due to the high treatment related mortality. Hypomethylating agents (HMA), such as azacitidine or decitabine, are standard therapies for patients with high-risk MDS, who are not eligible for an allo-HSCT. HMAs works by incorporating themselves into the DNA by competitively binding at cytidine nucleotides. After DNA incorporation, the drug covalently attaches to DNA methyltransferase (DNMT), resulting in a loss of methylation and subsequently re-expression of the affected genes as the cell divides (**Figure 2A**) (40).

Several possible synergies may be achieved by combining HMA with therapeutic cancer vaccination. Firstly, a group of genes called cancer testis antigens (CTA) not usually expressed in healthy tissue due to gene methylation, has been found to be expressed by neoplastic cells (41). Treatment with HMA has shown to enhance the expression of CTA (42–46), while not affecting the expression in healthy tissue (47–49). Since healthy cells do not express CTA, the immune system has not developed central tolerance to these antigens, and they can be exploited as targets for immunotherapy. Secondly, HMA induces transcription of DNA from endogenous retroviruses resulting in an inflammatory response in tumor cells (50–53). Double stranded RNA from the viruses activates viral defense pathways, which causes the cell to produce interferon's and upregulate HLA class I molecules (**Figure 2A**). This inflammatory response makes the cancer cells more susceptible to immune mediated killing. Thirdly, the bone marrow of MDS patients has an immunosuppressive microenvironment with an increased amount of myeloid derived suppressor cells (MDSCs) (54). HMA has been shown to deplete MDSCs (55), thus potentially making it easier for T cells to exert an effective tumor-specific immune response.

Vaccination against CTA as monotherapy has previously been tested in many cancer types with varying success (56–58), and trials combining CTA-derived epitopes with HMA are now emerging (59, 60). In NCT02750995 we are targeting four CTAs (NY-ESO-1, PRAME, MAGE-A3, and WT-1) in combination with azacitidine, and another study is investigating a dendritic cell directed vaccine targeting NY-ESO-1 in combination with decitabine and a PD-1 checkpoint inhibitor (NCT03358719). The use of checkpoint inhibitors is expected to further enhance the potency of the combination therapy, since HMA also induces upregulation of PD-L1 on tumor cells and PD-1 on T cells (61, 87).

TARGETING NEO-ANTIGENS IN MYELOPROLIFERATIVE NEOPLASMS

Chronic myeloproliferative neoplasms (MPN) are cancer diseases of the hematopoietic stem cells of the bone marrow and are characterized by an increased production of peripheral blood cells. MPNs display a very homogenic mutational landscape, as 50% of patients harbor the Janus Kinase 2 (*JAK2*)V617F driver mutation (62, 63), and 20–25% have a driver mutation in exon 9 of the calreticulin (*CALR*) gene (64, 65). Recently, both of these mutations were shown to be targets of specific T cells (**Figure 2B**) (66–68). These findings have opened an avenue for therapeutic cancer vaccination with peptides derived from the *JAK2*- or *CALR*-mutations for patients with MPN. However, MPN-patients display several immune-regulatory mechanisms that may attenuate the tumor specific immune response induced by vaccination. Wang et al. showed that patients with MPN have increased numbers of MDSC in peripheral blood, and that mononuclear cells from MPN-patients express increased amounts of the immunoregulatory enzyme arginase-1 compared to healthy donors (69). Additionally, MDSCs from MPN patients are more suppressive to T cells compared to MDSCs from healthy donors. Prestipino and colleagues recently showed that the *JAK2*V617F-mutation enhances PD-L1 expression in mutant cells through activation of STAT3 and STAT5 (70). As described above, both arginase-I and PD-L1 are targets of specific T cells (23, 24, 71), and the immune mediated killing of arginase-I and PD-L1 expressing cells is believed to enhance the tumor specific immune response (72). Recently, strong and frequent spontaneous T-cell responses against both PD-L1 and arginase-1 were detected in patients with MPN (73, 74). We hypothesize that enhancing these already existing anti-regulatory T-cell responses through therapeutic cancer vaccination with arginase-I and PD-L1 derived epitopes can boost the neo-antigen specific immune response induced by vaccination with JAK2/CALR-mutant epitopes. This method of combinatorial cancer vaccination targeting both driver mutations and immunoregulation could potentially break the immune evasion leading to anti-tumor immunity and clinical effect. Another means to enhance the anti-tumor immune response would be to combine JAK2/CALR-vaccines with PD-1 specific mAbs, as treatment with these drugs have been shown to enhance the amount of neo-antigen specific T cells in peripheral blood (75).

Apart from the obvious combination of JAK2/CALR mutant vaccines with immune checkpoint blocking antibodies, the combination of vaccines with interferon-alpha (IFN-α) is a most interesting option. IFN-α is a potent immunostimulatory cytokine and has been used for years for the treatment of MPN (76). IFN-α has been shown to induce complete hematological responses and major molecular remissions in a substantial proportion of patients (77–79). Concurrently, treatment with IFN-α induces marked alterations in immune cell subsets and in the expression of HLA-related genes (80–83), and the mechanism beyond the clinical effect of IFN-α is believed to rely partially on the induction of an anti-tumor immune response (84). Previous reports on therapeutic cancer vaccination in other malignancies have underscored the importance of a low tumor burden at the time of vaccine initiation in order to obtain a proper clinical response (85). As IFN-α is the only drug, which is able to reduce the tumor burden in a substantial part of the patients, it is most apparent to reduce the tumor burden with IFN-α, and after attainment of a major molecular

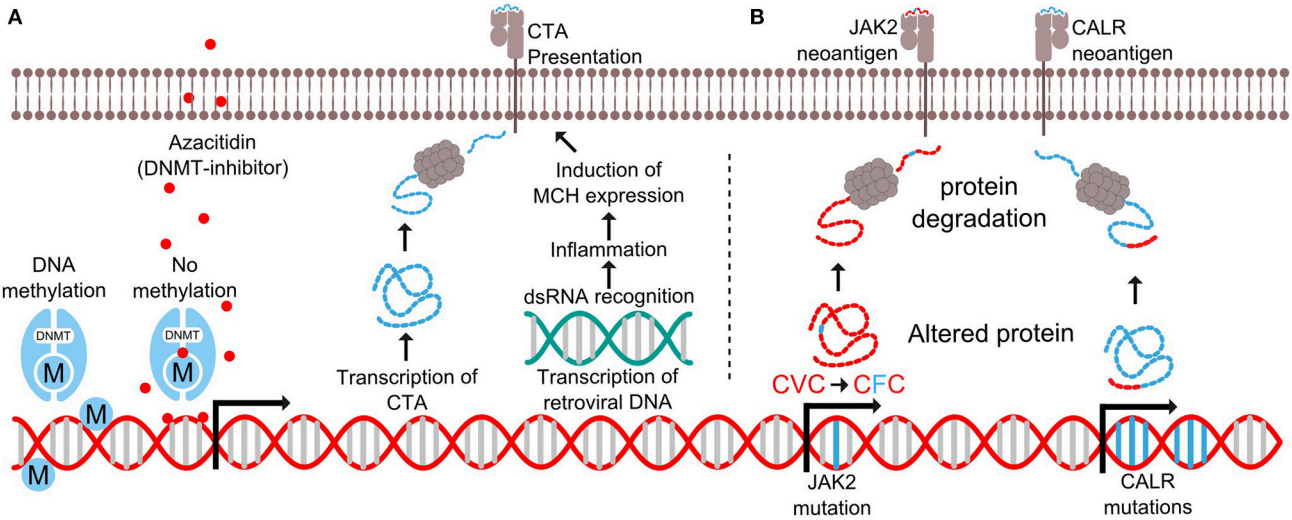

FIGURE 2 | Expression of antigens in myelodysplastic syndrome and myeloproliferative neoplasms. **(A)** DNA methyltransferase (DNMT) add methyl (M) groups to parts of the genome to prevent transcription. The drug azacitidine binds to cytidine nucleotides where it covalently attaches to DNMT to prevent further methylation. This results in the transcription of otherwise suppressed genes such as cancer testis antigens (CTA) and retroviral DNA. The CTA are processed as proteins and presented by MHC molecules on the cell surface, while the double stranded RNA (dsRNA) trigger intracellular pattern recognition receptors causing inflammation and increased MHC expression. **(B)** Mutations in the *JAK2* gene results in the substitution of valine (V) to phenylalanine (F) in position 617 of the JAK2 protein. This results in the generation of a mutant antigen. Likewise, the *CALR* exon 9 mutations generate a novel mutant C-terminus in the CALR protein, thus generating several mutant antigens.

remission, initiate therapeutic cancer vaccination against the targets described above. This could hopefully eradicate the malignant clone and ultimately cure the patient. However, as exposure of cells to interferon increases the expression of PD-L1 on the exposed cells it could be worthwhile to explore the combination of neo-antigen vaccines and IFN-α with either PD-1 blocking mAbs and/or PD-L1 vaccine in order to counteract the increased amounts of PD-1 ligands induced by IFN-α treatment (86).

CONCLUSION

The trials described above represent novel approaches to overcome some of the challenges in peptide vaccination including the suppressive mechanisms protecting the tumor cells from an effective anti-tumor immune response. Targeting the immune checkpoints such as the PD-1 ligands or other immune suppressive molecules such as arginase-1 could shift the immunological balance in the tumor microenvironment and ultimately induce an adequate anti-tumor immune response—a strategy that is currently being explored in FL and MM. Combining this approach with tumor specific antigens such as the neoantigens described in MPN could further enhance the anti-tumor response. Finally, combining vaccination against shared antigens, such as CTA, with HMA treatment in MDS is a promising approach to increase immunogenicity of the malignant cells. If the peptide vaccines prove safe and ultimately effective, they will become welcome additions to the toxic treatment options currently available for patients with hematological cancers.

ETHICS STATEMENT

All undergoing studies mentioned in the review are approved by the ethical committee of the capital region of Denmark and conducted according to national ethical guidelines and the Helsinki declaration.

AUTHOR CONTRIBUTIONS

MA, IS, and UK contributed to the conception and design of the review. UK wrote the first draft of the manuscript and provided the figures. SH, MH, NJ, and JG wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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Conflict of Interest Statement: MA and MH have filed a patent application on the *JAK2* and *CALR* mutations for therapeutic cancer vaccination. MA has filed patent applications on the use of PD-L1, PD-L2, and arginase-1 for therapeutic cancer vaccines. The rights of the patents have been transferred to the Capital Region and Zealand Region according to Danish law on inventions made at public research institutions. The capital region has licensed some of these patents to the company IO Biotech ApS. MA is a shareholder and board member of the IO Biotech ApS, which has the purpose of developing immune-modulating vaccines for cancer treatment.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Vaccine Strategies to Improve Anti-cancer Cellular Immune Responses

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More than many other fields in medicine, cancer vaccine development has been plagued by a wide gap between the massive amounts of highly encouraging preclinical data on one hand, and the disappointing clinical results on the other. It is clear now that traditional approaches from the infectious diseases' vaccine field cannot be borrowed as such to treat cancer. This review highlights some of the strategies developed to improve vaccine formulations for oncology, including research into more powerful or "smarter" adjuvants to elicit anti-tumoral cellular immune responses. As an illustration of the difficulties in translating smart preclinical strategies into real benefit for the cancer patient, the difficult road of vaccine development in lung cancer is given as example. Finally, an outline is provided of the combinatorial strategies that leverage the increasing knowledge on tumor-associated immune suppressive networks. Indeed, combining with drugs that target the dominant immunosuppressive pathway in a given tumor promises to unlock the true power of cancer vaccines and potentially offer long-term protection from disease relapse.

Keywords: cancer vaccine, adjuvant, dendritic cell, TLR, STING, checkpoint

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INTRODUCTION

The aim of a vaccine is to induce an *in vivo* adaptive immune response against a defined antigen or set of antigens. This implies leveraging specific functions of professional antigen-presenting cells in order to trigger T-helper cell responses to support production of antibody production and induce cytotoxic effector T-cells.

The remarkable clinical responses observed with immune checkpoint inhibitors and CAR-T cell therapy have put a definitive end to the discussion whether the human immune system, and T-cells in particular, is capable of controlling or even eradicating cancer. The problem is that vaccination approaches have largely been successful when it comes to inducing humoral immunity, while no major breakthrough has been reached in diseases where cellular responses are also required, such as tuberculosis, HIV, or cancer. For cancer, the bar is raised even higher as vaccines are primarily developed in a therapeutic setting, i.e., with the aim of controlling clinically evident or, at best, minimally residual disease.

The purpose of this review is not to give an exhaustive account of all attempts at cancer vaccination so far, but to provide the reader with the necessary concepts to understand where the field is going, specifically focusing on strategies to elicit clinically meaningful cellular immune responses. Finally, this review will give a perspective of potential combinatorial strategies that could unlock the unique power of vaccines in cancer.

In order for vaccination to deliver unequivocal clinical benefit for cancer patients, improvements must be achieved at two levels: (1) maximizing the induction of a T-cell response with optimal amplitude, specificity and effector profile, (2) ensuring that vaccine-induced T-cells can reach the tumor site and perform their function without any restraint.

The first level involves optimization of the choice of antigenic target(s), of adjuvant potency, and of delivery system. The main principles and some representative preclinical examples in this field will be highlighted in the following section, followed by clinical data ("reality check") using lung cancer as an illustrative case. In a last section we will outline combinatorial strategies that could herald a revival of cancer vaccines. Molecular formulation of antigens and specific antigen delivery systems constitute a wide domain on their own and will not be handled in detail in this review.

OPTIMIZING ANTIGENIC TARGETS

The antigenic landscape in cancer is far more complex than that of viral or bacterial pathogens, where adaptive immunity to well-defined epitopes can drive long term disease protection. In cancer vaccines, it seems rational to target the broadest repertoire of antigens possible in order to avoid selection of escape variants. Approaches that can address this need are the use of autologous tumor lysates, whole tumor-derived mRNA, irradiated autologous tumor cells or allogeneic tumor cell lines (3, 4). All of these pose challenges in terms of logistics, standardization and compliance to regulatory demands including Good Manufacturing Practice (GMP) requirements. Many efforts have been devoted in developing vaccines targeting one or a restricted set of cancer antigens. These can be either differentiation antigens (e.g., MelanA, gp100, tyrosinase), cancer-testis antigens (e.g., MAGE/LAGE/XAGE family, NY-ESO1), or virus-derived antigens (e.g., HPV or EBV-derived proteins) (5). On one hand, this is motivated by practical considerations, including simplicity of vaccine manufacturing and monitoring of immune responses. On the other hand, it is anticipated that effective responses to one antigen, through tumor cell destruction, can lead to an immunogenic release of additional endogenous antigens and spark a broader immune response, a phenomenon known as "epitope spreading" (6).

Mutanome-derived epitopes are the most recent addition to defined tumor antigens for use in cancer vaccines. The idea originates from the observation that objective responses to immune checkpoint blockade are proportional to the mutational burden of a given tumor, a number which is the highest in carcinogen-induced cancers (7). This is why the top targets for immune checkpoint inhibition are melanoma, lung cancer and bladder cancer, along with tumors with DNA mismatch repair defects (8). It is now thought that among the total bulk of non-synonymous mutations, a subset that is clonally distributed within the tumor gives rise to mutation-containing peptides (neo-epitopes) that can be recognized by cytotoxic T-cells (9). In addition to single-nucleotide variants, indels have been shown to be strongly predictive of response to immune checkpoint inhibition as well (10). Complex bioinformatic pipelines have been developed to extract a list of candidate immunogenic neo-epitope for a given patient's cancer. This requires deep genomic sequencing of a tumor sample to list all single nucleotide variations (SNVs) and indels. In parallel, RNA sequencing on the same material allows to narrow down on the genomic aberrations that are effectively expressed. Next, *in silico* algorithms are called into action to predict which of the mutations will be presented to T-cells based on proteasome processing and binding affinity for human leucocyte antigen (HLA) molecules. The resulting coding sequences can be synthesized either as peptides as synthetic mRNA. This methodology has been validated in preclinical experiments, showing that vaccination with mutanome-derived neo-antigens can induce protective and therapeutic immune response to autologous tumors (11). Today, this ambitious approach, entirely patient-individualized has entered clinical development with recent phase 1 data demonstrating the feasibility, safety and immunogenicity of neo-antigen-targeted vaccine in metastatic melanoma (12). Notwithstanding the sophistication of this approach, two concerns can be brought forward: (1) several algorithms exist for the prediction of neo-epitopes, and the list of candidate antigens produced for a given tumor can be influenced by the bioinformatic pipeline used, (2) the whole process from next-generation sequencing until manufacturing and release of a GMP-compliant mutanome-derived mRNA vaccine currently takes around 100 days (12), implying that only patients with maximally debulked or relatively indolent tumors are optimally eligible.

THE (VERY CROWDED) ROAD TOWARD OPTIMAL CANCER VACCINE ADJUVANTS

The benefit of adjuvants are best described by the operational definition of Gaston Ramon, better known as the father of the diphtheria vaccine (13): "substances used in combination with a specific antigen that produce more immunity than the antigen alone." Finding adjuvant formulations that can unlock clinically relevant immune responses against cancer antigens has remained a challenging task: for one, cancer antigens are often poorly immunogenic due to partial homology with self-antigens; on top of that, the optimal cancer vaccine adjuvant must succeed in driving a type 1-polarized, cell-mediated immunity rather than a type 2-polarized and/or humoral response.

Adjuvants can be subdivided in two major classes: (1) immunostimulatory molecules that trigger innate immune receptors, and (2) particulate adjuvants which mainly act either as antigen depots or as delivery systems. Immunostimulatory

Abbreviations: ASC, Apoptosis-Associated Speck-Like Protein Containing CARD; CCL, CC chemokine ligand; cGAS, Cyclic GMP-AMP synthase; CSF-1R, Colony-stimulating factor receptor-1; CTLA-4, Cytotoxic T-Lymphocyte Associated Protein 4; IFN, Interferon; IKK, IκB kinase; IL, Interleukin; IRF3, Interferon regulatory factor 3; ISCOM, Immune stimulating complexes; LMP-2, Epstein–Barr virus (EBV) latent membrane protein 2; NFκB, Nuclear Factor kappa-light-chain-enhancer of activated B cells; TAA, Tumor-associated antigen; TAP-1, Transporter 1, ATP Binding Cassette Subfamily B Member; TBK1, TANK Binding Kinase 1; TCR, T-cell receptor; TGF-β, Transforming growth factor beta; TRAIL, TNF-related apoptosis-inducing ligand.

adjuvants mostly consist of molecules that mimic pathogen-associated molecular patterns and engage Toll-like receptors (TLRs) on antigen presenting cells (APCs) including B-cells, macrophages and most importantly dendritic cells (DCs). In the case of DCs this results in a complex and highly coordinated cellular response aimed at sparking adaptive immunity: (1) switch from antigen uptake mode to antigen processing and presentation, upregulation of a whole array of T-cell costimulatory molecules, upregulation of chemokine receptors mediating migration into T-cell areas of draining lymphoid tissues, and release of specific cytokines and chemokines to polarize the resulting T-cell response. Due to their immunostimulatory power and the capacity to prime naïve T-cells, properly activated DCs are also referred to as "nature's adjuvants." The use of *ex vivo*-generated and antigen-loaded **DCs as cellular vaccines** will be reviewed in a different article of this Special Edition. The following paragraphs provide a non-exhaustive overview of some of the most notable acellular adjuvant systems optimized for use in cancer vaccines.

Immunostimulatory Adjuvants: TLR Ligands and Beyond

Among **immunostimulatory adjuvants**, TLR4 ligands constitute some of the most potent members in terms of APC activation. Lipopolysaccharide (LPS), the prototype TLR4-ligand, cannot be used as such in clinical formulations due to toxicity issues. MPL (3-O-desacyl-4′-monophosphoryl lipid A) is a chemically detoxified form of LPS derived from strain R595 of *Salmonella minnesota*, while still retaining immunostimulatory properties (14). It is the only defined TLR ligand approved as part of a vaccine in humans to this day and is a key ingredient of the AS04 adjuvant formulation used in the commercially available HPV and HBV vaccines. However, what makes MPL especially attractive with respect to anti-cancer vaccination is its capacity to induce robust Th1-polarized and cell-mediated immunity. MPL is also an ingredient of the DETOX adjuvant system, when combined with cell wall peptidoglycans from Mycobacteria (15). DETOX is the adjuvant used in the Melacine® vaccine formulation, which incorporates lysate from two allogeneic melanoma cell lines and has shown some modest clinical benefit in resected stage III melanoma patients (16). Likewise, CG-enriched oligodeoxynucleotides (CpG), by triggering the intracellular TLR9, have also been described as powerful inducers of Th1 and cytolytic T-cell responses. These properties have led the incorporation of MPL together with CpG as part of the proprietary adjuvant formula AS15 in the MAGE-A3-targeted cancer vaccine developed by GSK Biologicals (17). Because of biosynthetic variability in the structure of bacterial-derived LPS and downstream hydrolytic steps, MPL is a heterogenous mix of closely related structures ("congeners"). Hence, synthetic TLR4 agonists have been designed, i.e., aminoalkyl glucosaminide 4-phosphates (AGPs) such as glucopyranosyl lipid A and RC-529 (18). The latter has shown its capacity to induce Th1 responses equivalent to MPL, and still with much lesser *in vivo* toxicity than LPS (19). Several other extra- and intracellular TLR-ligands have been the subject of intensive research efforts [reviewed in (20)], and all have shown value to varying degrees in diverse preclinical tumor models. Although some molecules such as the TLR7/8 agonist imiquimod or the TLR2/4-stimulating preparation Bacille-Calmette-Guérin (BCG) are used routinely in the clinic as standalone therapies, no TLR agonist has so far successfully entered standard of care as an ingredient of a cancer vaccine.

It should be noted that triggering TLR signaling also activates homeostatic counterregulatory mechanisms. These include release of IL-10 by myeloid cells, induction of regulatory T-cells (Tr1), and upregulation of the T-cell checkpoint molecule programmed death ligand-1 (PD-L1) on APCs: all of which contribute to the further induction of T-regs and the dampening of anti-tumor cellular immune responses [reviewed in (21)]. The TLR ligands Pam2Cys (TLR2), LPS (TLR4), imiquimod (TLR7) and CpG (TLR9) all induce IL-10 production, and blockade of IL-10/IL10R axis in these settings augments immune responses (17, 18) Similarly, the TLR3-ligand poly I:C induces PD-L1 on DCs, while PD-L1 blockade boosts effector CD8+ T-cell expansion after a tumor vaccine involving poly I:C as adjuvant (22). Another counterregulatory mechanism after TLR stimulation is the upregulation of indoleamine 2,3-dioxigenase expression in DCs, a side-effect observed with CpG oligodeoxynucleotides (23). IDO is a well-described mediator of immunological tolerance: by depleting tryptophan and generating toxic catabolites, IDO enzymatic activity suppresses T-cell activation and promotes T-reg induction in the tumor micro-environment (discussed in more detail below).

A different class of immunostimulatory adjuvants does not belong to bacterial or viral pathogen-associated molecules but consists of extracts from plant origin. **Saponins** derived from the bark of the South American soapbark tree (*Quillaja saponaria*) contain a family of water-soluble, structurally diverse molecules with strongly pro-inflammatory properties. **QS21** is one of the RP-HPLC fractions of *Q. saponaria* extracts that has been used the most in vaccine development (24). The triterpene aldehyde group is considered as the adjuvant active site, resulting in preclinical models in a strong mixed T-helper 1 (Th1), CD8 T-cell and humoral response. QS21 was shown to primarily activate the ASC/NALP3 inflammasome pathway, which converts pro-IL-1β and pro-IL-18 into their bioactive forms (25). This provides the rationale to combine with a TLR4 ligand in order to induce upstream expression of the pro-forms. Still, it appears that the magnitude and quality of the resulting immune response is not proportional to the degree of inflammasome activation, and high doses of QS21 can cause cell membrane lysis and apoptosis of APCs (25). QS21 has been tested extensively in therapeutic cancer vaccine formulations involving ganglioside antigens (GD2, GD3, or GM2) (24). Although robust and humoral responses were invariably observed, there was no convincing evidence of cell-mediated immunity in humans. QS21 is also combined with MPL as part of the AS01 and AS15 adjuvant formulation (GSK), as evaluated in the MAGE-A3 cancer vaccines (discussed below).

STING agonists are a recent addition to the arsenal of candidate vaccine adjuvants. STING (STimulator of INterferon Genes) is a transmembrane protein located in the endoplasmic reticulum that belongs to the family of nucleic acid sensors

(26). STING activation triggers robust type 1 IFN responses in a TBK1-IRF3-dependent way as well as IKK/NFkB-dependent upregulation of inflammatory cytokines and chemokines. STING can be activated in two ways. The presence of cytosolic double-stranded DNA (e.g., originating from invading DNA viruses or self-DNA from stressed/damaged cells) is first detected by the cGAS molecule which generates cyclic 2′3′-GMP-AMP (cGAMP) from ATP and GTP. As a second messenger, 2′3′-cGAMP then goes on to bind and activate STING, triggering both IRF3- and NFkB-dependent immune/inflammatory gene expression. cGAS expression is by itself inducible by type I interferon, which provides a positive feedback mechanism when relevant ligands persist. Alternatively, STING can be directly triggered by bacterial cyclic dinucleotides such as c-di-GMP. In preclinical models, high doses of c-di-GMP injected intratumorally can directly induce caspase 3-dependent apoptosis of tumor cells and release of tumor-associated antigens, while lower exposure to c-di-GMP can lead to activation of DCs and promote CD8+ T-cell responses against those antigens (27). Other preclinical studies have demonstrated the value of STING agonists in the setting of therapeutic cancer vaccination (28). Caution must be paid however as among immune cells, STING expression is the highest in T lymphocytes. STING activation has been shown to lead to T-cell apoptosis, a phenomenon that appeared cell-specific as macrophages and DCs did not display such sensitivity (29). Hence, implementation of STING agonists in cancer vaccines should ideally be combined with adjuvant/antigen delivery systems that specifically target myeloid cells *in vivo*, as already reported (30). A potential bonus with this type of approach is that STING agonists can reprogram myeloid-derived suppressor cells toward a DC-like immune-stimulating phenotype expressing IL-12 and T-cell costimulatory molecules (27). Another difficulty in translating preclinical data to clinical development strategies is the fact that STING agonists can have differential binding properties in murine vs. human cells. The flavonoid compound DMXAA for instance can bind mouse STING and induced anti-tumor immunity, but fails to activate human STING (31). Still, based on its unique properties, the STING pathway has become a "hot" candidate in the pipeline of several biotech and larger pharmaceutical companies (IFM Therapeutics, Selvita, iTeos, MSD). To date few compounds have reached the stage of early clinical development: ADU-S100 (Novartis) and MK-1454 (MSD). Due to systemic toxicity, both require accessible lesions for intratumoral injection, and both are (quite rationally) combined with systemic administration of an immune checkpoint inhibitor (NCT03172936, NCT03010176).

Next to pathogen-derived molecules, specific host proteins have been shown to perform adjuvant-like functions as well. Immunostimulatory cytokines such as IL-2, IFN-γ, IL-12 and **granulocyte-macrophage colony stimulating factor** (**GM-CSF**) represent an obvious choice as an ingredient for a vaccine. By far the most used in clinical trials is GM-CSF. Based on preclinical studies, GM-CSF helps in the recruitment of dendritic cells to the vaccine injection site, promotes DC maturation and antigen-presentation, resulting in enhanced adaptive immune responses (32). GM-CSF is also the essential ingredient for the *ex vivo* generation of monocyte-derived DCs for vaccination purposes, as discussed elsewhere in this edition. GM-CSF has been incorporated in vaccine formulations either as a standalone adjuvant, or in the shape of allogeneic tumor cell lines engineered for stable expression of GM-CSF (GVAX®) (32). A concern still persists as to the optimal dosage of GM-CSF however, with preclinical studies indicating the potential of this cytokine to expand MDSCs, with paradoxical suppression of T-cell mediated anti-tumor responses *in vivo* as a consequence (33). This effect on MDSCs was also observed in clinical trials, where a low-dose GM-CSF added to a cancer vaccine caused a systemic expansion of an immunosuppressive CD14-positive HLA-DR-low/-negative myeloid cell subset. In an another controlled clinical trial, including GM-CSF as part of an incomplete Freund's adjuvant formula resulted in significantly lower T-cell responses to vaccine antigens compared to adjuvant *without* GM-CSF (34). Still, a surprisingly large number of trials using GM-CSF as an adjuvant component are active (listed in **Supplementary Table**); their results will need to be interpreted with caution.

A different class of endogenous proteins with immunogenic activity are **heat-shock proteins** (**HSPs**). HSPs are chaperones that are released from stressed or dying (cancer) cells, with the unique property of binding cell-derived peptides (35). These peptides can be delivered to DCs resulting in cross-presentation and induction of efficient CD8+ T-cell-mediated immunity (36). The transfer of peptides from HSPs to the APC's MHC class I molecules is not passive but requires uptake by the HSP receptor CD91 expressed by the APC and internal processing. The repertoire of peptides bound by the HSPs reflects the antigenic make-up of the cell of origin, a property which can be leveraged to induce a broad T-cell-mediated protective immunity. In addition, HSP carrier molecules by themselves act as innate immune stimuli, triggering essential events in APCs including release of TNF-α, IL-1β, IL-12, GM-CSF, inflammatory chemokines, and upregulation of costimulatory molecules (37). This effect could be due to binding of HSPs to TLR4, which reinforces the notion that HSPs constitute *bona fide* endogenous adjuvants. Immunization with tumor cell-derived HSPs such as HSP70 and GP96 has demonstrated impressive protective immunity in several preclinical studies [reviewed in (38)]. This has led to the clinical development of autologous HSP96-based vaccines formulation (e.g., vitespen / Oncophage®). Clinical trials have shown that this therapy is feasible and non-toxic, although clinical benefit was low except maybe in subset analyses including early-stage renal cell cancer (RCC) and a trend toward benefit in M1a/M1b melanoma patients (39, 40). With these results, vitespen failed to obtain approval from the European Medicines Agency (EMA). Also, one major limitation for further development of HSP-based vaccines is the manufacturing process itself which requires access to sufficient amounts of autologous tumor material. Still, a number of combination clinical trials implementing HSP-based vaccines are ongoing (**Supplementary Table**).

Particulate Matter Adjuvants

The most widely used particulate adjuvants historically have been aluminum salts, mostly in the shape of aluminum hydroxide ("alum"). Alum triggers innate immune responses in a TLR-independent way but rather stimulates the NALP3

inflammasome. Being very potent in inducing pure T-helper 2 (Th2) and antibody responses, alum salts are by themselves unfit for use in cancer vaccines. However, when associated with type-1 polarizing ingredients such as ISA 51 (Montanide, see below) and recombinant IL-12, alum was shown to enable a more sustained immune response to tumor-associated antigens probably due to a depot / slow release effect (41). Likewise, combining alum with MPL (GSK's AS04 adjuvant formula) enables a more sustained type-1 polarized cytokine response (42). Other particulate adjuvants have been tailored to better respond to the demands of a cancer vaccine (43). The oldest prototype, Freunds adjuvant, is a water-in-oil emulsion containing heat-killed Mycobacteria. Although being very immunogenic in preclinical models, it is much too toxic for human use. A less toxic formulation that incorporates squalene and oleate, **Montanide ISA-51** ("Incomplete Freunds Adjuvant") has been used in many therapeutic cancer vaccines. This includes a pivotal trial using the melanoma TAA gp100 as target, in which the clinical activity of ipilimumab alone or in combination with a vaccine vs. vaccine alone was assessed in metastatic melanoma patients (44). Despite induction of robust antibody and CTL responses and signals of clinical benefit in small patient cohorts, none of the Montanide-adjuvanted cancer vaccines has reached advanced clinical development in oncology so far. Adjuvants based on oil-in-water emulsions have been subsequently developed and show a superior safety profile, excellent depot properties, but produce strongly Th2-biased and humoral immune responses (15).

It has been observed by many research groups that a key to induce cellular immunity is the capacity to exploit the cross-presentation capacity of dendritic cells. An efficient way to achieve this goal is by packaging antigens in non-soluble particles, such as virosomes, liposomes, ISCOMs, and microspheres (45). **Virosomes** and **virus-like particles** (VLP) are 20–100 nm size and consist of the membrane envelop of a virus (including embedded proteins) but devoid of a replication-competent genome. Nevertheless, VLPs can efficiently fuse with the membrane of the target cell (ideally an APC), simultaneously delivering an antigenic cargo and any PAMP that can be incorporated in the design. A successful VLP-based vaccine is Gardasil®, which contains capsid proteins of HPV serotypes 6, 11, 16, and 18. The vaccine uses aluminum hydroxide phosphate sulfate as adjuvant and is hence a potent inducer of long-lasting and very protective humoral immune responses.

Considerable experience has also been gathered with **ISCOMs**, which are 40 nm micellar structures in which a saponin adjuvant (QS21) and protein antigen is incorporated. ISCOMATRIX consists of just the micellar components and adjuvant, with the flexibility of adding an antigen of choice. ISCOMs differ from liposomes as the latter contain an internal aqueous space confined by a lipid bilayer. As a consequence of the built-in saponin, ISCOMs exert their adjuvant activity by activating the NALP3 inflammasome, while delivering antigenic cargo to dendritic cells to cross-prime CD8+ T-cells (46). *In vivo*, tumor antigen-specific cellular and humoral immune responses were observed after vaccination with NY-ESO1-containing ISCOMs (47). Further intensive research efforts are being devoted to engineer **novel synthetic particles** with the aims of maximizing vaccine potency while specifically targeting cross-presenting APCs. The wide spectrum of physico-chemical parameters that can be varied in the manufacturing such next-generation nanoparticles offers great flexibility in terms of targeting and immunostimulatory properties (see (48) for a comprehensive overview).

OPTIMIZING CANCER VACCINE FORMULATIONS: A REALITY CHECK

The solid preclinical rationale upon which several types of vaccine designs are based stands in sharp contrast to the sobering clinical results observed. Here, we summarize vaccine development in non-small cell lung cancer (NSCLC) as a good example of the limited clinical benefit of cancer vaccines as monotherapy. Many of the strategies described in the previous section have been tested clinically in lung cancer, be it protein-, liposome-, VLP-based or genetically engineered whole cell vaccine platforms.

One of the largest clinical trials ever undertaken in NSCLC was a randomized, double-blind, placebo-controlled phase 3 study using GSK Biological's recombinant MAGE-A3 vaccine (49). The formulation contains full-length recombinant MAGE-A3 protein, a cancer-testis antigen expressed in about 40% of NSCLC patients, combined with the AS15 adjuvant system described earlier. Despite the cancer-specificity of MAGE-A3, notwithstanding the strong type-1 polarizing activity of the AS15 adjuvant formulation and promising phase 2 trial data, the phase 3 trial showed no benefit at all in terms of overall and disease-free survival in early-stage NSCLC patients vaccinated after surgical resection (49). Moreover, an "immune-activated" predictive gene expression signature identified in the melanoma MAGE-A3 vaccine trials failed to identify a MAGE-A3+ NSCLC patient subset who might benefit from vaccination. The vaccine produced strong and long-lasting antibody responses, in line with early clinical data (50), but no convincing evidence for the induction of cytotoxic T-cell responses was provided in this trial. In part due to these results, development of a similar vaccine targeting the cancer-testis antigen PRAME in NSCLC was stopped prematurely (51).

L-BLP25 (Stimuvax®) is a liposomal formulation incorporating as antigen a synthetic lipopeptide coding for 25 amino acids of the Muc-1 protein (tecemotide), and MPL as adjuvant. Muc-1 is a glycoprotein that is overexpressed and typically aberrantly glycosylated in a several adenocarcinomas, among which a large subset of NSCLC. L-BLP25 failed to demonstrate a benefit in overall survival in the intention to treat population in a phase III trial involving locoregionally advanced NSCLC patients after chemo-radiotherapy (START trial, NCT00409188) (52). However, a major increase in median OS was observed in the subgroup of patients who received concurrent rather than sequential chemoradiotherapy. These results were meant to be verified in a follow-up phase 3 trial (START2, NCT02049151), however based on negative results of a trial in Asian NSCLC patients (INSPIRE, NCT01015443) (53) the

sponsor decided to stop development of L-BLP25 ("Stimuvax") in all indications.

TG4010 is another Muc-1-targeting vaccine evaluated in NSCLC. It consists of a replication-deficient viral vector, modified vaccinia Ankara (MVA), expressing both Muc-1 as well as IL-2 to support T-cell proliferation. In preclinical models, MVA induces expression of the incorporated antigen sequence in target tissues at equivalent levels compared to replication-competent virus, albeit with a faster kinetic (54). MVA can trigger type-1 IFN production in a TLR-independent fashion. This, combined with the induction of not only humoral but also of type-1-polarized cellular immune response makes MVA theoretically an attractive tool for cancer vaccination purposes. A first trial in advanced NSCLC gave indication of benefit when combined with 1st line chemotherapy, vs. chemotherapy + placebo (55). This prompted a confirmatory phase 2b/3 trial that included a candidate predictive biomarker (the percentage of activated NK-cells in peripheral blood). Results of the phase 2b part showed a significant increase in progression-free survival (PFS; primary endpoint) that was most pronounced in non-squamous NSCLC (where Muc-1 expression is expected to be the highest) and with biomarker value in the lower 3 quartiles (56). Results of the phase 3 part are still pending.

As a final example, in an attempt to target a broad repertoire of antigens, a vaccine was designed containing four irradiated NSCLC allogeneic cell lines (belagenpumatucel-L, Lucanix®). In addition, the cell lines where genetically engineered to express an antisense gene vector that inhibits TGF-β2 expression. TGF-β2, along with IL-10, is a prototypical mediator of tumor-induced immune suppression and T-reg induction, and introduction of TGF-β2 antisense plasmid was shown to increase vaccine immunogenicity in preclinical studies (57). It must be stressed though that while the production of TGF-β2 by the vaccine cells themselves is suppressed, this does not affect the levels of this suppressive cytokine emanating from the tumor microenvironment. Belagenpumatucel-L has been evaluated as consolidation therapy in locally advanced and metastatic NSCLC patients that had not progressed on their last line of chemotherapy. Data from a phase 2 trial appeared promising with a clear dose-dependent increase in overall survival (58). However, in a follow-up phase 3 study, no benefit in OS was observed except in a subgroup of patients that had received radiation and chemotherapy <6 months prior to randomization (59). Patient numbers in this subgroup were very small though and to this day it remains unsure whether this analysis will prompt a confirmatory phase 3 study focusing on this subpopulation.

The impossibility or at best difficulty to demonstrate unequivocal clinical benefit in these vaccination trials raises many questions. When it comes to cancer immunotherapy, the avalanche of robust and positive data coming from the immune checkpoint inhibitor field represents today's benchmark. Patient outcomes after vaccination highlight the difficulty of inducing productive cytolytic responses against cancer in humans. It is clear that a careful choice of antigenic target, adjuvant formula and delivery platform are not sufficient to elicit therapeutic or protective immunity against cancer. This warrants more attention to the tumor-associated tolerogenic or immunosuppressed climate that reigns in the cancer patient.

UNLEASHING IMMUNE EFFECTOR MECHANISMS DOWNSTREAM OF VACCINE ACTION

The immune response against cancer cells is a series of critical steps, also described as the "cancer immunity cycle" (60). As a consequence, the strength of the response at the end of this chain of events will be determined by its weakest link (see **Figure 1**). Each of the obstacles to successful antitumor immune responses have been studied in detail and offers opportunity for therapeutic modulation. Clinical trials exploring combinatorial strategies are summarized in **Table 1**. The underlying principles will be discussed below.

Improving Effector T-Cell Access Into the Tumor

Following successful expansion and adequate polarization of tumor-antigen specific T-cells, the latter acquire the capacity of exiting the lymph node and recirculate through the bloodstream to scan for antigens in peripheral tissues. Unfortunately, penetration of effector lymphocytes into tumoral beds is hampered in many ways. Tumor-induced angiogenesis results in a network of aberrant blood vessels in which proper adhesion and extravasation of cytolytic T-cells is impaired. The endothelium of tumoral vasculature is known to be poor in leukocyte adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Overactivity of the endothelin-endothelin receptor axis on tumoral endothelia further limits T-cell extravasation by decreasing ICAM-1 expression while further boosting the production of angiogenetic factors such as vascular-endothelial growth factor (VEGF) (61). Similar to physiological immune-privileged organs, the endothelium of tumoral vessels also overexpresses T-cell checkpoint ligands including PD-L1, death receptors such as FasL and TRAIL, and IDO. All of these factors do not seem to hamper the recruitment of T-regs, and together contribute in shielding tumor cells from immune attack. Hence, the clinical benefit obtained with commonly used anti-angiogenic compounds such as the VEGF blocker bevacizumab potentially relies on boosting immune infiltration into tumors (62). Also, inhibition of endothelin receptor signaling has been shown to restore endothelial ICAM-1 expression, increase T-cell infiltration and importantly, act synergistically together with a cancer vaccine (63). Regardless of its prototypical role in angiogenesis, VEGF is also known as a cytokine that suppresses T-cell function and DC activation. Hence VEGF-targeted anti-angiogenic therapy can also exert positive immunomodulatory effects in a cancer immunotherapy setting (64–66).

Fighting Suppressive Immune Cells in the Tumor Microenvironment

A next obstacle for vaccine elicited T-cells is the influence of several immune suppressive leukocytes that populate

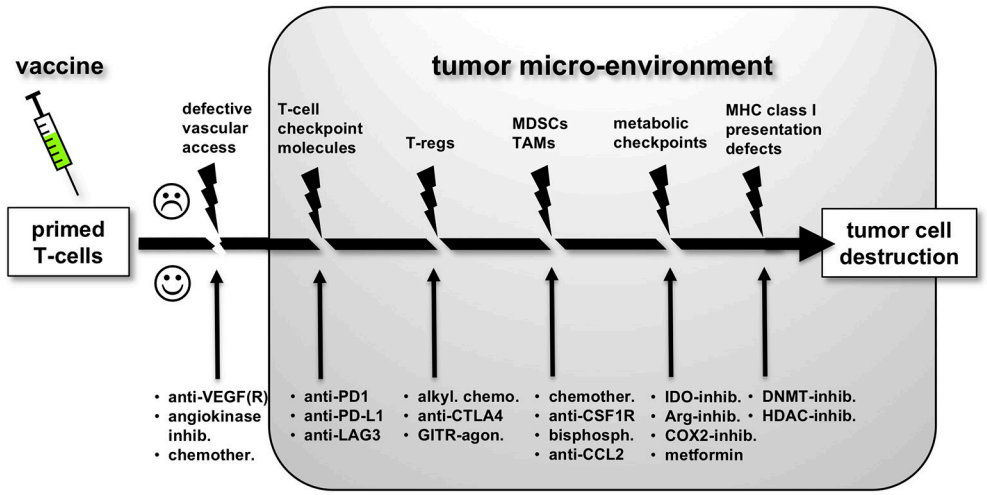

FIGURE 1 | The multiple obstacles to effective anti-tumor immune responses following successful priming of tumor antigen-specific T-cells by a vaccine. Each obstacle offers opportunities for therapeutic intervention in order to increase vaccine efficacy, as discussed in more detail in the main text.

the tumor micro-environment, foremost **regulatory T-cells (T-regs)** and **myeloid-derived suppressor cells (MDSCs)**. **T-regs** are known to be preferentially recruited into tumors and inhibit the functions of antitumoral T-cells by producing immunosuppressive mediators such as interleukin-10 (IL-10), transforming growth factor-beta (TGF-β) and adenosine or by consuming interleukin-2 (IL-2) which is critical for cytolytic T-lymphocyte (CTL) proliferation. In a clinical trial involving a NY-ESO1-ISCOMATRIX vaccine in melanoma, absence of clinical efficacy and cellular immune responses was correlated to increased T-reg activity in metastatic compared to early stage patients (67). Preclinical exploration of this phenomenon in a mouse model of pancreatic cancer showed that impaired responses to ISCOM vaccine can be restored by anti-CD25 mAb-mediated depletion of T-regs, or interestingly by adding low-dose CpG-ODN to the ISCOM formulation (68). Numerous other preclinical studies have shown that therapeutic vaccine efficacy can be boosted by depleting T-regs *in vivo* (69). However, selectively eliminating T-regs in a clinical setting is not a straightforward task. As an example the alkylating agent cyclophosphamide can decrease the number of T-regs in cancer patients (70), however this effect is not easily reproducible and is only achieved within a narrow dose range ("metronomic scheduling"). The development of new clinical-grade compounds that can specifically interfere with the suppressive function of T-regs enables interesting combinatorial approaches with vaccines. T-regs typically express high levels of CTLA-4, and the anti-CTLA4 checkpoint inhibitor ipilimumab, being an IgG1-class antibody, can mediate Fc-dependent depletion of these cells in the tumor micro-environment (TME) (71). Glucocorticoid-induced tumor necrosis factor (TNF) receptor related gene (GITR) is another receptor that is highly expressed on T-regs. Engaging GITR with an agonist has the capacity to shut down the immunosuppressive functions of T-regs, while also stimulating CD8+ T-cell function (72). GITR agonists are currently in clinical development as an add-on to anti-PD-1 checkpoint blockade. Preclinical experiments also indicate a clear synergism between GITR agonists and therapeutic cancer vaccines (73, 74), yet to date no clinical trials are investigating this avenue in cancer patients.

MDSCs constitute another potential obstacle to vaccine success. This heterogenous population of immature monocytic and granulocytic leukocytes are released from the bone marrow in advanced cancer patients and can severely disrupt CD8+ T-cell function through several mechanisms. For instance, MDSCs produce high levels of nitrogen monoxyde (NO) and reactive oxygen species (ROS), combining to form nitrosamines that impair TCR function (75). MDSCs also typically overexpress arginase 1 which depletes arginine in the TME, thereby depriving effector T-cells with an essential "fuel" for proliferation (76). Tumor-associated macrophages are myeloid cells which share several T-cell suppressive properties with MDSCs. Tumor-associated macrophages (TAMs) release TGF-β, IL-10, pro-fibrogenic, and pro-angiogenetic factors (77).

Several classes of compounds can be "repurposed" to achieve a reduction of MDSCs both systemically and intratumorally, and/or interfere with these cell's suppressive capacity (78). In many cases this results in enhancement of T-cell responses in a therapeutic cancer vaccine setting. This is true for myeloablative **chemotherapeutics** such as platinum salts, taxanes, and anti-metabolites (gemcitabine, 5-FU) (79–81), which are known to decrease systemic MDSC numbers in metastatic cancer patients. In preclinical vaccination models, this has been shown to translate into a boosted in T-cell response to vaccination (82, 83). Alternative strategies to target suppressive myeloid cells include administration of all-trans retinoic acids, triterpenoids, phosphodiesterase inhibitors (e.g., sildenafil), tyrosine kinase inhibitors (e.g., sunitinib), amino-bisphosphonates, recombinant IL-12 and anti-IL-6R monoclonal antibodies (84–89). Anti-CSF-1R and anti-CCL2 can both reduce the recruitment of

TABLE 1 | Current clinical trial landscape exploring combinatorial approaches to improve therapeutic cancer vaccine efficacy.

Clinical trial I.D.	Study title	Interventions	Phase
(A) Cancer Vaccine + Angiogenesis-Targeting			
NCT03050814	Standard of Care Alone or in Combination With Ad-CEA Vaccine and Avelumab in People With Previously Untreated Metastatic Colorectal Cancer QUILT-2.004	Drug: Avelumab|Biological: Ad-CEA vaccine|Drug: Bevacizumab|Drug: 5-FU|Drug: Leucovorin|Drug: Oxaliplatin|Drug: Capecitabine	Phase 2
NCT02754362	A Toll-like Receptor Agonist as an Adjuvant to Tumor Associated Antigens (TAA) Mixed With Montanide ISA-51 VG With Bevacizumab for Patients With Recurrent Glioblastoma	Drug: Bevacizumab|Biological: Peptide Vaccine|Drug: Poly-ICLC as immune adjuvant|Drug: Keyhole limpet hemocyanin (KLH)	Phase 2
NCT02432846	Intratumoral Vaccination With Intuvax Pre-nephrectomy Followed by Sunitinib Post-nephrectomy vs. Sunitinib Post-nephrectomy in Newly Diagnosed Metastatic Renal Cell Carcinoma (mRCC)	Biological: Intuvax (ilixadencel)|Drug: Sunitinib	Phase 2
NCT02010606	Phase I Study of a Dendritic Cell Vaccine for Patients With Either Newly Diagnosed or Recurrent Glioblastoma	Biological: Dendritic cell vaccination, in addition to standard temozolomide chemotherapy and involved field radiation therapy|Biological: Dendritic cell vaccination, with optional bevacizumab treatment for patients previously treated with bevacizumab	Phase 1
NCT01814813	Vaccine Therapy With Bevacizumab vs. Bevacizumab Alone in Treating Patients With Recurrent Glioblastoma Multiforme That Can Be Removed by Surgery	Biological: HSPPC-96|Drug: bevacizumab	Phase 2
NCT01551745	Salvage Ovarian FANG Vaccine + Bevacizumab	Biological: Vigil,Ñc Vaccine|Drug: Bevacizumab	Phase 2
NCT01312376	Autologous T-Cells Combined With Autologous OC-DC Vaccine in Ovarian Cancer	Biological: OC-DC vaccine|Drug: Bevacizumab|Drug: cyclophosphamide 300 mg/m^2/d for 3 days|Drug: fludarabine 30 mg/m^2/d for 3 days|Drug: *ex vivo* CD3/CD28-costimulated vaccine-primed peripheral blood autologous T cells	Phase 1
NCT01223235	Polyvalent Vaccine-KLH Conjugate + Opt-821 Given in Combination With Bevacizumab	Biological: bevacizumab and the polyvalent vaccine-KLH conjugate + OPT-821	N/A
NCT00913913	Bevacizumab, Autologous Tumor/DC Vaccine, IL-2 and IFNŒ±-2b in Metastatic Renal Cell Carcinoma (RCC) Patients	Biological: DC vaccine|Drug: Bevacizumab|Biological: IL-2|Biological: IFN	Phase 2
NCT00874588	Peptide Vaccine Targeting to Cancer Specific Antigen Combined With Anti-angiogenic Peptide Antigen in Treating Patients With Non-small Cell Lung Cancer	Biological: HLA-A*2402restricted URLC10, CDCA1, VEGFR1, and VEGFR2	Phase 1
NCT00828009	BLP25 Liposome Vaccine and Bevacizumab After Chemotherapy and Radiation Therapy in Treating Patients With Newly Diagnosed Stage IIIA or Stage IIIB Non-Small Cell Lung Cancer That Cannot Be Removed by Surgery	Biological: bevacizumab|Biological: emepepimut-S|Drug: carboplatin|Drug: cyclophosphamide|Drug: paclitaxel|Radiation: radiation therapy	Phase 2
(B) Cancer Vaccine + TAM/MDSC-Targeting			
NCT02544880	PDE5 Inhibition Via Tadalafil to Enhance Anti-Tumor Mucin 1 (MUC1) Vaccine Efficacy in Patients With HNSCC	Drug: Tadalafil|Biological: Anti-MUC1 Vaccine|Biological: Anti-Influenza Vaccine|Other: Tadalafil Placebo|Other: Vaccine Placebo|Procedure: Peripheral Blood Collection|Procedure: DTH Skin Test|Procedure: Tumor specimen collection	Phase 1/2
NCT02479230	Type I-Polarized Autologous Dendritic Cell Vaccine With Tumor Blood Vessel Antigen-Derived Peptides in Metastatic Breast Cancer Patients	Biological: tumor blood vessel antigen peptide-pulsed alpha-type-1 polarized dendritic cell vaccine|Drug: gemcitabine hydrochloride	Phase 1
NCT02432378	Intensive Locoregional Chemoimmunotherapy for Recurrent Ovarian Cancer Plus Intranodal DC Vaccines	Biological: Cisplatin + celecoxib + DC vaccine|Biological: Cisplatin + CKM + Celecoxib + DC Vaccine	Phase 1/2
NCT02275039	p53MVA Vaccine and Gemcitabine Hydrochloride in Treating Patients With Recurrent Ovarian Epithelial Cancer	Biological: modified vaccinia virus ankara vaccine expressing p53|Drug: gemcitabine hydrochloride|Other: laboratory biomarker analysis	Phase 1
NCT01876212	Dendritic Cell Vaccines + Dasatinib for Metastatic Melanoma	Biological: DC vaccine|Drug: Dasatinib	Phase 2
NCT01803152	Dendritic Cell Vaccine With or Without Gemcitabine Pre-Treatment for Adults and Children With Sarcoma	Biological: Dendritic Cells Vaccine|Biological: Lysate of Tumor|Drug: Gemcitabine|Drug: Imiquimod|Procedure: Leukapheresis	Phase 1

(Continued)

TABLE 1 | Continued

Clinical trial I.D.	Study title	Interventions	Phase
NCT01697800	A Phase II Trial of Tadalafil in Patients With Squamous Cell Carcinoma of the Upper Aero Digestive Tract	Drug: Tadalafil|Drug: Placebo	Phase 2
NCT02616185	A Phase 1 Study To Evaluate Escalating Doses Of A Vaccine-Based Immunotherapy Regimen For Prostate Cancer (PrCa VBIR)	Biological: PF-06755992|Biological: PF-06755990|Device: TDS-IM Electroporation Device|Biological: Tremelimumab|Drug: Sunitinib|Biological: PF-06801591	Phase 1
NCT02432846	Intratumoral Vaccination With Intuvax Pre-nephrectomy Followed by Sunitinib Post-nephrectomy vs. Sunitinib Post-nephrectomy in Newly Diagnosed Metastatic Renal Cell Carcinoma (mRCC)	Biological: Intuvax (ilixadencel)|Drug: Sunitinib	Phase 2
NCT03153410	Pilot Study With CY, Pembrolizumab, GVAX, and IMC-CS4 (LY3022855) in Patients With Borderline Resectable Adenocarcinoma of the Pancreas	Drug: Cyclophosphamide|Drug: GVAX|Drug: Pembrolizumab|Drug: IMC-CS4	Early Phase 1
NCT02432378	Intensive Locoregional Chemoimmunotherapy for Recurrent Ovarian Cancer Plus Intranodal DC Vaccines	Biological: Cisplatin + celecoxib + DC vaccine|Biological: Cisplatin + CKM + Celecoxib + DC Vaccine	Phase 1/2
(C) Cancer Vaccine + T-Reg-Targeting			
NCT03203005	IMA970A Plus CV8102 in Very Early, Early and Intermediate Stage Hepatocellular Carcinoma Patients	Drug: IMA970A plus CV8102 and Cyclophosphamide	Phase 1/2
NCT03066947	SV-BR-1-GM in Metastatic or Locally Recurrent Breast Cancer	Biological: SV-BR-1-GM|Drug: Cyclophosphamide|Biological: Interferon-alpha-2b	Phase 1/2
NCT02709993	Consolidation Therapy in Patients With Hematologic Malignancies	Biological: TAPA-pulsed DC vaccine	Phase 1/2
NCT02705703	Consolidation Therapy in Patients With Metastatic Solid Malignancies	Biological: TAPA-pulsed DC vaccine	Phase 1/2
NCT02390063	Vaccination in Prostate Cancer (VANCE)	Biological: ChAdOx1.5T4|Biological: MVA.5T4|Drug: Cyclophosphamide	Phase 1
NCT02224599	Treatment of Patients With Progressive and/or Refractory Solid Malignancies	Biological: TAPA-pulsed DC vaccine	Phase 1/2
NCT02223312	Therapy for Progressive and/or Refractory Hematologic Malignancies	Biological: TAPA-pulsed DC vaccine	Phase 1/2
NCT01696877	A Neoadjuvant Study of Androgen Ablation Combined With Cyclophosphamide and GVAX Vaccine for Localized Prostate Cancer	Drug: degarelix acetate|Drug: Cyclophosphamide|Drug: GVAX	Phase 1/2
NCT01192555	Allogeneic Tumor Cell Vaccination With Oral Metronomic Cytoxan in Patients With High-Risk Neuroblastoma	Biological: Neuroblastoma Vaccine (unmodified SKNLP, with gene-modified SJNB-JF-IL2 and SJNB-JF-LTN neuroblastoma cells)|Drug: Cytoxan	Phase 1/2
NCT00703105	Ovarian Dendritic Cell Vaccine Trial	Biological: Ontak DC|Biological: DC vaccination|Drug: Ontak	Phase 2
NCT00626483	Basiliximab in Treating Patients With Newly Diagnosed Glioblastoma Multiforme Undergoing Targeted Immunotherapy and Temozolomide-Caused Lymphopenia	Biological: RNA-loaded dendritic cell vaccine|Drug: basiliximab	Phase 1
NCT00515528	Vaccination Plus Ontak in Patients With Metastatic Melanoma	Drug: 4-peptide melanoma vaccine|Drug: 4-peptide melanoma vaccine plus Ontak|Drug: ontak	Phase 2
(D) Cancer Vaccine + Checkpoint inhibition			
NCT03548467	A Study to Evaluate Safety, Feasibility, Efficacy of Multiple Dosing With VB10.NEO Immunotherapy in Patients With Locally Advanced or Metastatic Cancer	Drug: VB10.NEO	Phase 1/2
NCT03532217	Neoantigen DNA Vaccine in Combination With Nivolumab/Ipilimumab and PROSTVAC in Metastatic Hormone-Sensitive Prostate Cancer	Biological: PROSTVAC-V|Biological: PROSTVAC-F|Drug: Nivolumab|Drug: Ipilimumab|Biological: Neoantigen DNA vaccine|Device: TriGrid Delivery System|Procedure: Tumor biopsy|Procedure: Peripheral blood|Procedure: Fecal samples	Phase 1
NCT03422094	Neoantigen-based Personalized Vaccine Combined With Immune Checkpoint Blockade Therapy in Patients With Newly Diagnosed, Unmethylated Glioblastoma	Biological: NeoVax|Biological: Nivolumab|Biological: Ipilimumab|Procedure: Research blood draw|Procedure: Leukapheresis for research	Phase 1

(Continued)

TABLE 1 | Continued

Clinical trial I.D.	Study title	Interventions	Phase
NCT03362060	PVX-410 Vaccine Plus Pembrolizumab in HLA-A2+ Metastatic Triple Negative Breast Cancer	Drug: Pembrolizumab|Biological: PVX-410	Phase 1
NCT03311334	A Study of DSP-7888 Dosing Emulsion in Combination With Immune Checkpoint Inhibitors in Adult Subjects With Advanced Solid Tumors	Drug: DSP-7888 Dosing Emulsion|Drug: Nivolumab|Drug: Atezolizumab	Phase 1
NCT02654587	Study of OSE2101 vs. Standard Treatment as 2nd or 3rd Line in HLA-A2 Positive Patients With Advanced NSCLC After Failure of Immune Checkpoint Inhibitor	Drug: OSE2101|Drug: Docetaxel|Drug: Pemetrexed	Phase 3
NCT03113487	P53MVA and Pembrolizumab in Treating Patients With Recurrent Ovarian, Primary Peritoneal, or Fallopian Tube Cancer	Other: Laboratory Biomarker Analysis|Biological: Modified Vaccinia Virus Ankara Vaccine Expressing p53|Biological: Pembrolizumab	Phase 2
NCT02977156	Immunization Strategy With Intra-tumoral Injections of Pexa-Vec With Ipilimumab in Metastatic / Advanced Solid Tumors.	Biological: Pexa-Vec|Drug: Ipilimumab	Phase 1
NCT02506114	Neoadjuvant PROSTVAC-VF With or Without Ipilimumab for Prostate Cancer	Biological: PROSTVAC V/F|Drug: Ipilimumab	Phase 2
NCT02432963	Vaccine Therapy and Pembrolizumab in Treating Patients With Solid Tumors That Have Failed Prior Therapy	Other: Laboratory Biomarker Analysis|Biological: Modified Vaccinia Virus Ankara Vaccine Expressing p53|Biological: Pembrolizumab	Phase 1
(E) Cancer Vaccine + Costimulation Agonists			
NCT03258008	Utomilumab and ISA101b Vaccination in Patients With HPV-16-Positive Incurable Oropharyngeal Cancer	Drug: Utomilumab|Biological: ISA101b	Phase 2
NCT01898039	Modified Melanoma Vaccine for High Risk or Low Residual Disease Patients	Biological: A2/4-1BBL melanoma vaccine|Procedure: DNP sensititzation|Drug: Cyclophosphamide	Phase 1/2
NCT01861938	Modified Melanoma Vaccine for High Risk or Low Residual Disease Patients	Biological: Melanoma vaccine modified to express HLA A2/4-1BB ligand	Phase 2/3
NCT01644968	Phase 1 Study of Anti-OX40 in Patients With Advanced Cancer	Drug: Cohort 1 anti-OX40|Drug: Cohort 2 anti-OX40|Drug: Cohort 3 anti-OX40|Biological: Tetanus Day 29|Biological: Tetanus Day 1|Biological: KLH Day 1|Biological: KLH Day 29	Phase 1
NCT00534209	Vaccine Therapy in Patients With Stages IIIB/IV Non-Small Cell Lung Cancer Who Have Finished First-Line Chemotherapy	Biological: Allogeneic B7.1/HLA-A1|Other: Placebo	Phase 1/2
NCT00031564	Phase II Study of a B7-1 Gene-Modified Autologous Tumor Cell Vaccine and Systemic IL-2	Biological: Interleukin-2|Biological: B7-1	Phase 2
(F) Cancer Vaccine + IDO-Inhibition			
NCT02166905	DEC-205/NY-ESO-1 Fusion Protein CDX-1401, Poly ICLC, and IDO1 Inhibitor INCB024360 in Treating Patients With Ovarian, Fallopian Tube, or Primary Peritoneal Cancer in Remission	Biological: DEC-205/NY-ESO-1 Fusion Protein CDX-1401|Drug: Epacadostat|Other: Laboratory Biomarker Analysis|Other: Pharmacological Study|Drug: Poly ICLC	Phase 1/2
NCT03047928	Combination Therapy With Nivolumab and PD-L1/IDO Peptide Vaccine to Patients With Metastatic Melanoma	Drug: Nivolumab|Biological: PD-L1/IDO peptide vaccine	Phase 1/2
(G) Cancer Vaccine + Epigenetic Modulation			
NCT02166905	DEC-205/NY-ESO-1 Fusion Protein CDX-1401, Poly ICLC, and IDO1 Inhibitor INCB024360 in Treating Patients With Ovarian, Fallopian Tube, or Primary Peritoneal Cancer in Remission	Biological: DEC-205/NY-ESO-1 Fusion Protein CDX-1401|Drug: Epacadostat|Other: Laboratory Biomarker Analysis|Other: Pharmacological Study|Drug: Poly ICLC	Phase 1/2
NCT02886065	A Study of PVX-410, a Cancer Vaccine, and Citarinostat +/- Lenalidomide for Smoldering MM	Drug: Hiltonol|Drug: Citarinostat|Drug: Lenalidomide|Biological: PVX-410	Phase 1

Combinations were structured in line with discussion in the text. Database searches were focused on combinations with agents that target ***(A)*** *angiogenesis,* ***(B)*** *MDSCs/TAMs,* ***(C)*** *T-regs,* ***(D)*** *immune checkpoint molecules,* ***(E)*** *costimulatory molecules,* ***(F)*** *IDO, and* ***(G)*** *epigenetic modifications. No trials were found combining vaccines with interventions targeting immunosuppressive cytokines (IL-10, TGF-β, IL-6), arginase activity, hypoxic metabolism or adenosine signaling. Notes: Database search restricted to clinical trials that are active or will be activated in the near future. Only antigen-specific vaccination protocols were retained (e.g., the use of radiotherapy or intratumoral injections of checkpoint inhibitors was excluded). Combinations with anti-CTLA4 were listed onder "Vaccine + checkpoint inhibition" even though CTLA-4 blockers such as ipilimumab may also directly deplete T-regs. Interventions targeted at hematological malignancies were omitted.*

MDSCs and monocyte-derived TAMs into the tumor bed and also contribute to revert the immunosuppressive climate within tumors (90, 91).

Finally, as noted earlier, next to their adjuvant property in itself, STING agonists have the interesting property of being able to reprogram MDSCs from a T-cell suppressive into a type-1 immune polarizing leukocyte (27).

Freeing T-Cells From Negative Checkpoint Signals

On a molecular level, tumor beds also maintain a climate of tolerance and immune suppression through the abundant expression of **T-cell checkpoint ligands** and a relative lack of costimulatory molecules. Fortunately, the field of immuno-oncology is currently driven forward by the development of several compounds that can disrupt this inhibitory climate: in a first wave of clinical trials, **immune checkpoint inhibitors (ICIs)** such as **CTLA-4**, **PD-1** and **PD-L1** blocking antibodies have demonstrated unequivocal clinical activity as monotherapy in many types of cancer. The performance plateau of immune checkpoint blockade is now being pushed upward by applying combinatorial strategies (e.g., ICI + chemotherapy or ICI + ICI). It can be expected that combinatorial approaches that include ICIs will be the major development that will unlock the full potential of cancer vaccines. Indeed, a robust activation of T-cells (as potentially achieved by a powerful vaccine) will induce expression of counterregulatory checkpoints such as CTLA-4 and PD-1. CTLA-4 can "steal the steam" of signaling through the B7-CD28 costimulatory axis, hereby shutting down T-cell activation by the APC. PD-1, when engaging PD-L1 which is abundantly expressed on cancer cells and intratumoral myeloid cells by exposure to IFN-γ and/or hypoxia, results in paralysis of T-cell effectors at the tumor front. As a clinical indication for this obstacle to vaccine efficacy, in the trial evaluating the TG4010 Muc-1 vaccine in lung cancer only patients whose tumor expressed low levels of PD-L1 had a marked benefit in progression-free survival (56).

Mechanistically, ICIs can potentiate vaccine responses in two main ways. Anti-CTLA-4 checkpoint inhibition will mainly act by boosting the amplitude of the priming phase, by broadening the repertoire of the T-cell response (92) and also by removing the suppressive activity of T-regs in the TME, as noted earlier. PD-1 or PD-L1 blockade will ensure that vaccine-elicited anti-tumoral T-cells can exert their function unhampered once inside the tumor micro-environment. Conversely, vaccination may be an additional combination partner to improve the performance of checkpoint inhibition, whose response rate as monotherapy across all tumors plateaus around 20% in biomarker-unselected patients.

The benefits of combining vaccines with ICIs have been demonstrated in numerous preclinical tumor models (93–96), and these proof-of-concepts have already led to the design of several clinical trials (summarized in **Table 1D**). Initial results in humans were not encouraging though, when a pivotal trial showed no benefit at all of combining an adjuvanted gp100 peptide vaccine with anti-CTLA4, compared with anti-CTLA4 alone (44). However, more advanced vaccine platforms may still benefit from combination with ICI, as illustrated by a more recent phase 2 trial exploring the combination of a DC vaccine plus ipilimumab: objective response rates and survival were markedly superior than historical data with ipilimumab as monotherapy (97).

The relative **timing** of vaccination and immune checkpoint blockade could be very critical for optimal anti-tumor effect. CTLA-4 blockade was found to synergize optimally with a prostate cancer GVAX vaccine when administered after vaccination (98). Likewise, responses to TG4010 (Muc-1-targeted MVA vaccine) were enhanced when PD-1 blockade was administered several days after the vaccine (99). By contrast McNeel et al. observed that responses to a PSA-targeted DNA vaccine against prostate cancer were only observed with concurrent rather than sequential PD-1 checkpoint blockade, both in murine models as well as in a small clinical trial (100). The sequencing could be different when it comes to PD-L1 blockade: PD-L1 upregulation is a physiological phenomenon upon DC activation which may serve to protect the DC from elimination during cognate interaction with the CD8+ T-cell. Hence, PD-L1 blockade at the time of vaccination/DC activation may result in abortive T-cell priming due to shortened APC survival and limit effector T-cell polarization and expansion.

Additional checkpoint molecules are currently being explored as clinical targets. Lymphocyte-activation gene 3 (**LAG3)** is the third immune checkpoint to have been targeted in humans after CTLA4 and the PD-1/PD-L1 axis. LAG-3 is expressed by "exhausted" TILs and T-regs. It shares high structural homology to CD4 and binds MHC class II on APCs. Besides keeping the T-cell itself in an inactive state, LAG3 can reverse-signal to the APC and maintain the latter in an immature/pro-tolerogenic state with impaired upregulation of costimulatory molecules and IL-12 secretion (101). LAG3 blockade as such shows limited effects, but it can roughly double the response rate to PD-1 blockade when used in combination, an added benefit that is clearly enhanced in LAG3-expressing tumor beds (NCT01968109, P. Ascierto et al presented at ESMO 2017). Interestingly, a soluble dimeric recombinant protein consisting of four LAG3 extracellular domains fused to the Fc portion of human IgG1 (LAG3-Ig) has been shown to act as an "APC activator" (102). A possible concern however is that it also stimulates release of the chemokines CCL17 and CCL22, which are known to preferentially attract Th2 lymphocytes and T-regs. The clinical compound, IMP321, is now being evaluated in patients in combination with cancer vaccines in different tumor settings (**Table 1D**).

Besides an abundance in negative checkpoint molecules, the tumor milieu also fosters immune tolerance through a lack in costimulatory molecules. Agonists of T-cell costimulatory pathways are in clinical development, notably monoclonal antibodies that bind to TNF-superfamily receptors such as OX40 and 4-1BB. Preclinical experiments indicate that costimulation agonists can synergize with vaccination to break tolerance toward

poorly immunogenic tumors (103, 104), with several clinical trials now underway (**Table 1E**).

Dealing With the Immunosuppressive Metabolic Tumor Environment

Next to defined molecular axes, the global metabolic climate within solid tumors provides a hostile environment for proper effector T-cell function as well. An important counterregulatory mechanism in response to an IFN-γ-dominated T-cell attack is the upregulation of **IDO** (indoleamine-2,3-dioxygenase). Also, activation of DCs results in IDO expression in these cells and promotes paradoxical induction of T-regs (105). Prostaglandin E2, generated by COX2-expressing TAMs, is also an inducer of IDO (106, 107). Originally identified as a major contributor to immune tolerance at the maternofoetal interface (108), IDO enzymatic activity is now recognized as one of the "metabolic checkpoints" in tumors such as melanoma and lung cancer: IDO catabolises tryptophan, which is also a "fuel" for proper T-cell activation and proliferation, into kynurenines that act as T-cell toxic metabolites. Tryptophan depletion will also favor the induction of T-regs (109). IDO inhibitors have demonstrated positive effects in many preclinical models of cancer immunotherapy (109). Clinical development of IDO inhibitors took a hit recently with negative phase 3 results in combination with ICI in melanoma, despite promising phase 2 data (NCT02752074, results presented at ASCO 2018). Nevertheless, results in other tumors are still pending, and combining IDO-inhibition with a vaccine may still be an effective strategy (110) (**Table 1F**). **Arginase** activity is also increased in tumors in proportion to myeloid cell infiltration and induces T-cell paralysis by depleting arginine (as described above). Arginase inhibitors are currently in early clinical development [NCT02903914 (111)], with preclinical data showing clear synergism with anti-PD-L1 checkpoint inhibition (112). No clinical trials combining arginase inhibitors with a cancer vaccine have been reported to date.

More difficult to correct through therapeutic intervention are the consequences of **aberrant energy metabolism** in tumors, where cancer cells out-compete TILs for glucose availability and establish a high lactate/low-pH milieu that blocks T-cell proliferation and IFN-γ release (113). These conditions are further exacerbated by the poor quality of the tumor vasculature which prevents proper clearance of toxic metabolites and exacerbates intratumoral **hypoxia**. The latter induces upregulation of glucose transporters on tumor cells, further decreasing extracellular glucose availability for effector T-cells.

Metformin, better known as a therapy for insulin-resistant diabetes, also inhibits cancer cell oxygen consumption. This has been shown to decrease tumoral hypoxia, hereby augmenting intratumoral CD8+ T-cell activation and unlocking synergistic effects with checkpoint blockade in otherwise immunotherapy-resistant tumors (114).

Hypoxia also increases expression of **ectonucleotidases** on the cell membrane of cancer cells and myeloid cells, resulting in degradation of ATP to **adenosine**. Adenosine triggers A2AR, the most predominant adenosine receptor on immune cells, leading to an increase in intracellular cAMP levels which mediates a plethora of immunosuppressive effects: inhibition T-cell and NK-cell functions, suppression of DC maturation and IL-12 secretion, increase in IL-10 production, induction of T-regs (115).

A2AR antagonists have been developed, with preclinical studies showing promising activity. In a phase I trial the A2AR antagonist CPI-444 produced marked CD8 T-cell infiltration when comparing pre- vs. post-treatment biopsies (116). Preliminary clinical data suggests synergism with PD-L1 blockade, however it is clear from their biological effect that adenosine receptor or ectonucleotidase inhibitors could be attractive add-ons in a therapeutic vaccine setting.

Improving Tumor Visibility to the Immune System

For vaccine-induced T-cells to fulfil their final role, in addition to intratumoral penetration and surmounting suppressive mechanisms, tumor cells must expose sufficient levels of relevant antigen on their surface. This cannot be taken for granted as cancer cells can reduce expression of tumor-associated antigens or downregulate critical components of the antigen-processing and MHC presentation machinery. Interestingly, this loss of "visibility" to the immune system seems to be mediated by **epigenetic mechanisms**, i.e., DNA hypermethylation and histone deacetylation, which opens up opportunity for therapeutic modulation (117). Expression of cancer-testis antigens is in particular regulated through epigenetic mechanisms, and treatment with DNA methyl transferase (DNMT) inhibitors can increase cancer-testis antigen (CTAG) expression levels on cancer cells. Components of the antigen-processing machinery (APM) such as TAP-1, TAP-2, LMP-2 and Tapasin can be increased by treatment of cancer cells with histone deacetylase (HDAC) inhibitors, which ends up increasing surface expression of MHC class I molecules as well (118, 119).

In addition, epigenetic drugs can help create a more favorite immunological climate within tumors. HDAC inhibitors have been shown to induce Th1, CD8 and NK-cell-attracting chemokines and boost response to anti-PD1 immune checkpoint blockade (120). The combination of DNMT and HDAC-inhibition can also potentiate ICI efficacy by reducing granulocytic MDSC levels (121). Another fascinating discovery is the fact that DNMT-inhibitors can awaken expression of endogenous retroviral vectors (also known as long terminal repeat retro-transposons), thus generating intracellular dsRNAs that can be sensed by the MAD5/MAVS cytosolic sensor and trigger type 1 interferon responses (122).

A large number of clinical trials are now combining checkpoint inhibitors with epigenetic modulators, however only 1 trial exploring the combination a DNMT-inhibitor with a DC-based cancer vaccines in pediatric sarcoma has been completed:

remarkably 1 patient of the 10 included experienced a complete response (123). A few other trials combining vaccination with epigenetic modulation are active at the time of this writing (**Table 1G**).

CONCLUSION

Given the daunting complexity of tumor-associated immune suppressive networks, it comes as no surprise that vaccination in a therapeutic setting has delivered so little benefits to cancer patients so far. Still, the overwhelming amount of preclinical data supports the notion that vaccination can control or even eradicate tumors, just as preclinical work showed the value of immune checkpoint blockade many years ago. Given the multiple obstacles to T-cell mediated cancer cell destruction, it is clear that the success of a vaccine will depend on our capacity to accurately map the dominant immunosuppressive pathway for each individual patient. An essential aspect when it comes to therapeutic modulation of these pathways is to delineate the hierarchy of obstacles to effective immune responses. For instance, combining a vaccine with immune checkpoint blockade is an effort in vain when a large part of the tumor has acquired defects in MHC class I presentation. An important challenge will be to develop technologies that can deliver comprehensive tumor "immunomics" in a timely and cost-effective fashion. The aim is to provide clinicians with robust biomarkers to guide therapeutic decision making especially when it comes to the wide repertoire of possible combination therapies. An additional challenge is to take into account both the spatial and the temporal heterogeneity of a tumor for a given patient, i.e., are different metastatic sites sensitive/resistant to immunotherapy to the same extent, and how does this evolve over time during the course of specific treatments? As the field of cancer immunology further evolves, several additional questions are raised: what is the role of CD4+ T-cells in vaccine-induced anti-tumor responses? Which could be the optimal chemotherapy or radiotherapy regimen in combination with a cancer vaccine? Does the gut microbiome impact on cancer vaccine efficacy the same way as it influences responses to checkpoint inhibitors? As difficult as these challenges may be, the reward is considerable given the excellent tolerability of vaccines and the promise of long term protective immunological memory, which may transform disease control into cure.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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SUPPLEMENTARY MATERIAL

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Design of Peptide-Based Nanovaccines Targeting Leading Antigens From Gynecological Cancers to Induce HLA-A2.1 Restricted CD8$^+$ T Cell Responses

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Gynecological cancers are a leading cause of mortality in women. CD8$^+$ T cell immunity largely correlates with enhanced survival, whereas inflammation is associated with poor prognosis. Previous studies have shown polystyrene nanoparticles (PSNPs) are biocompatible, do not induce inflammation and when used as vaccine carriers for model peptides induce CD8$^+$ T cell responses. Herein we test the immunogenicity of 24 different peptides, from three leading vaccine target proteins in gynecological cancers: the E7 protein of human papilloma virus (HPV); Wilms Tumor antigen 1 (WT1) and survivin (SV), in PSNP conjugate vaccines. Of relevance to vaccine development was the finding that a minimal CD8$^+$ T cell peptide epitope from HPV was not able to induce HLA-A2.1 specific CD8$^+$ T cell responses in transgenic humanized mice using conventional adjuvants such as CpG, but was nevertheless able to generate strong immunity when delivered as part of a specific longer peptide conjugated to PSNPs vaccines. Conversely, in most cases, when the minimal CD8$^+$ T cell epitopes were able to induce immune responses (with WT1 or SV super agonists) in CpG, they also induced responses when conjugated to PSNPs. In this case, extending the sequence around the CD8$^+$ T cell epitope, using the natural protein context, or engineering linker sequences proposed to enhance antigen processing, had minimal effects in enhancing or changing the cross-reactivity pattern induced by the super agonists. Nanoparticle approaches, such as PSNPs, therefore may offer an alternative vaccination strategy when conventional adjuvants are unable to elicit the desired CD8$^+$ T cell specificity. The findings herein also offer sequence specific insights into peptide vaccine design for nanoparticle-based vaccine carriers.

Keywords: nanoparticles, HPV, WT1, survivin, CD8 T cell epitopes, vaccine, immunogenicity, HLA-A2.1

INTRODUCTION

Gynecological malignancies, including ovarian, endometrial, vulvar, fallopian tube and cervical cancers, are the leading cause of mortality in women (∼9.8% of cancer related deaths in women) (1), with the most lethal malignancy being ovarian cancer (2, 3). There are many factors that cause gynecologic cancers. Although oncogenes and tumor suppressor genes promote the growth of cancer, almost all cervical cancers and some cancers of the vagina and vulva are caused by a virus known as Human Papillomavirus (HPV). The development of a preventive vaccine to limit the infectivity and transmission of the HPV, working primarily through the induction of virus neutralizing antibodies, is a tremendous positive step forwards, but is not able to be used therapeutically (4–6). Moreover, there are also no licensed vaccines to target and treat the other gynecological malignancies, such as to ovarian cancer.

High levels of tumor infiltrating $CD8^+$ T cells are associated with increased survival in patients with diverse gynecological malignancies, notably, with ovarian cancer (7, 8). Emerging immunotherapies which can re-establish full functionality for $CD8^+$ T cells in the local tumor microenvironment, based primarily on disrupting immunosuppressive PD1/PDL1 interactions, are showing great promise in multiple clinical trials, and have been touted as a game-changer for cancer treatment (9). These advances are bringing renewed interest in the development of practical methods to increase initial $CD8^+$ T cell numbers to relevant tumor antigens by vaccination. An additional major emerging trend for cancer immunotherapy is the ability to use high-throughput analysis "omics" techniques, such as transcriptomics, to define tumor subtypes and cancer cell heterogeneity (10, 11). These findings are being used to identify subtypes and hence patients most able to respond clinically to specific chemotherapies, an aspect of "precision" or "personalized" medicine. These omics techniques are also resulting in databases rich in antigen sequences, and are potentially able to define the best target antigens expressed by cancer cells within each patient, and to develop personalized vaccines.

Peptides offer a practical source of antigen for personalizing therapeutic cancer vaccines to induce high levels of $CD8^+$ T cells. They are also non-infectious, completely defined, relatively easy to produce, and are generally considered to be safe. The design of peptide-based vaccines, particularly those involving new generation nanoparticle-based delivery systems, involves the challenge of ensuring correct antigen processing into MHC class I (MHC I) restricted epitopes to promote $CD8^+$ T cell priming. Controversy remains in the literature on the nature of the peptides to be used in such vaccines in the context of cancer, ranging from (1) peptides representing only minimal native $CD8^+$ T cell epitopes; (2) their agonist variants (to help break potential tolerance, or enhance MHC I binding or immunogenicity of peptides representing weak natural epitopes); (3) minimal peptide epitopes with added amino acids at either end, to promote stability in micro-environments which contain exopeptidases, as well as potentially promote appropriate cleavage or processing if the minimal epitopes are covalently conjugated to a nanoparticle; 4) the inclusion of $CD4^+$ T cell epitopes, either by replicating in a peptide region from a protein that contains both $CD4^+$ and $CD8^+$ T cell epitopes, or constructing artificial constructs encompassing in one peptide containing $CD8^+$ and $CD4^+$ epitopes from different proteins. Further in this context, another limitation of peptide-based vaccines/immunotherapy is the need for each immune dominant epitope to match the patient's human leukocyte antigen (HLA). HLA polymorphisms in patients make it difficult to develop a peptide-based vaccine that are broadly applicable across the patient population.

The usually low immunogenicity of cancer associated antigens (which are often overexpressed or variant self-antigens) also needs the selection of powerful vaccine adjuvants and carriers able to promote strong immune responses. We have previous reported that nanoparticles at a specific size (∼50 nm) induce strong immune responses when covalently linked to an antigen (12–14). As a platform technology, the specific size defined polystyrene nanoparticles (PSNPs) have shown powerful self-adjuvanting properties when used to deliver protein model antigens such as ovalbumin (OVA) (12), DNA plasmids expressing OVA (15), as well as high affinity peptides (13, 16), including strong antigens from respiratory syncytial virus (RSV) (17) and malaria liver stage antigens (16, 18). In these studies, PSNPs showed superior adjuvancity to conventional pro-inflammatory adjuvants such as Aluminum hydroxide (Alum), Quil A and monophosphoryl lipid A (MPL) for the induction of antigen specific $CD8^+$ T cell and $CD4^+$ T cells, particularly IFN-γ producing T cells, as well as long lasting antibody levels. A unique feature of the PSNP adjuvanting system is that, in contrast to other adjuvants which work by promoting inflammation via toll-like-receptors (TLRs) or pathogen-recognition-receptors (PRRs) signaling, PSNPs do not induce conventional inflammation (mediated by Erk or Akt signaling) (19), or the induction of conventional pro-inflammatory cytokines such as IL-6 and TNF (20), or the expansion of inflammation reactive regulatory T cells (Tregs) (18). These features could make these, and other systems with similar properties, particularly useful for the development of cancer therapeutic vaccines, where both inflammation and Treg induction are associated with tumor progression (21, 22).

Furthermore, our PSNPs-peptide vaccine formulations have also shown protective and therapeutic efficacies in various murine tumor models with multiple diverse peptide antigens [(12, 13, 15) and unpublished]. However, a major challenge in translation remains in understanding the rules by which to select useful peptides that can be appropriately processed and presented to stimulate CD8 T cell immunity. In this paper we specifically explore this challenge by testing >20 different peptide formulations in HLA-A2.1 transgenic animals. We hypothesized here that PSNPs could be effectively linked (covalently conjugated) to peptide antigens derived from gynecological tumors and generate immunogenic constructs capable of inducing HLA-A2.1 restricted $CD8^+$ T cells. Moreover, herein we explore the diverse formulation challenges using peptides in vaccines generally, and specifically differences in processing into minimal $CD8^+$ T cell epitope using

nanoparticle-based vaccine such as PSNPs. To explore this issue, we studied diverse peptides derived from three different antigens associated with major and diverse gynecological malignancies: the E7 protein from HPV16, a demonstrated major target for $CD8^+$ T cells in cervical cancer (23–25); Survivin (SV), an oncogenic inhibitor-of-apoptosis protein expressed in cervical and ovarian malignancies (26–32); and Wills Tumor antigen 1 (WT1), a well-studied antigen in the context of diverse tumor types such as leukemia and ovarian cancer (33) [reviewed by (34–36)]. WT1 has recently been listed among the top of the 75 ideal cancer antigens in immunotherapies by the U.S. National Cancer Institute (37).

MATERIALS AND METHODS

Peptides and Carrier/Adjuvants

Table 1 lists all the peptides synthesized for this study. Peptide HPV01, HPV05, HPV08, SV01, SV02, and WT1B were synthesized by Auspep (Tullamarine, VIC, Australia); peptides HPV12, SV03 to SV09, WT1A, WT1C, WT1D, and WT1E were synthesized by CS Bio (Menlo Park, CA, United States). The purity (>95%) and identity of peptides were determined by HPLC and mass spectrometry, respectively.

Conjugating Peptide Antigen Onto Nanoparticles (PSNPs)

Selected antigen peptides (from **Table 1**) were chosen as peptide-based vaccine targets to form nanovaccine formulations. Each of the individual peptides were covalently conjugated to 40–50 nm carboxylated polystyrene nanoparticles (PSNPs, Polysciences Inc., Warrington, PA, United States) to form peptide-PSNPs vaccine formulations (e.g., HPV08-PSNPs, WT1B-PSNPs, or SV10-PSNPs etc.). Peptide conjugations were optimized for each peptide in order to achieve the best conjugation efficiency and size. In brief, following the conjugation procedures described previously (20), PSNPs at a final of 1% solids were pre-activated by gently mixing on a rotation wheel for 1 h at room temperature in a mixture containing 2-*N*-Morpholino-ethanesulfonic acid (MES) (50 mM final, pH = 6), 1-ethyl-3-(3-dimethylaminopropryl) carbodiimide hydrochloride (EDC) (4 mg/mL final) (Sigma-Aldrich, St. Louis, United States), *N*-hydrosulfosuccinimide (Sulfo-NHS) (50 mM final) (Pierce™, Thermo Fisher Scientific, Waltham, MA, United States) with final pH adjusted to be 5.5–6. After pre-activation, the excess activation agents (EDC and Sulfo-NHS) were removed from the pre-activation mix using a gel filtration column (Zeba spin desalting column following manufacturer's instruction, Thermo Fisher Scientific), and buffer exchanged at the same time via the column (buffer concentration and pH were optimized for each peptide antigen) before adding the peptide antigen for a further 2 h. The final conjugation mix was then dialysed against phosphate buffer (PBS, ∼pH 7.2–7.4) in 1 kDa dialysis membrane (if non-PBS buffer was used as conjugation buffer). Final conjugation efficiency was determined by BCA™ protein assay (Pierce™ Micro BCA protein assay, Thermo Fisher Scientific) or amino acid analysis via HPLC (performed by Auspep). Particles sizing and polydispersity of the final peptide conjugated PSNPs (peptide-PSNPs) formulation were measured by dynamic light scattering (Zetasizer, Malvern Instruments Ltd, Worcestershire, United Kingdom). Each vaccine dose (100 μL) contained ∼50 μg peptides and ∼0.8–1% solid of PSNPs in PBS. The amounts of peptide antigen injected were matched for all formulations by adjusting the injection volume for each experiment. Those formulations were directly compared to the bench mark adjuvant CpG by direct mixing the testing peptides with CpG (20 μg/injection) (ODN 1826, InvivoGen, San Diego, CA, United States).

Mice and Immunizations

The vaccine study was carried out in accordance with the recommendations of the "Institutional Guidelines and the Animal Welfare Assurance Act, Alfred Medical Research and Education Precinct (AMREP)." The protocol was approved by the AMREP animal ethics committee, Melbourne Australia. Immunogenicity of peptide-PSNPs vaccine formulations were tested in HLA-A2/Kb [A2KbC57BL/6JTgN(A2KbH2b)6Hsd)] transgenic mice (Animal Resources Centre, Western Australia). Briefly, mice (3–5/group) were immunized with testing formulations (∼50–200 μl/injection) multiple times (as per experimental design) intradermally (i.d.) at the base of tail, 1–2 weeks apart (as per experimental design). Details of each immunization schedules are listed in the respective figure legends. Ten to Fourteen days following the last immunization, mice were euthanized by CO_2 asphyxiation and spleens were removed and splenocytes were harvested and tested for antigen specific immunogenicity on an enzyme-linked immunospot (ELISpot) assay.

ELISpot Assay

Antigen specific $CD8^+$ T cell responses were evaluated by IFN-γ ELISpot assays (38). Briefly, 96-well filtration plates (MAHA, MSIP or MAIP plates, Millipore, Billerica, MA) were coated with 100 μl/well of anti-mouse IFN-γ (AN18, 5 μg/ml, MABTech, Stockholm, Sweden). Following overnight incubation at 4°C, the wells were washed and blocked with RPMI 1640 completed medium (CM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, 0.1 mM β-mercaptoethanol and 20 mM Hepes (all from Gibco, Life Technologies, CA, United States). Splenocytes (50 μl) from immunized mice (2×10^7 cells/ml, either individual or pooled) were added to triplicate wells and incubated with 50 μl of recall antigens (see figure legends for specific details for respective experiment) at various concentrations (2.5–25 μg/ml final for all potential $CD8^+$ epitopes and 25–100 μg/ml final for long peptides and protein) at 37°C incubator filled with 5% CO_2 for a minimum of 16 h. Concanavalin A (Con-A) (1 μg/ml final, Amersham Biosciences, Uppsala, Sweden) was used as a positive control and background wells were added with CM only. The plates were then washed 6 times in PBS and incubated with 100 μl biotinylated detection antibodies [anti-mouse IFN-γ biotinylated mAb R4-6A2 (Mabtech) at 1 μg/ml final] at room temperature for 2 h. After washing as above, streptavidin-alkaline phosphatase was added (final at 1 μg/ml) and incubated for

TABLE 1 | Peptides and sequences.

Peptide code	Sequence	Amino acid position
HPV PEPTIDE ANTIGENS		
HPV01	LLMGTLGIVCPICKQQLLRREVYDFAFRDLCIVYRDGN	HPV16-E7$_{82-94}$ and HPV16-E6$_{41-65}$
HPV05	TLGIVCPI	HPV16-E7$_{86-93}$
HPV08	VQSTHVDIRTLEDLLMGTLGIVCPI	HPV16 E7$_{69-93}$
HPV12	KQQLLRREVYDFAFRDLCIVYRDGN	HPV16-E6$_{41-65}$
WT1 PEPTIDE ANTIGENS		
WT1A	RMFPNAPYL	WT1$_{126-134}$
WT1B	YMFPNAPYL	WT1$_{126-134}$ variant
WT1C	SGQAYMFPNAPYLPSCLES	WT1$_{122-140}$
WT1D	AAYYMFPNAPYL	AAY+ WT1$_{127-134}$
WT1E	AAYYMFPNAPYLPSCLES	AAY+WT1$_{127-134}$ +PSCLES
SURVIVIN (SV) PEPTIDE ANTIGENS		
SV01	KKQFEELTLGEFLKLDRERAKNKIAKETNNKKKEF	SV$_{90-124}$
SV02	GAPTLPPAWQPFLKDHRISTFKNWPFLEGCACTPE	SV$_{2-36}$
SV03	ELTLGEFLKL	SV$_{95-104}$
SV04	LTLGEFLKL	SV$_{96-104}$
SV05	TLPPAWQPFL	SV$_{5-14}$
SV06	RISTFKNWPFL	SV$_{18-28}$
SV07	LTLGEFLKLDRERAKN	SV$_{96-111}$
SV08	WQPFLKDHRISTFKN	SV$_{10-24}$
SV09	HRISTFKNWPFLEGCACT	SV$_{17-34}$
SV10	LMLGEFLKL	SV$_{96-104}$ variant
SV11	ELMLGEFLKL	SV$_{95-104}$ variant
SV12	DLAQMFFCFKELEGW	SV$_{53-67}$ variant
SV13	KKQFEELMLGEFLKL	SV$_{90-104}$ variant
SV14	KKQFEELMLGEFLKLDRERAK	SV$_{90-110}$ variant
SV16	AAYLMLGEFLKL	AAY+SV10 (SV$_{96-104}$ variant)

another 1.5 h at room temperature. Plates were then washed again, with a final wash using Reverse Osmosis (RO) water to remove residual PBS. The spots were developed using a colorimetric AP kit (Bio-Rad, Philadelphia, USA) following the manufacturers' instructions. Spot counting was performed using an AID ELISPOT Reader System (Autoimmun Diagnostika GmbH, Germany). The magnitudes of the IFN-γ induction in response to the recall antigen were compared either directly for its spot forming unit (SFU) or normalized against the background response (media alone response) from the same treatment group, calculated as stimulation index (SI) of SFU over background (SI = [SFU from the recall antigen stimulation in mice under the same treatment] / [SFU from the media alone stimulation in mice under the same treatment] for each corresponding recall antigens).

Statistical Analysis

All statistical analyses were performed using Graph Pad Prism v6.04 software (Graph Pad Software, Inc., La Jolla, CA, United States) and Microsoft Excel (Microsoft Corporation, Redmond, WA, United States). Comparisons were performed using one or two-way ANOVA analysis as appropriate. Differences were considered statistically significant when $p < 0.05$. Values are expressed as mean $\pm$ standard deviation (SD).

RESULTS

The primary selection parameter for antigens capable of inducing $CD8^+$ T cells in peptide-based cancer vaccine formulations is the ability of the peptide binding to MHC I molecules, and hence potential to be presented by appropriate antigen presenting cells (APC) to prime a $CD8^+$ T cell response. The HLA-A2.1 molecule is the most common MHC-I molecule in humans (in ~44–50% of Caucasians and Asian) (39), and hence most initial vaccine development aims to identify suitable HLA-A2.1 restricted $CD8^+$ T cell epitopes. $CD4^+$ T cells may help to promote sustained $CD8^+$ T cell reactivity, therefore when extending the peptide sequences around the desired $CD8^+$ T cell minimal epitope, we took the opportunity to incorporate them together with $CD4^+$ T cell epitopes with predicted broad binding affinity to HLA-DR, to offer a potential downstream powerful combination vaccine (40). However, the present study has only focused on the key issue of the generation of $CD8^+$ T cell epitopes capable of inducing HLA-A2.1 restricted $CD8^+$ T cell immunity in transgenic mice, since if this is not confirmed the

vaccine combination would not go forwards into development for use in humans. Apart from epitope design, we also have considered that the peptides selected would need to be feasibly manufactured, as well as retain solubility and stability during the conjugation process (using EDC chemistry) to the vaccine carrier nanoparticles (PSNPs). To further help promote synthetic peptides being effectively processed into $CD8^+$ or $CD4^+$ T cell epitopes after attachment to the nanoparticles, as well as to help protect the peptide ends from the action of exoproteases present and also to improve the epitope recognition *in vivo*, in some cases, an extra region of amino acids was added at either or both ends (amino and carboxy) in the designed peptides.

Based on the above matrix of selection criteria, multiple peptides from HPV, Survivin and WT1 were designed, conjugated to nanoparticles and evaluated for their ability to induce antigen specific T cell responses, in particular $CD8^+$ T cell responses. Further details that led to the design of specific peptides being synthesized, derived from each one of the three proteins, are expanded upon in each corresponding protein section below in results.

HPV Peptide-Based Nanovaccine Formulations and Immunogenicity

HPV Peptide Antigen Design and Selection

HPV type 16 (HPV16) is responsible for up to 50% of all cervical cancers (41). HPV16 E7 is a protein of 98 amino acid (aa); highly immunogenic with good indications of clinical relevance and immunogenicity in cervical cancer (23–25). Based on extensive literature search (42–47), clinical trials (24, 25, 48) and manufacturing feasibility, as well as with the aids of epitope prediction programs (the predictive algorithm of the SYFPEITHI database: http://www.syfpeithi.de.), we designed and finalized three HPV peptide candidates as nanovaccine targets (**Table 2**): 1) HPV05: a HLA-A2.1-restricted minimal $CD8^+$ T cell epitope (HPV16-$E7_{86-93}$); 2) HPV01: a chimeric peptide consisting of two HLA-A2.1-restricted $CD8^+$ T cell epitopes from HPV16-E7 ($E7_{82-94}$) and a $CD4^+$ T cell helper construct from HPV16-E6 ($E6_{41-65}$) (HPV12); 3) HPV08: peptide fragment HPV16 $E7_{69-93}$, containing both a $CD4^+$ helper epitope and two HLA-A2.1-restricted $CD8^+$ T cell epitopes. We also designed a peptide containing promiscuous $CD4^+$ T cell epitopes (HPV12) as a helper peptide to be incorporated in some of the nanovaccine formulations when necessary.

Covalently Linking the HPV Peptide Candidates to Nanoparticles (PSNPs) and Optimization of Peptide-PSNPs Formulations

We have developed a procedure to covalently link the peptide antigens to nanoparticles and produce uniformly sized with single layer antigen attached nanovaccine formulations (20). The conjugation process requires the use of activating agents such as 1-ethyl-3-(3-dimethylaminopropryl) carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (Sulfo-NHS) which cleaves the carboxyl groups and creates intermediate amine reactive ester bonds that allow covalent coupling of the peptide/proteins to the nanoparticles. This is best achieved in a condition of pH 5–6; however, at such pH, some peptides can be insoluble and form peptides/PSNPs aggregates, subsequently not suitable as nanovaccine formulations as particle size is crucial in particle-adjuvancity (38). Therefore, based on the standard procedure (see Material and Methods section), we altered conjugation conditions in the "conjugation step" and tested for a range of pH (5.5, 6, 6.5, 7 and 7.5) and buffers (PBS and $NaHCO_3$) for each peptide candidate to ensure high conjugation efficiency as well as to minimize aggregations, since each peptide has its own physiochemical characteristics. The quality of the peptide conjugated nanoparticle formulations (peptide-PSNPs) were determined by sizes and polydispersity index (Pdl), as well as conjugation efficiency and antigen loading per particle.

Conjugations of HPV peptides to the PSNPs were tested in PBS (for HPV01 and HPV08) and $NaHCO_3$ (for HPV05) at the various pH. As results shown in **Figure 1**, at a lower pH 5.5–6.5 during the conjugation step, HPV(peptide)-PSNPs formulations tended to aggregate and increased in size, though the aggregations were reduced with the increasing pH, optimal at pH 7–7.5. The final pH range to generate acceptable sizes for all HPV(peptide)-PSNPs conjugates were selected on the basis of conditions which produce particle-conjugates in the range of 40–60 nm with nanoparticle polydispersity (Pdl) <0.2 (**Table 3**).

To determine the conjugation efficiency under the selected optimal buffer and pH conjugation condition for each peptide

TABLE 2 | HPV peptide antigens (the predicted $CD8^+$ T cell epitopes are underlined).

Peptide code	Sequence	Amino acid position	Function
HPV01	LLMG<u>TLGIVCPI</u>CKQQLLRREVYDF AFRDLCIVYRDGN	HPV16-$E7_{82-94}$, HPV16-$E6_{41-65}$	Chimeric peptide consisting two HLA-A2.1-restricted $CD8^+$ T cell epitopes from HPV16-$E7_{82-90,86-93}$ (47), and promiscuous HLA-DR restricted $CD4^+$ T cell epitopes $E_{42-56,\ 52-62,54-68}$ (49, 50)
HPV05	<u>TLGIVCPI</u>	HPV16-$E7_{86-93}$	HLA-A2.1-restricted $CD8^+$ T cell epitope.
HPV08	VQSTHVDIRTLEDLLMG<u>TLGIVCPI</u>	HPV16-$E7_{69-93}$	Consists two HLA-A2.1-restricted $CD8^+$ T cell epitopes HPV16-$E7_{82-90,86-93}$ (47) and a HLA-DRB1 $CD4^+$ T cell epitope (HPV16-$E7_{73-87}$) (50)
HPV12	KQQLLRREVYDFAFRDLCIVYRDGN	HPV16-$E6_{41-65}$	Promiscuous HLA-DRB1 and HLA-DP0201 restricted $CD4^+$ T cell epitopes (50)

tested here, the remaining non-binding peptide material in each formulation after the conjugation process was determined by BCA™ protein assay or analysis via HPLC where possible. The final conjugation efficiency was determined as the percentage of antigen successfully conjugated to PSNPs (the targeted antigen concentration was 0.5 mg/ml for all antigen peptides). **Table 3** below summarizes the optimal conjugation conditions for each of the HPV peptide candidates evaluated in the study. The HPV05 peptide, representing the native HLA-A2.1-restricted minimal $CD8^+$ T cell epitope (HPV16-$E7_{86-93}$), achieved the highest antigen loading per PSNP (2.72×10^3 peptide molecules/particle) compared to the other peptides, 4.36×10^3/particle for HPV01 peptide loading and 9.34×10^2/particle for the HPV08 peptide loading. For consistency, the matching amount of each antigens across each experimental groups were used for immunogenicity studies.

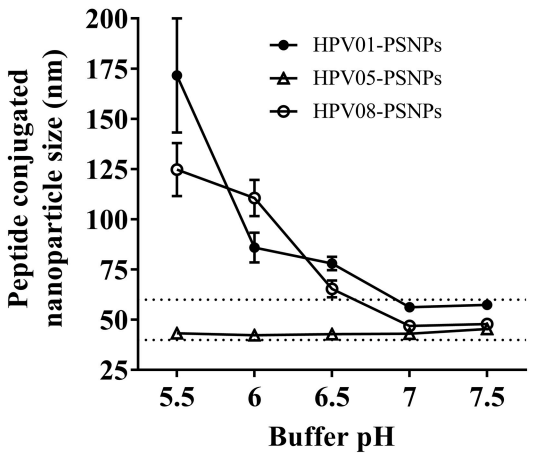

FIGURE 1 | Optimization of conjugation conditions to covalently conjugate HPV peptides to PSNPs to produce uniform HPV(peptide)-PSNPs nanovaccine formulations. PSNPs (1% solid final) were pre-activated following the standard procedure (detailed in Materials and Methods), and then re-conditioned in different buffer and pH solutions before mixing with each peptide antigen (0.5 mg/ml final) for conjugation. After conjugation, the final particle sizes for each peptide-PSNPs formulation was assessed using a Zetasizer. Data presented as peptide-PSNPs conjugate size (nm) ± SD (3 repeated measurements) under each conjugation conditions for each peptide. The dotted lines indicated the acceptable nanovaccine formulation size range at 40–60 nm.

Antigen Specific Immunogenicity Induced by HPV(peptide)-PSNPs Nanovaccine Formulations

HPV peptide-based nanovaccine formulations HPV01-PSNPs, HPV05-PSNPs or HPV08-PSNPs were injected into different groups of HLA-A2.1/Kb transgenic mice (i.d. at the base of tail), to evaluate their immunogenicity. The HPV HLA-A2.1-restricted minimal $CD8^+$ T cell epitope HPV05 (HPV16-$E7_{86-93}$, TLGIVCPI) peptides alone was the first to be tested for their capacity to induce antigen specific $CD8^+$ T cell responses in HLA-A2.1/Kb mice, when directly conjugated to PSNPs, or when mixed together with CpG with/without the additional peptide from a $CD4^+$ T cell epitope (HPV12). This peptide was selected as it has the predicted capacity to induce MHC class II restricted immunity in either mice or humans (**Table 1**). Results showed that after one immunization, HPV05 either mixed with CpG or conjugated to PSNPs alone, did not induce a HPV05 antigen specific $CD8^+$ T cell response (**Figure 2A**). Upon mixing with the addition of a $CD4^+$ T cell helper epitope (HPV12), high IFN-γ production was observed to the $CD4^+$ T cell peptide epitope HPV12 itself, but no $CD8^+$ T cell response could be elicited (**Figure 2A**). These results indicated that the HPV minimal $CD8^+$ T cell epitope alone, or with added $CD4^+$ T cell help, was not capable of provoking an antigen specific $CD8^+$ T cell response.

HPV01 (consisting of HPV16-$E7_{82-94}$ and HPV16-$E6_{41-65}$) and HPV08 (HPV16-$E7_{69-93}$) are long peptide antigens which both include the $CD8^+$ T cell epitope HPV05 (HPV16-$E7_{86-93}$), but in a different surrounding amino acid context, by including different $CD4^+$ T epitopes into their sequence (**Table 2**). Nanovaccine formulations with either of these two peptides conjugated to PSNPs were used to immunize animals (mice). Antigen specific response to the HPV16-$E7_{86-93}$ HLA-A2.1-restricted $CD8^+$ T cell epitope (HPV05) were observed upon HPV08-PSNPs, but not HPV01-PSNPs vaccination in HLA-A2.1/H2Kb transgenic mice, even after one immunization (**Figure 2B**), indicating that the minimal HLA-A2.1-restricted $CD8^+$ T cell epitope (TLGIVCPI) contained in HPV08 was efficiently processed and presented on HLA-A2.1 molecules. By contrast, the formulations with CpG for either of these two peptides (HPV01 and HPV08) did not elicit a $CD8^+$ T cell TLGIVCPI-specific responses, despite being generally immunogenic as full-length sequences (**Figure 2B**). These data suggest differences in antigen processing by CpG and nanovaccines for $CD8^+$ T cell epitopes, which in this case have identified HPV08 as a suitable peptide target to be used for

TABLE 3 | Optimal conjugation conditions for the HPV(peptide)-PSNPs formulations.

Peptide-PSNPs	Buffer	pH	Size (nm)	Polydispersity (PdI)	Conjugation efficiency (%)	Antigen loading (peptide molecules/particle)
HPV01-PSNPs	PBS	7.1	56.28 ± 0.68	0.23 ± 0.02	80*	4.36×10^2
HPV05-PSNPs	50 mM $NaHCO_3$	7.5	42.97 ± 0.34	0.08 ± 0.02	78*	2.72×10^3
HPV08-PSNPs	PBS	7.5	48.34 ± 0.81	0.14 ± 0.01	100*	9.34×10^2

**Conjugation efficiency determined by HPLC amino acid analysis.*

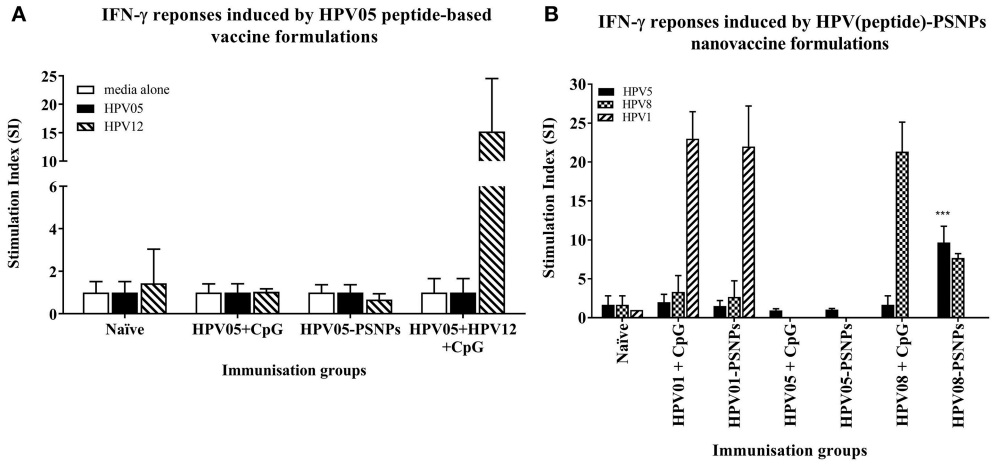

FIGURE 2 | Antigen-specific T cell responses in HLA-A2.1/Kb mice induced by HPV peptides with CpG or PSNPs. HPV01, -HPV05 and –HPV08 peptides were either mixed with CpG or covalently conjugated to PSNPs forming nanovaccine formulations. Each formulation was injected with matching amount of target peptide antigen (all contained 0.5 μg/peptide antigen/injection in 100–200 μl volume). Matching amount of HPV01, HPV05 and HPV08 peptides were also mixed with CpG (20 μg/injection) as comparison. Mice were immunized once intradermally. 15 days after the immunization, antigen specific T cell responses were evaluated by IFN-γ ELISpot assay upon stimulations with different concentration of antigen specific peptides (5, 10, 20, and 50 μg/ml) or controls (media alone, or Con A). Each condition was tested in triplicate on splenocytes from pooled cells within each group of mice ($n = 3$). Results were expressed as Stimulation Index (SI) of the antigen-induced IFN-γ responses (measured by SFU) over the background levels (media alone responses) (± SD triplicated in assay) upon stimulation with HPV05, HPV08 and HPV01 peptide at 20 μg/ml. ***$p < 0.001$ **(A):** HPV05-PSNPs formulation vs. HPV05+CpG ± HPV12 formulations (representative 1 of 3 experiments); **(B):** HPV01-, HPV05-, and HPV08-PSNPs formulations vs. each peptide adjuvanted by CpG formulations (summarized from multiple experiments) in comparison.

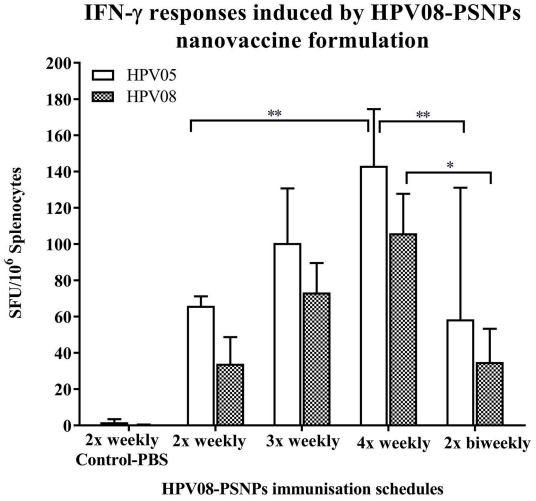

FIGURE 3 | Impact of immunization schedules and time interval on HPV08-PSNPs immunogenicity. HPV08 peptides were covalently conjugated to PSNPs forming HPV08-PSNPs nanovaccine formulation (final containing 0.37 mg/ml of HPV08 conjugated to PSNPs, 100 μl (or 37 μg)/injection). Mice were immunized following the schedules listed in the figure. Twelve days after the last immunization, antigen specific T cell responses were evaluated by IFN-γ ELISpot assay upon stimulations with antigen specific peptides (HPV05 and HPV08, all at 25 μg/ml) or controls (media alone, or Con A). Each condition was tested in triplicate on splenocytes from individual mouse ($n = 4$). Results are expressed as net spot-forming-unit (SFU)/million splenocytes/mouse upon each peptide recall ± SD ($n = 4$ individual mice). Two-way ANOVA analysis indicated the significance of HPV05 and HPV08 peptides induced specific responses in the HPV08-PSNPs formulations *$p < 0.05$, **$p < 0.01$.

the development a peptide based nanovaccine to elicit HPV05 responses against cancers induced by HPV16-E7.

Optimization of Immunization Schedules

We further explored the potential for changes in immunization schedule to improve the potency of the HPV08-PSNPs nanovaccine formulation. Specifically, we assessed the impact of changing the time interval between each immunization (**Figure 3**). The HLA-A2.1 transgenic mice were injected with the same batch of HPV08-PSNPs (i.d. at the base of tail) following the schedules of 2x-weekly, 3x-weekly, 4x-weekly and 2x-biweekly. The overall levels of the immune responses to the native HLA-A2 epitope (HPV05) and to the immunogen itself (HPV08) were generally increased with each additional immunisations scheduled from 2x to 4x weekly immunisations (**Figure 3**); although the 2x-weekly immunisations were also similar to the 2x-biweekly injections in the overall induction of HPV05 and HPV08 immune responses. The 2x-weekly immunization schedules produced more consistent levels (less "mouse-to-mouse" variability) of the immune responses to HPV05 than the 2x-biweekly immunization schedules. This clearly showed that shortening the time between immunizations to 7 days was not detrimental for $CD8^+$ T cell immune response induction upon HPV-PSNPs vaccination (no T cell response exhaustion) and might even be beneficial. Therefore, intradermal immunization with HPV08-PSNPs induced antigen-specific IFN-γ responses against the minimal HLA-A2.1-restricted $CD8^+$ T cell epitopes HPV05 in HLA-A2.1/Kb transgenic mice. Increasing number of immunisations positively increased the overall immune

responses with the strongest immune response observed after 4x weekly immunizations.

WT1 Peptide-Based Nanovaccine Formulations and Immunogenicity

WT1 Peptide Antigen Design and Selection

The Wilms' tumor antigen 1 (WT1) has been shown to be highly expressed and plays an oncologic role in various hematological and solid malignancies (51), but is negligibly expressed in normal tissues, thus making WT1 an ideal target for cancer immunotherapy strategies (52). WT1 has been listed among the top of the 75 ideal cancer antigens in immunotherapies by the U.S. National Cancer Institute (37). In humans, peptide-based vaccines with HLA-A24-restricted $WT1_{235-243}$ epitopes have been well characterized in the literature to elicit WT1-specific $CD8^+$ T cell responses in adult and children cancer patients with the HLA-A24 allele (52–56). Although the $CD8^+$ T cell responses toward the HLA-A2.1-restricted $WT1_{126-134}$ epitope "RMFPNAPYL" (herein called WT1A, **Table 4**) have been identified in various HLA-$A2^+$ cancer patients, research and clinical trials using WT1A peptide vaccination strategies have been disappointing (57, 59, 60). The WT1A-specific $CD8^+$ T cell responses were either short-lived with repeated vaccinations enriching for lower avidity populations (59) or could not be further expanded *in vitro* and may have been functionally impaired following WT1A vaccination (60). A modified version to substitute an arginine (R) to tyrosine (Y) at position 1 (***Y***MFPNAPYL, herein called WT1B, **Table 4**) has been shown to increase the peptide binding and stability to the HLA-A2.1 molecule (58). WT1B has been shown to be recognized by the native WT1A in humans (58). Our previous studies (61) also demonstrated that both WT1A and WT1B vaccination (adjuvanted by CpG) generated functionally similar $CD8^+$ T cell responses to the cognate antigen *ex vivo*, and both vaccination regimens could be readily expanded in response to the cognate peptide. While WT1A generated greater WT1A-specific $CD8^+$ T cell responses, WT1B showed greater potential to generate a proportion of dual responses that cross-reacted with WT1A, and could be expanded by the WT1A peptide (61). To further potentially promote better responses to WT1B (that would further be able to cross-react with the native epitope WT1A), based on our findings with HPV05 and HPV08, we designed variant peptides which could contain WT1B within an extended peptide (WT1C, WT1D, and WT1E, **Table 3**), conjugated them to the PSNPs to form WT1 peptide-PSNPs nanovaccine formulations, and evaluated their ability at inducing antigen specific $CD8^+$ T cell responses. In this case, we also extended the sequence at both the carboxy and amino ends with what would have been the native WT1A context (WT1C). Additionally, we followed recent literature suggesting that flanking amino acids with aromatic (tyrosine, Y), basic (lysine, K), and small aliphatic side chains (alanine, A) supported efficient cytotoxic T lymphocyte (CTL) recognition epitopes (62), and an additional AAY amino acid sequence was included at the amino end of WT1B to generate the WT1D peptide in the attempt to increase the $CD8^+$ T cell epitopes processing and recognition. To further explore providing processing context to both side of the epitopes, we generated WT1E, which is WT1D plus the same extension at the carboxy end as WT1C (**Table 4**).

Covalently Linking the WT1 Peptide Candidates to Nanoparticles (PSNPs) and Optimization of the Peptide-PSNPs Formulations

Conjugations of WT1 peptides to the PSNPs were tested in PBS at the various pH ranges. As shown in **Figure 4**, WT1A and WT1B peptides were conjugated over a range of pH conditions in PBS during the conjugation step, WT1A-PSNPs formulation aggregated in pH=5.5 buffer condition, but were stable when pH>6; whereas WT1B-PSNPs formulation were stable and no aggregation was observed over the pH ranges tested. Therefore, the optimal pH range for all WT1 peptides candidates was 6.5–7.5. All other WT1 peptides (WT1C, WT1D, and WT1E) were conjugated to PSNPs at pH 7.1, and final conjugated nanovaccine formulations were uniform in sizes (ranging between 40 and 60 nm, with $Pdl < 0.2$). **Table 5** summarizes the optimal conjugation conditions for each of the WT1 peptide candidates evaluated in the study. The overall conjugation efficiency was excellent (up to 100% by HPLC analysis), and antigen loadings (number of peptide molecules/particle) were also high (**Table 5**). For consistency,

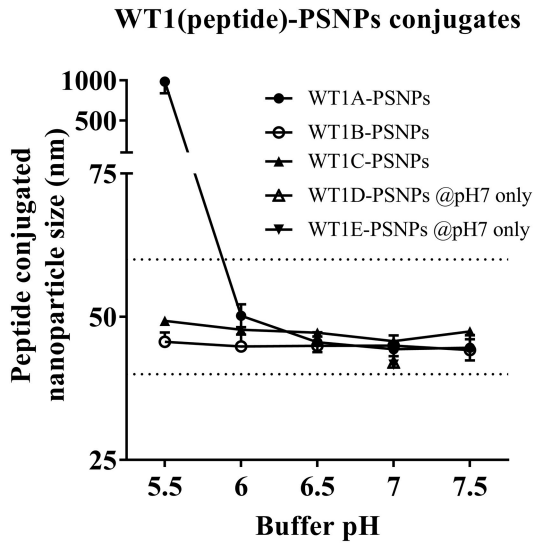

FIGURE 4 | Optimization of conjugation conditions to covalently conjugate WT1 peptides to PSNPs to produce uniform WT1(peptide)-PSNPs nanovaccine formulations. PSNPs (1% solid final) were pre-activated following the standard procedure (detailed in Materials and Methods), and then re-conditioned in different buffer and pH solutions before mixing with each peptide antigen (0.5 mg/ml final) for conjugation. After conjugation, the final particle sizes for each peptide-PSNPs formulation was assessed using a Zetasizer. Data presented as peptide-PSNPs conjugate size (nm) ± SD (3 repeated measurements) under each conjugation conditions for each peptide. The dotted lines indicated the acceptable nanovaccine formulation size range at 40–60 nm.

the matching amount of each antigens across each experimental groups were used for immunogenicity studies.

Antigen Specific CD8+ T Cell Responses Induced by WT1(peptide)-PSNPs Nanovaccine Formulations

The WT1 peptide-based nanovaccine formulations (WT1A-PSNPs, WT1B-PSNPs, WT1C-PSNPs, WT1D-PSNPs, and WT1E-PSNPs) were injected into HLA-A2.1/Kb transgenic mice (i.d. at the base of tail) to evaluate their immunogenicity (see material and methods section and figure legends for details). Results in **Figure 5** show that intradermal immunization with WT1B-, WT1C-, or WT1D-PSNPs formulations, but not with WT1A-PSNPs, induced antigen-specific IFN-γ responses to the HLA-A2.1-restricted $CD8^+$ T cell epitopes WT1A (RMFPNAPYL, native sequence) and its variant WT1B (***Y***MFPNAPYL) ($^{**}p < 0.01$, $^{*}p < 0.05$, $^{*}p < 0.05$, respectively). Despite the fact that the WT1C-PSNPs formulation contained both $CD8^+$ and $CD4^+$ T cell epitopes, there were negligible differences in the $CD8^+$ T cell specific responses elicited, between the two formulations, although there was a trend for a better induction of antigen-specific T cell responses to the native epitope WT1A in WT1B-PSNPs vaccinated animals. Additional of the amino acid sequence (AAY) at the flanking region of the WT1B peptide has been reported to promote appropriate processing and recognition of the minimal epitope (62), but this was not observed in our study, as the incorporation of this sequence did not enhance responses to the minimal epitope WT1B, and even decreased the cross-reactive $CD8^+$ T cell responses to the native WT1A antigen, when comparing WT1D-PSNPs and WT1E-PSNPs induced responses to the other formulations ($^{*}p < 0.05$ and $^{**}p < 0.01$, respectively) (**Figure 5**). Therefore, in the case of WT1 peptide antigen, substituting an amino acid [arginine (R) to tyrosine (Y)] generated strong immune responses to itself as well as cross-reactive responses to the native WT1A epitope, but extending the minimal $CD8^+$ T cell epitope by incorporating amino acids derived from its natural context, or predicted to potentially promote processing, did not enhance the $CD8^+$ T cell immune responses being induced.

Survivin Peptide-Based Nanovaccine Formulations and Its Immunogenicity

Survivin Peptide Antigen Design

Survivin (SV) is an oncogenic inhibitor-of-apoptosis protein (142 aa) crucial for the survival of tumor cells. It is generally expressed at low to negligible levels in normal tissue but is over expressed in a wide variety of cancers including lung, breast, pancreatic, colorectal, stomach and ovarian tumors as well as hematological malignancies (63). It is the fourth most highly expressed transcript in human cancer cells (26), and has been

TABLE 4 | WT1 peptide antigens (the predicted $CD8^+$ T cell epitopes are underlined).

Peptide code	Sequence	Amino acid position	Function
WT1A	**R**MFPNAPYL	$WT1_{126-134}$	Minimal native HLA-A2-restricted $CD8^+$ epitope (57)
WT1B	**Y**MFPNAPYL	$WT1_{126-134}$	WT1A variant with higher binding affinity. One amino acid substitution at position 1 by tyrosine (Y) instead of arginine (R) (58).
WT1C	SGQAYMFPNAPYLPSCLES	$WT1_{122-140}$	Consisting both $CD8^+$ ($WT1_{126-134}$, HLA-A2-restricted) and $CD4^+$ ($WT1_{124-138}$, HLA-DRB1 and DR15, DR53-restricted) epitopes
WT1D	AAYYMFPNAPYL	AAY+ $WT1_{126-134}$	Modified WT1B sequence including an extended sequence (AAY) at flanking region to increase epitope recognition, still consisting of $WT1_{127-134}$, an HLA-A2-restricted $CD8^+$ epitope
WT1E	AAYYMFPNAPYLPSCLES	AAY+ $WT1_{126-140}$	Modified WT1D sequence with additional sequence for $CD4^+$ epitope at C-terminal; consisting of both HLA-A2-restricted $CD8^+$ epitope and $CD4^+$ epitope (HLA-DRB1, -DR15 and DR53-restricted)

TABLE 5 | Optimal conjugation conditions for the WT1(peptide)-PSNPs formulations.

Peptide-PSNPs	Buffer	pH	Size (nm)	Polydispersity (PdI)	Conjugation efficiency (%)	Antigen loading (peptide molecules/particle)
WT1A-PSNPs	PBS	7.1	44.67 ± 0.45	0.09 ± 0.01	100*	2.24×10^3
WT1B-PSNPs	PBS	7.1	47.11 ± 1.42	0.10 ± 0.02	100*	1.41×10^3
WT1C-PSNPs	PBS	7.1	45.80 ± 2.17	0.07 ± 0.02	100*	1.61×10^3
WT1D-PSNPs	PBS	7.1	42.00 ± 0.19	0.05 ± 0.00	44#	7.51×10^2
WT1E-PSNPs	PBS	7.1	41.66 ± 0.45	0.05 ± 0.00	60#	7.12×10^2

Conjugation efficiency determined by HPLC amino acid analysis.

#conjugation efficiency determined by BCA assay. The overall conjugation efficiencies were low, and this was due to the specific amino acid contents interfering with the BCA assay, subsequently also impacting the calculation for the antigen loading/particle.

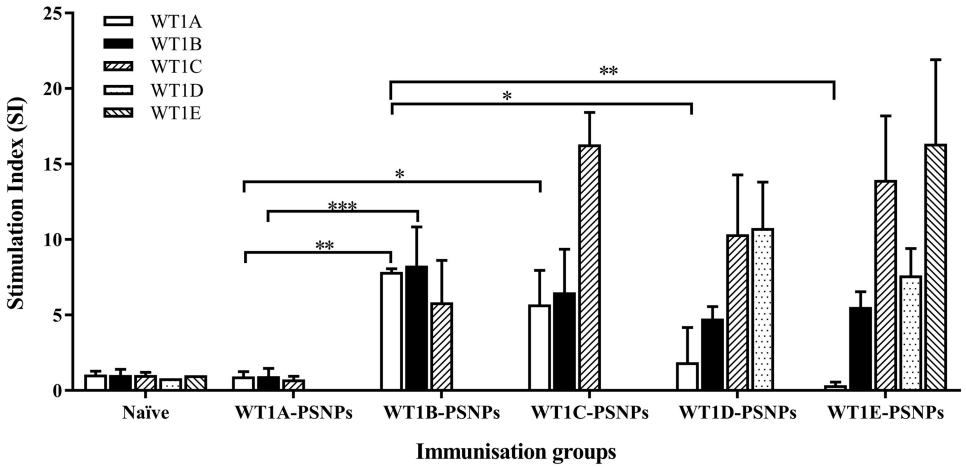

FIGURE 5 | Induction of IFNγ-producing antigen specific $CD8^+$ T cells following i.d. administrations of WT1(peptide)-PSNPs candidates in HLA-A2.1/Kb mice. WT1 derived peptides (WT1A, WT1B, WT1C, WT1D, and WT1E) were covalently conjugated to PSNPs to constitute PSNPs vaccine formulations (containing 0.5 mg/ml of each peptide in each of the conjugation mix). Mice were immunized 3 times with each formulation (100 μl or 50 μg (including both conjugated and non-conjugated peptide)/injection) intradermally, 10 days apart. 11 days after the last immunization, antigen specific T cell responses were evaluated by IFN γ ELISpot assay upon stimulations with WT1 peptides (5 μg/ml) or controls (media alone or Con A). Each condition was tested in triplicate on splenocytes from individual mouse ($n = 4$). Results are expressed as stimulation index (SI) of the SFU over the background (media alone) ± SD ($n = 4$ individual mice). Two-way ANOVA analysis indicated the significance of WT1A and WT1B peptide processing in the WT1peptide-PSNPs formulations. *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$. Figure was summarized from multiple experiments.

TABLE 6 | Survivin peptide antigens (the predicted $CD8^+$ T cell epitopes are underlined).

Peptide code	Sequence	Amino Acid position	Function
PEPTIDES SELECTED AS ANTIGEN TARGETS FOR NANOVACCINES			
SV01	KKQFEELTLGEFLKLDRERAKNKIA KETNNKKKEF	SV_{90-124}	Containing both HLA-A1 and A2.1 restricted $CD8^+$ ($SV_{92-101,\ 95-104}$) and HLA-DR1, 3, 4-restricted $CD4^+$ T cell epitopes ($SV_{97-111,110-124}$)
SV02	GAPTLPPAWQPFLKDHRISTFKNWPFL EGCACTPE	SV_{2-36}	Containing both HLA-A2.1-restricted $CD8^+$ ($SV_{5-14,18-28}$) and HLA-DR1, 15, 3,7,13,11-restricted $CD4^+$ T cell epitopes ($SV_{10-24,22-36}$)
SV10	L**M**LGEFLKL	SV_{96-104} variant	As above, SV_{97}: T to M
SV12	DLAQ**M**FFCFKELEGW	SV_{53-67} variant (SV_{57}: M)	Containing multiple $CD8^+$ T cell epitopes (cross-reactive to both H2Kb and HLA-A2) and promiscuous HLA-DR-restricted $CD4^+$ T cell epitopes (68)
SV16	AAYLMLGEFLKL	AAY+SV10 (SV_{96-104} variant)	As above, SV_{97}: T to M
PEPTIDES FOR RECALL ANTIGEN SPECIFIC REACTIVITY IN ELISpot ASSAY:			
SV03	ELTLGEFLKL	SV_{95-104}	HLA-A2.1-restricted $CD8^+$ T cell epitope (70)
SV04	LTLGEFLKL	SV_{96-104}	HLA-A2.1-restricted $CD8^+$ T cell epitope (71)
SV05	TLPPAWQPFL	SV_{5-14}	HLA-A2.1-restricted $CD8^+$ T cell epitope (70)
SV06	RISTFKNWPFL	SV_{18-28}	HLA-A2.1-restricted $CD8^+$ T cell epitope (68)
SV07	LTLGEFLKLDRERAKN	SV_{96-111}	HLA-DR1, DR3, DR4-restricted $CD4^+$ T cell epitopes (73)
SV08	WQPFLKDHRISTFKN	SV_{10-24}	Promiscuous HLA-DR1, DR15, DR3, DR7, DR13, DR11-restricted $CD4^+$ epitopes (72)
SV09	HRISTFKNWPFLEGCACT	SV_{17-34}	$CD4^+$ T cell epitope (74)
SV11	ELMLGEFLKL	SV_{95-104} (SV03) variant	SV_{97}: T to M, consists $CD8^+$ T cell epitope
SV13	KKQFEELMLGEFLKL	SV_{90-104} variant	SV_{97}: T to M, consists $CD8^+$ T cell epitope (extended SV11)
SV14	KKQFEELMLGEFLKLDRERAK	SV_{90-110} variant	SV_{97}: T to M, consists both $CD8^+$ and $CD4^+$ T cell epitopes (SV07)

found to be over-expressed in up to 90% of ovarian cancers (64, 65), making it potentially a good target for vaccine based treatment for ovarian cancer. However, despite the fact that Survivin peptides have been studied in multiple clinical trials, confirming their safety (66, 67), Survivin has been only weakly immunogenic, and hence not protective, across most studies (63, 68). A different choice of antigen delivery and adjuvant system could potentially enhance the immunogenicity of this protein.

Both $CD4^+$ and $CD8^+$ T cells epitopes from Survivin protein are important for induction of effective anti-tumor immune response (63). Given the PSNP nanoparticle vaccine approach has been successful in delivering peptide antigens [see above and previous publications (13, 69)], we explored how to increase the immunogenicity of a lead Survivin peptide containing $CD8^+$ T cell epitope, using these nanoparticle formulations. A number of Survivin-derived candidate peptides were identified based on an extensive literature search and clinical trials (70–73) and manufacturing feasibility (**Table 6**). The HLA-A2.1 restricted $CD8^+$ T cell native epitope peptide SV03 (SV_{95-104}) and SV04 (SV_{96-104}) were mostly cited by literature (70, 71, 75–78). In order to increase the minimal $CD8^+$ T cell epitope binding affinity to the HLA-A2.1 allele and subsequently to increase the immune responses, modified versions of SV03 and SV04 peptides were made by substituting the amino acid Threonine (T) to Methionine (M) at the position 97 (EL***M***LGEFLKL, herein named SV11 and SV10) as an agonist for use with PSNP vaccines. To further potentially encourage appropriate antigen processing and the epitope recognition to the HLA-A2.1 molecule, "AAY" amino acid sequence at the amino flanking region of the SV10 was also included (*AAY*LMLGEFLKL, named SV16). Additional panel of peptides were also designed to incorporate both $CD8^+$ and $CD4^+$ T cell epitopes (for potential downstream use in humans) in the peptide antigen sequences and evaluated for immunogenicity in PSNPs nanovaccine formulations in this study, such as SV01 (SV_{90-124}), SV02 (SV_{2-36}), and SV12 (**Table 6**). SV01 and SV02 contained both $CD8^+$ and $CD4^+$ T cell epitopes. SV01 (SV_{90-124}) covers multiple HLA-A2.1 and HLA-A1-restricted $CD8^+$ T cell epitopes ($SV_{92-101,\ 95-104}$) (70, 79), as well as HLA-DR1, DR3, DR4-restricted $CD4^+$ T cell epitopes ($SV_{97-111,110-124}$) (72, 73), good coverage for both MHCI and MHC II recognition. SV02 (SV_{2-36}) contains HLA-A2.1-restricted $CD8^+$ T cell epitopes ($SV_{5-14,18-28}$) (68, 70) and promiscuous HLA-DR-restricted (HLA-DR1, 15, 3,7,13,11) $CD4^+$ T cell epitopes ($SV_{10-24,22-36}$) (72). SV12 (SV_{53-67} variant: M57) contains multiple $CD8^+$ T cell epitopes (cross-reactive to both H2Kb and HLA-A2) and promiscuous HLA-DR-restricted $CD4^+$ T cell epitopes (68).

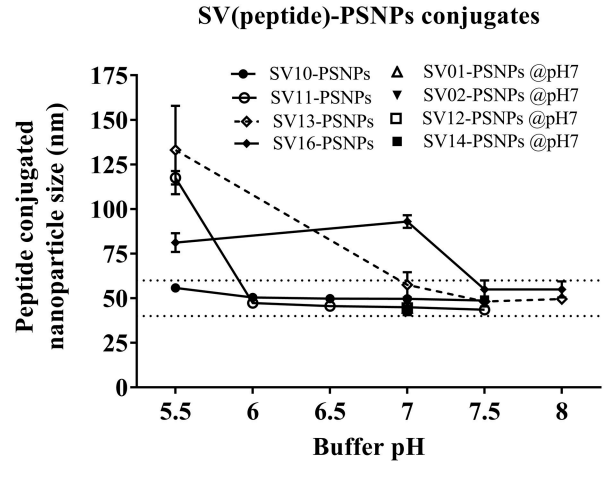

FIGURE 6 | Optimization of conjugation conditions to covalently conjugate Survivin peptides to PSNPs to produce uniform SV(peptide)-PSNPs nanovaccine formulations. PSNPs (1% solid final) were pre-activated following the standard procedure (detailed in Materials and Methods), and then re-conditioned in different buffer and pH solutions before mixing with each peptide antigen (0.5 mg/ml final) for conjugation. After conjugation, the final particle sizes for each peptide-PSNPs formulation was assessed using a Zetasizer. Data presented as peptide-PSNPs conjugate size (nm) ± SD (3 repeated measurements) under each conjugation conditions for each peptide. The dotted lines indicated the acceptable nanovaccine formulation size range at 40–60 nm.

Covalently Linking the Survivin Peptide Candidates to Nanoparticles (PSNPs) and Optimization of SV(peptide)-PSNPs Nanovaccine Formulations

Conjugations of Survivin peptides to the PSNPs were tested in PBS at the various pH. As results shown in **Figure 6**, SV10, SV11, SV13, and SV16 peptides were conjugated over a range of pH conditions in PBS during the conjugation step, apart from SV10, the SV11-, SV13-, and SV16-PSNPs formulations aggregated at pH=5.5 buffer condition and aggregations were

TABLE 7 | Optimal conjugation conditions for the SV(peptide)-PSNPs formulations.

Peptide-PSNPs	Buffer	pH	Size (nm)	Polydispersity (PdI)	Conjugation efficiency (%)	Antigen loading (peptide molecules/particle)
SV01-PSNPs	PBS	7.1	44.48 ± 0.12	0.1 ± 0.01	85.4[#]	6.07×10^2
SV02-PSNPs	PBS	7.1	43.68 ± 0.52	0.06 ± 0.00	87.8[#]	5.67×10^2
SV10-PSNPs	PBS	7.1	45.94 ± 0.88	0.17 ± 0.02	64.7*	1.56×10^3
SV11-PSNPs	PBS	7.1	44.96 ± 0.61	0.09 ± 0.01	ND	-
SV12-PSNPs	PBS	7.1	42.37 ± 0.22	0.09 ± 0.00	64.2	8.33×10^2
SV13-PSNPs	PBS	7.1	43.15 ± 0.14	0.09 ± 0.01	ND	-
SV14-PSNPs	PBS	7.1	42.49 ± 0.13	0.08 ± 0.00	ND	-
SV16-PSNPs	PBS	7.5	43.48 ± 0.14	0.09 ± 0.01	86.91[#]	1.54×10^3

**Conjugation efficiency determined by HPLC amino acid analysis.*

[#]conjugation efficiency determined by BCA assay.

ND: not determined due to the specific amino acid content interfering with the BCA assay.

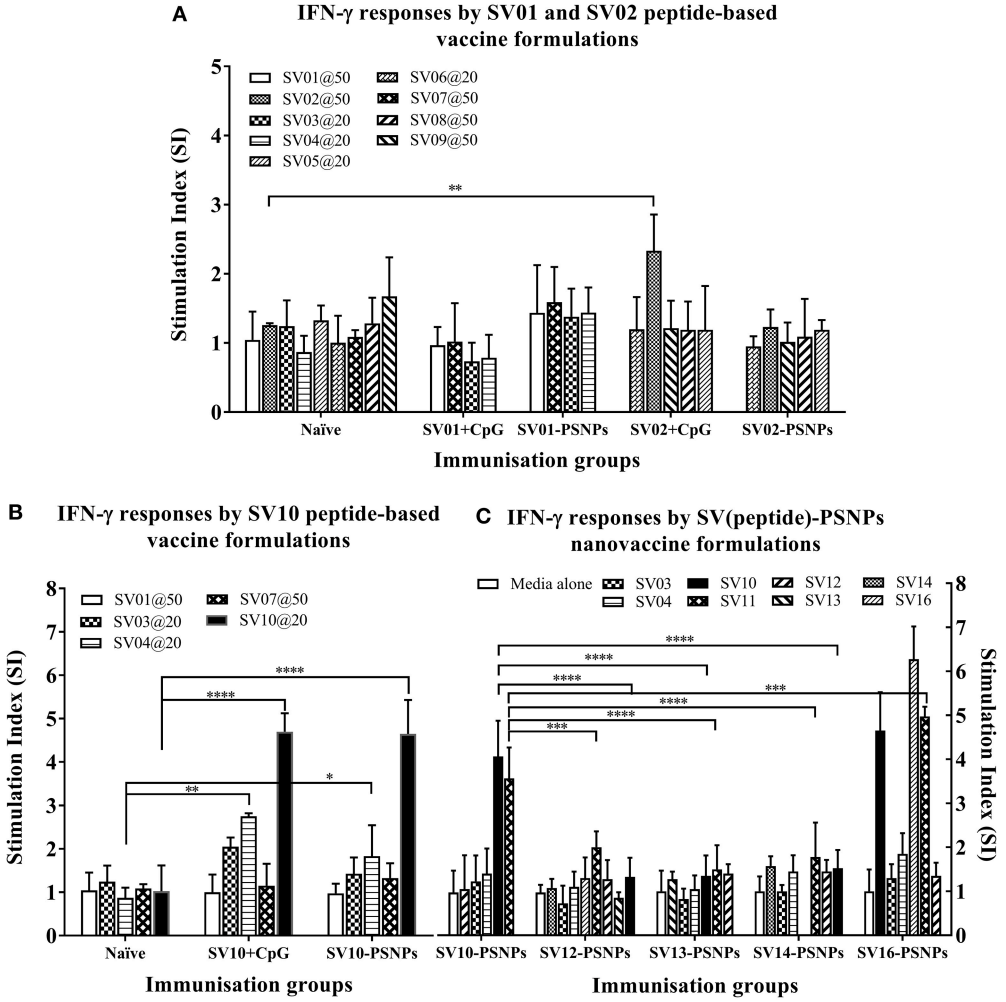

FIGURE 7 | Antigen-specific T cell responses in HLA-A2.1/Kb mice induced by SV peptides with CpG or PSNPs. SV-derived peptides: **(A)** SV01 and SV02, **(B)** SV10, **(C)** SV10, SV12, SV13, SV14, and SV16 were covalently conjugated to PSNPs forming PSNPs vaccine formulations. Each formulation contained equal amount of each SV peptide target and PSNPs (all at 0.5 mg/ml per peptide, 1% solid for PSNPs; 100 μl/injection). Equivalent amount of SV01, SV02, and SV10 peptides were also mixed with CpG (20 μg/injection) as comparison. For each immunization group, mice were immunized 3 times intradermally, 10 days apart. 11 days after the last immunization, antigen specific T cell responses were evaluated by IFN-γ ELISpot assay upon stimulations with antigen specific peptides (dosages on the figure (μg/ml) except C all at 25 μg/ml) or controls (media alone, or Con A). Each condition was tested in triplicate on splenocytes from individual mouse ($n = 3$–4). Results are expressed as the Stimulation Index (SI) of the antigen-induced IFN-γ responses (measured by SFU) over the background levels (media alone responses) ± SD ($n = 4$ individual mice) upon stimulation for each peptide conditions assayed in triplicated wells. Two-way ANOVA analysis indicated the significance of antigen specific responses induced by specific peptides in the SVpeptide-PSNPs or SVpeptide+CpG formulations. $^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$, and $^{****}p < 0.0001$. Figure was summarized from multiple experiments.

reduced with the increasing pH, optimal at pH 7–8. The SV10-PSNPs formulation were stable and there was no aggregation over the pH ranges tested. Therefore, the optimal pH range for all SV peptides candidates were 7–7.5. All other SV peptides (SV01, SV02, SV12, and SV14) were conjugated to PSNPs at pH 7.1, and final conjugated nanovaccine formulations were uniform in sizes (range between 40 and 60 nm, with $Pdl < 0.2$). **Table 7** below summarizes the optimal conjugation conditions for each of the SV peptide candidates evaluated in this study. All SV peptides were able to be conjugated to the PSNPs with high conjugation efficiency, and ultimately high levels of antigen loading represented by the number of peptide molecules per particle (**Table 7**). For consistency, the matching amount of each antigens across each experimental groups were used for immunogenicity studies.

Antigen Specific Immunogenicity Induced by SV(peptide)-PSNPs Nanovaccine Formulations

The Survivin peptide-based nanovaccine formulations were injected into HLA-A2.1/Kb transgenic mice (i.d. at the base of tail) to evaluate their immunogenicity (see material and methods section and figure legends for details). The long 35aa peptides SV01 (SV_{90-124}) and SV02 (SV_{2-36}) which contain multiple $CD8^+$ and $CD4^+$ T cell epitopes as well as SV10 (minimal $CD8^+$

T cell epitope SV_{96-104} variant,), were the first to be evaluated in the PSNPs conjugated nanovaccine formulations. Results in **Figure 7A**, showed that when SV01 peptides were conjugated to PSNPs or mixed with CpG and tested for antigen specific immune responses against the recall peptides SV03, SV04, SV07, or itself (SV01), none of them induced antigen specific IFN-γ T cell responses. When SV02 peptides were conjugated to PSNPs or mixed with CpG, and tested against the recall peptides SV05, SV06, SV08, SV09 or itself (SV02), only the SV02 peptide was able to induce a very weak IFN-γ responses in the SV02+CpG formulation (SI = ∼2, $^{**}p < 0.01$), but not SV02-PSNPs, when compared to the background. Therefore, both SV01 and SV02 peptides were not able to substantial $CD8^+$ T cell responses to the native HLA-A2.1 restricted epitopes SV_{95-104}, $SV_{96-104,}$ SV_{5-14} and SV_{18-28} (SV03, SV04, SV05, and SV06, respectively) either in formulations conjugated to PSNPs or adjuvated by CpG. No $CD4^+$ T cell mediated IFN-γ responses observed to any of the other recall $CD4^+$ T cell epitopes SV_{96-111}, SV_{10-24} and SV_{17-34} (SV07, SV08, and SV09, respectively) (**Figure 7A**).

However, the SV10 peptide (an agonist L***M***LGEFLKL peptide epitope for the natural epitope SV04 (SV_{96-105}) antigen conjugated to PSNPs (SV10-PSNPs) was able to generate strong IFN-γ responses to itself ($^{****}p < 0.0001$, **Figure 7B**) with responses equivalent to those elicited by the CpG adjuvated SV10 peptide formulation. Meanwhile, very weak but significant responses were also induced to the SV04 peptide in both formulations compared to the naïve group ($^{**}p < 0.01$ and $^{*}p < 0.05$ for CpG and PSNPs groups, respectively).

Based on the immunogenicity of the SV10 peptide formulations, we further designed Survivin peptides SV12 (SV_{53-67} agonist variant), SV13 (SV_{90-104} agonist variant), SV14 (SV_{90-110} agonist variant) and SV16, an extended sequence (AAY) at flanking region of SV10 to potentially help increase the epitope processing. We then evaluated their immunogenicity when conjugated to PSNPs. As shown in **Figure 7C**. However, none of these longer peptides (SV12, SV13 and SV14) containing both $CD4^+$ and $CD8^+$ T cell Survivin derived natural or agonist epitopes were able to induce antigen specific $CD8^+$ T cell responses. By contrast, the $CD8^+$ T cell epitope variant SV10, and SV16 (which contains SV10) were able to induce the HLA-A2.1 restricted $CD8^+$ T cell responses to SV10 and SV11 (a $SV03/SV_{95-104}$ variant) upon immunization with SV10-PSNPs or SV16-PSNPs vaccine formulations (**Figure 7C**). Disappointingly however, none of the native or agonist formulations were able to induce strong to the natural SV3 and SV4 Survivin $CD8^+$ T cell epitopes.

DISCUSSION

This comprehensive study assessed the impact of minor relative changes in peptide length and sequence for the induction $CD8^+$ T cell responses in HLA-A2.1 transgenic mice to antigens relevant to the development of gynecological cancer vaccines, based on the lead vaccine antigens HPVE7, Survivin and WT1. It focused specifically on their potential to be used in nanoparticle-based vaccine formulations such as PSNPs.

The minimal $CD8^+$ T cell peptide epitope HPV05 did not elicit significant immunity using a conventional adjuvant (CpG 1826) or when delivered as a conjugate with PSNPs nanoparticle carriers. This result contrasts previous studies using PSNPs to deliver very high affinity minimal $CD8^+$ T cell epitopes such as SIINFEKL (from OVA) (12, 13) or SYIPSAEKI (from *Plasmodium berghei* circumsporozoite protein) (18). Differences in antigen loading would not explain this finding, as there was excellent loading and nanoparticle size retention in an immunogenic range comparable to our previous studies. It has been suggested that lower affinity epitopes may be more dependent on $CD4^+$ T cell help (80–82). To address whether our observed lack of response was because of lack of $CD4^+$ T cell help, we mixed HPV05 with a known HPV derived $CD4^+$ T cell helper epitope (HPV12). However, this approach did not facilitate $CD8^+$ T cell induction. By contrast, HPV05 specific responses were elicited when the HPV05 sequence was lengthened at the amino end within its natural context to further include a $CD4^+$ T cell epitope, and used to formulate nanoparticle based vaccines. To note, this same extended sequence (HPV08), by contrast, when CpG adjuvanted, elicited responses to the full-length peptide, but failed to induce $CD8^+$ T cell responses to HPV05. It is likely that delivering this extended peptide conjugated to PSNPs promoted uptake and helped in the intracellular processing by cross-priming DC, specialized for the induction of $CD8^+$ T cells. Indeed previous studies with PSNPs have shown uptake by cross-priming $CD8^+$ DC (83) as well as TAP dependency for the priming of $CD8^+$ T cells to epitopes contained in PSNP-protein conjugated vaccines (12), indicating further the use of alternative intracellular cross-priming processing pathways (84). Furthermore, $CD4^+$ T cell responses could also be elicited to HPV08 in naïve T cell priming cultures from human peripheral blood mononuclear cells (PBMC) (unpublished data).

The minimal HLA-A2.1 binding $CD8^+$ T cell epitope WT1A from the WT1 protein conjugated to nanoparticles (PSNPs) similarly failed to induce $CD8^+$ T cells by itself, but in this case, it was sufficient to generate a high affinity agonist (WT1B) to produce a bioactive vaccine PSNPs conjugate which was able to induce immune responses to WT1B, which were further cross-reactive with WT1A. Such results suggested that mutated antigens derived from described antigens and upon conjugation with nanoparticles can induce higher grade of immunogenicity. Further extending the sequence at either end of WT1B, modeling it on either the natural peptide context for WT1A, or incorporating the sequence AAY at the amino end [described in the literature as being able to promote better antigen processing and recognition (62)], failed to further enhance $CD8^+$ T cell responses generated by vaccines including these formulations. In this specific case therefore, the optimal vaccine may be, simply a minimal high affinity agonist $CD8^+$ T Cell epitope conjugated directly to the nanoparticle, similarly to our previous studies using malaria high affinity agonist peptides with PSNPs (18). Similarly, initially negative results were observed using the unmodified Survivin derived minimal $CD8^+$ T cell epitopes, SV03 and SV04, and extending the peptide length alone and

conjugating to PSNPs was not able to rescue $CD8^+$ T cell induction. SV02 and SV04 are particularly weak binders to MHC class I (68, 70, 71), and known to be difficult epitopes in that there is a level of endogenous tolerance as self-antigens (85). In this case, we also trialed the testing of a super agonist variant (SV10), which has been used in human clinical trials in the context of other adjuvants, to explore its potential utility in nanoparticle-based formulations. Similarly to what we observed with WT1, using the agonist SV10 coupled directly to the nanoparticles was able to induce substantial $CD8^+$ T cell responses to SV10. Disappointingly, these responses were not cross-reactive to the native SV03 and SV04 sequences. Further extending the SV10 sequence within the natural SV03/04 context to generate longer peptides, did not increase or broaden, and even decreased reactivity to SV10 itself. By contrast, adding the AAY sequence at the amino end did result in enhanced immune responses to SV10, but these enhanced responses were not accompanied by a broadening of reactivity to include cross-reactivity with SV03 or SV04. Expanding the spectrum of cross-reactivities may be explored in future studies by further methodically changing the amino acid sequence of SV10 to generate more complex agonists. This approach has been used successfully to expand the spectrum of recognized variant $CD8^+$ T cell epitopes in the circumsporozoite protein from *P. berghei* (16) in the context of malaria.

The magnitude of immune responses induced by the formulations in the present study is comparable to our previous studies which have shown tumor protection in diverse animal models [(12, 13, 15) and unpublished]. However, as with any vaccine aiming to induce $CD8^+$ T cells, this does not really translate into certainty in obtaining high or tumor protective $CD8^+$ T cell responses in humans, as, at best, tumor protection studies in animals, even transgenic animals, can only be indicative of vaccine potential. The aim of this study was not to progress any particular formulation to human trials. If this was an objective in the future it will be important to perform challenge experiments in appropriate transgenic models.

Together the findings presented herein demonstrate nanoparticle carriers such as PSNPs which do not induce conventional inflammation, are capable of generating and enhancing $CD8^+$ T cell immune responses, not just to model antigens in mice, but to vaccine relevant HLA-A2.1 restricted peptide epitopes from multiple proteins relevant to gynecological cancers. Furthermore, for specific peptide epitopes, PSNPs nanovaccines were shown to elicit $CD8^+$ T cell responses even when other strong adjuvants failed to induce such responses. This study, however, suggests that for some particularly weak natural epitopes, neither conventional inflammatory adjuvants (CpG), or nanoparticle vaccine approaches may by themselves convert them into strong immunogens, and it will be necessary to optimize the use of super-agonist epitopes.

AUTHOR CONTRIBUTIONS

SX and MP: designed and supervised all experiments; SX: performed some of the experiments, analyzed and interpreted all the data; MP, AG, and AH: also analyzed and interpreted some of the data; KW: performed some of the experiments and analyzed some of the data; SX and MP: wrote the manuscript. All authors reviewed and agreed on the contents of the final version of the manuscript.

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REVIEW
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Adjuvants Enhancing Cross-Presentation by Dendritic Cells: The Key to More Effective Vaccines?

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Over the last decades, vaccine development has advanced significantly in pursuing higher safety with less side effects. However, this is often accompanied by a reduction in vaccine immunogenicity and an increased dependency on adjuvants to enhance vaccine potency. Especially for diseases like cancer, it is important that therapeutic vaccines contain adjuvants that promote strong T cell responses. An important mode of action for such adjuvants is to prolong antigen exposure to dendritic cells (DCs) and to induce their maturation. These mature DCs are extremely effective in the activation of antigen-specific T cells, which is a pre-requisite for induction of potent and long-lasting cellular immunity. For the activation of $CD8^+$ cytotoxic T cell responses, however, the exogenous vaccine antigens need to gain access to the endogenous MHCI presentation pathway of DCs, a process referred to as antigen cross-presentation. In this review, we will focus on recent insights in clinically relevant vaccine adjuvants that impact DC cross-presentation efficiency, including aluminum-based nanoparticles, saponin-based adjuvants, and Toll-like receptor ligands. Furthermore, we will discuss the importance of adjuvant combinations and highlight new developments in cancer vaccines. Understanding the mode of action of adjuvants in general and on antigen cross-presentation in DCs in particular will be important for the design of novel adjuvants as part of vaccines able to induce strong cellular immunity.

Keywords: adjuvants, dendritic cell, cross-presentation, aluminum, saponin, TLR, vaccine

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INTRODUCTION

Since the development of the first successful vaccine by Edward Jenner in 1796 against smallpox, a lot of research has been done on the development of vaccines against other diseases. Current vaccines against infectious agents can be divided into live attenuated vaccines (where their virulent properties are weakened, e.g., yellow fever, measles), subunit vaccines (containing a fragment of the pathogen, e.g., Hepatitis B), toxoid vaccines (with inactivated toxic compounds, e.g., tetanus, diphtheria), and conjugated vaccines (linking polysaccharide coats to protein, e.g., *Haemophilus influenzae* type B) (1). While especially prophylactic vaccines against infectious diseases have been developed successfully and are clinically applied, development of therapeutic vaccines against

persistent infections or cancer is lagging behind. For the development of new vaccines many aspects should be taken into consideration such as the nature of the antigenic material, the type of immune memory responses that needs to be induced, but also the administration and delivery routes, which might reduce the risk of side effects. Next generation vaccines like subunit vaccines for infectious diseases mostly aim for higher safety with less side effects, which is often detrimental for their immunogenicity. Therefore, adjuvants are usually required to enhance vaccine potency. Similarly, tumor neoantigen vaccines are devoid of immune activation potential and are fully dependent on strong adjuvants to induce protective immune responses. Adjuvants generally act by activating innate and adaptive immune responses, but can also function to create an antigen depot, slowly releasing the antigen for prolonged presentation and stimulation of the immune system (2). One of the first licensed carrier-adjuvants was alum, an inorganic adjuvant widely used in vaccines against e.g., hepatitis B virus, human papillomavirus, and diphtheria. Like most of the early adjuvants, they were mainly aimed at inducing protective antibody responses and hence strongly Th2 biased immunity. The discovery of microbe sensing pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors, has boosted research into vaccine adjuvants aiming to induce cellular immune responses that are essential to fight intracellular pathogens and cancer cells. Interaction of PRR with their corresponding ligands potentiate and shape the adaptive immune responses (3). Since then, several types of immune potentiating adjuvants (e.g., TLR agonists and saponin QS-21) have been licensed and used in the clinic against various diseases (**Table 1**).

Each adjuvant has a unique immunological signature that can be used in highly different types of diseases. Choosing the right adjuvant to combine with the best target antigen for a given disease is a challenging task (12). Next generation vaccine adjuvants are now mostly designed to contain both the function of a carrier and a potent immune response inducer to boost the efficacy of the vaccine. Although many prophylactic vaccines rely on neutralizing antibody responses, especially diseases such as cancer, HIV, tuberculosis, and malaria are in need of a vaccine eliciting strong T cell responses (13–17). As a consequence, many studies investigated the potency of next generation adjuvants for their capacity to induce antigen specific $CD8^+$ and $CD4^+$ T cell responses. An important characteristic of adjuvants able to induce cellular immunity is the efficient delivery of the target antigen into professional antigen presenting dendritic cells (DCs) and its potency in activating these DCs. In general, DC maturation enhances their antigen presentation capacity and ability to activate T cells and is a prerequisite for induction of potent and long-lasting immunity. One of the best studied DC maturation stimuli are TLR ligands, including poly(I:C), LPS, CpG, R848, and Pam_3CSK_4, which can activate DCs to upregulate co-stimulatory molecules such as CD40, CD80, and CD86 (18). TLRs can be expressed extracellularly (TLRs 1, 2, 4, 5, and 6) and intracellularly (TLRs 3, 7, 8, and 9) (3). All TLRs, except TLR3, utilize the adaptor molecule MyD88 to trigger activation of TGF-β Activated Kinase 1 which activates MAPK and NF-κB signaling resulting in TNF-α, IL-12, and IL-6 production (19, 20). Intracellular TLRs, which are mostly found in endosomes, require internalized ligands such as nucleic acids to activate downstream signaling. Currently, only the TLR4 agonist monophosphoryl lipd (MPL), a non-toxic LPS-derived TLR4 ligand, is approved for human applications (**Table 1**). Other TLR ligands showed effective tumor immunity in animal models or clinical trials (21–23).

Alternative pathways for DC maturation include intracellular receptors, such as Nucleotide binding domain-Like Receptor Protein 3 (NLRP3), which forms a caspase-1 activating complex (inflammasome) together with Cardinal and apoptosis-associated speck-like protein containing a caspase recruitment domain (24). This pathway results in cleavage and release of the pro-inflammatory cytokines IL-1β, IL-18, and IL-33 (25). A very important characteristic of adjuvants that has received much less attention is their ability to induce presentation of exogenous antigens not only in MHCII to $CD4^+$ T cells but also in MHCI to $CD8^+$ T cells. This latter process is essential for efficient $CD8^+$ T cell priming and is called antigen cross-presentation. In this review, we will focus on recent insights in clinically relevant adjuvants that impact DC cross-presentation. Understanding DC cross-presentation will be important to design novel adjuvants able to induce strong cellular immunity for future vaccine development.

MOLECULAR MECHANISMS OF DENDRITIC CELL CROSS-PRESENTATION

Dendritic cells are the professional APCs of our immune system that are key in linking innate and adaptive immunity. DCs are especially known for their ability to cross-present, as they process and present exogenous antigens on MHCI molecules much more efficiently than other phagocytes. The efficiency of $CD8^+$ T cell priming called cross-priming by DCs is dependent on both antigen cross-presentation efficiency (number of a given MHCI/peptide complex on the cell surface) and the level of DC maturation (expression levels of co-stimulatory molecules and cytokines). It has been reported that cross-presentation is important for inducing T cell responses specific for tumor antigens and infectious diseases (26–28). How exogenous antigens are processed in DCs and presented on MHCI to $CD8^+$ T cells is still not fully understood. Two main pathways of antigen cross-presentation in DCs have been proposed: the cytosolic pathway and the vacuolar pathway. In the cytosolic pathway, exogenous antigens or protein fragments derived from it are transported from endosomal vesicles into the cytosol where they are degraded by the proteasome. The trimmed peptides are then transported by the transporter associated with antigen processing (TAP) to the endoplasmic reticulum (ER) where they are loaded on MHCI molecules (29–31). However, there have been suggestions that the protein fragments can be transported back into endocytic compartments and trimmed by insulin-regulated aminopeptidase (IRAP) and loaded on MHCI (32). In the vacuolar pathway antigens are degraded by proteases in endo/lysosomal compartments and directly loaded on MHCI

TABLE 1 | Clinically approved adjuvants.

Adjuvant	Description	Proposed immune mechanism	Clinical application
Aluminum salts	Hydroxide, phosphate, alum	Activation of NLRP3 inflammasome and caspase-1 in DCs, induces Th2 response (4, 5).	HBV, HPV, diphtheria, and tetanus
AS01	Liposome (containing MPL and QS-21)	Activates APCs expressing TLR4, stimulates cytokine and co-stimulatory molecules production, promotes antigen-specific antibody responses and stimulates $CD8^+$ T cells (6).	Malaria, Herpes Zoster
AS02	Oil-in-water emulsion (containing MPL and QS-21)	Antigen specific $CD8^+$ and $CD4^+$ T cell responses and antibody responses (7)	Malaria
AS03	Oil-in-water emulsion (containing squalene, polysorbate 80 and α-tocopherol)	NF-κB activation, production of cytokines and chemokines in muscle and draining LN, provoke migration of monocytes, DCs and granulocytes into draining LN, enhancing $CD4^+$ T cell immune responses (8).	Pandemic influenza
AS04	MPL formulated in aluminum salt	Activates TLR4 on DCs, induction of cytokines and antigen specific T cell activation (9).	HBV, HPV
MF59	Oil-in-water emulsion	Rapid influx of $CD11b^+$ cells, upregulation of inflammatory cytokines and chemokines, recruitment of APCs (10).	Seasonal and pandemic influenza
Virosomes	Lipid vesicle containing inactivated viral proteins	Virosomal-adjuvanted influenza vaccine (Inflexal®V) increases antibody titer (11).	Influenza, Hepatitis A

NLRP3, nucleotide binding domain-like receptor protein 3; DCs, dendritic cells; HBV, Hepatitis B virus; HPV, human papillomavirus; MPL, monophosphoryl lipid; LN, lymph node.

molecules (33, 34). A comprehensive overview of these and alternative cross-presentation pathways in DCs has recently been reviewed (35).

How antigens are transported from the endosomes to the cytosol is still under debate. Extensive studies in murine models identified the ER-associated degradation (ERAD) member, Sec61, as a possible translocator for antigen from the endosomes into the cytosol. Applying a Sec61-specific intracellular antibody, Zehner et al. showed that they could trap Sec61 in the ER and prevent its transport toward endosomes, thereby blocking antigen translocation and cross-presentation (36). However, a more recent study using mycolactone, which binds specifically to Sec61α, showed severe inhibition of protein import into the ER but no inhibition of ERAD or protein export from endocytic compartments (37). Although, both studies showed inhibition of DC cross-presentation upon blocking of Sec61, it seems that Sec61 plays a more dominant role in inhibiting protein translocation into the ER and altering antigen cross-presentation at a different level than antigen export to the cytosol.

Another ongoing debate is how ER proteins are translocated to endosomes in DCs for efficient cross-presentation. The group of Amigorena proposed that recruitment of ER and ER-Golgi intermediate compartment (ERGIC) components to phagosomes is mediated by the ER-resident SNARE Sec22b (38). Silencing of Sec22b uncovered that phagosome-lysosome interactions were delayed, thereby limiting proteolysis and preserving antigenic fragments for cross-presentation, which was recently also confirmed in conditional Sec22b-knockout DCs (39). Conflicting results were found using similar Sec22b-knockout DCs (40) and based on a review of both studies with respect to technical differences, a role for Sec22b as well as for unidentified new regulators of cross-presentation was suggested (41). Although Sec22b seems to regulate antigen cross-presentation in the vacuolar pathway, it is not ruled out that it can play a role in the cytosolic pathway.

Two recent studies report on regulation of antigen cross-presentation in DCs by stromal interaction molecule 1 (STIM1), a calcium sensor that conveys the calcium content of the ER to store-operated channels of a cell (42, 43). Nunes-Hasler and colleagues showed that STIM1 can promote the contact sites between the ER and phagosomes (42). This induces Ca^{2+} signaling and thereby the migration and fusion of phagosomes with endosomes or lysosomes to enable efficient cross-presentation in DCs. In a companion study it was shown that the ER membrane protein uncoordinated 93 homolog B1 (UCN93B1) interacts with STIM1 and can control cross-presentation in DCs (43). Ablation of UCN93B1 impairs phago-lysosomal fusion, proteolytic activity, and antigen export to the cytosol, resulting in a decrease of antigen degradation and cross-presentation. Others showed that antigen transportation into the cytosol is enhanced by NADPH-oxidase complex (NOX2) and reactive oxygen species (ROS) production in the endosomes (44). Reactive oxygen species causes lipid peroxidation, which disrupts the endosomal membrane, resulting in antigen leakage from endosomes. Furthermore, it has been shown that NOX2 can be recruited to the endosomes to induce alkalization upon ROS release (45). This will cause an increase of endosomal pH thereby preventing rapid antigen degradation, resulting in enhanced antigen cross-presentation. The group of Guermonprez suggested that lipid bodies (LBs) are involved in DC cross-presentation (46). They showed that the Immunity-related GTPase family member 3 (Irgm3) controls accumulation of LBs induced by cell activation stimuli including INF-γ and Poly(I:C). LBs are organelles composed of a central core of

cholesteryl esters and triglycerides that are surrounded by a single layer of phospholipids also containing LB proteins (47). The Irgm3 protein is localized in the ER and in LBs where it interacts with the LB coat protein adipose differentiation-related protein (ADRP). Mice deficient in either Irgm3 or ADRP showed defects in LB formation and impaired cross-presentation in DCs. Further research is needed to understand how LBs control antigen cross-presentation by DCs and to determine the molecular pathways that control the involvement of LBs.

ANTIGEN CROSS-PRESENTATION AND DC SUBSETS

An important aspect to take into account when choosing an adjuvant to induce DC cross-presentation is the type of DC that will be affected. Intensive research has shown that there are many DC subsets present in mice as well in human, with still room for newly unidentified subsets. Murine DCs in secondary lymphoid organs can be divided roughly into conventional DCs (cDCs) and plasmacytoid DCs (pDCs). cDCs can be further divided into cDC1 ($CD8\alpha^+$ and $CD103^+$) and cDC2 ($CD8\alpha^-$, $CD11b^+$, and $CD172a^+$) DCs (48). The development of $CD8\alpha^+$ DCs is regulated by the transcription factors including inhibitor of DCN binding 2 (Id2), interferon regulatory factor (IRF) 8, basic leucine zipper ATF-like 3 transcription factor (BATF3), and the nuclear factor interleukin 3 regulated (NFIL3) (49). The development of $CD8\alpha^-$ DCs is orchestrated by the transcription factors including RelB, NOTCH2, RBP-J, IRF2, and IRF4. Deletion of either of these genes can lead to developmental defects of the DC subsets. Mice in which a given DC subset has been selectively depleted, e.g., $Batf3^{-/-}$ mice or zinc finger transcription factor knockout studies, have provided important insight in the functional role of DC subsets in antigen presentation (50, 51). However, the interpretation of the data in these mice regarding cross-presentation is not always straightforward due to incomplete depletion, depletion associated side effects, and DC cross-talk. In general, $CD8\alpha^+$ DCs are considered to be the most potent cross-presenting subset of antigens including proteins, antibody-bound-, cell-associated, and other types of antigens *in vivo* and *ex vivo* (50, 52–55). The explanations for the superior cross-presentation ability of $CD8\alpha^+$ DCs include lower degradation of antigen in endosomes by ROS production (56), more efficient transfer of exogenous antigens into the cytosol (57), and higher expression of components that are associated with MHCI processing pathway (55). Emerging data, however, suggest that the cross-presenting ability of each DC subset is tuned by and dependent on factors such as DC location and activation status, the type of antigen, and local inflammatory signals (58). Indeed, the main DC subset responsible for cross-presentation in lung, intestine and skin is the migratory $CD103^+$ DCs (59, 60). Although $CD8\alpha^-$ DCs are generally considered to be the most potent MHCII antigen presenting subset to $CD4^+$ T cells, it has been shown that $CD8\alpha^-$ DCs can efficiently cross-present antibody-bound antigen, antigens from *Salmonella typhimurium* and S. *cerevisiae*, or antigen in the presence of saponin adjuvants (61–65). $CD8\alpha^-$ DCs have been shown to cross-present antibody-bound antigen efficiently after activation of Fcγ-receptors (66), but a more recent study showed that complement factor C1q plays a dominant role in antibody-bound antigen uptake and cross-presentation in DCs (67). Although, some studies have shown the ability of pDCs to cross-present *in vitro* or *ex vivo* (34, 68, 69), their role in cross-presentation *in vivo* seems lacking during viral infections despite the fact that they are known for their ability in producing large amounts of type I interferons (70, 71). However, a recent study showed that upon TLR ligand activation, mitochrondial ROS production is increased independently of NOX2 in pDCs (72). Increased ROS production resulted in high endosomal pH, antigen protection from endosomal degradation, and induced export to the cytosol, ultimately leading to enhanced antigen cross-presentation and $CD8^+$ T cell priming.

In human, the cDC subset in blood can roughly be divided into $BDCA1^+$ ($CD1c^+$) and $BDCA3^+$ ($CD141^+$) DCs (73). The $BDCA1^+$ and $BDCA3^+$ subsets are proposed as the human counterparts of murine $CD8\alpha^-$ and $CD8\alpha^+$ DCs, respectively. It has been shown that $BDCA1^+$ DCs are capable of cross-presentation of extracellular antigen (74). Upon activation with TLR ligands, $BDCA1^+$ DCs showed similar efficiency in cross-presentation compared to $BDCA3^+$ DCs (75). A recent study showed that *in vivo* generated monocyte-derived DCs (moDCs) and monocyte-derived macrophages can both cross-present efficiently in a vacuolar-dependent pathway (76). In contrast to murine pDCs, the human counterpart has been reported to cross-present soluble, cell-associated antigen efficiently (77). However, recent work by the group of Ginhoux has identified a pre-DC subset that bears the classical pDC markers, including CD123, CD303, and CD304 (78). This pre-DC subset can be distinguished from the classical pDCs by additional markers, such as CD33, CX3CR1, CD2, CD5, and CD327. Importantly, they showed that only pre-DCs could induce $CD4^+$ T cell proliferation and IL-12 production compared to classical pDCs. These data imply that the antigen presenting ability of pDCs might be a result of "contaminating" pre-DCs. Whether these pre-DCs can also cross-present to $CD8^+$ T cells is currently unknown. It will be important to use additional markers to isolate pure pDC subset for future analysis of their antigen presenting capacity.

So far, most of the aforementioned studies investigating the molecular mechanisms of antigen cross-presentation make use of murine DC model systems and require confirmation in the human DC setting. Nevertheless, it seems that choosing specific antigen targeting routes can determine the outcome of DC cross-presentation efficiency of different subsets. Deciphering the molecular mechanisms of cross-presentation in the different DC subtypes in mice and human is needed for the optimal design of therapeutic vaccines.

CLINICALLY RELEVANT ADJUVANTS AND ANTIGEN CROSS-PRESENTATION

During the last years, many groups have been developing adjuvants that facilitate uptake by APCs, protect antigens against

degradation and stimulate strong immune memory responses (79). Here, we will focus on new insights in the mode of action of clinically relevant adjuvants on antigen cross-presentation by DCs and subsequent induction of cellular immunity. Many studies analysing adjuvants show an enhancement of $CD8^+$ T cells, but most studies do not differentiate between enhanced antigen cross-presentation by DCs or enhanced DC maturation, e.g., expression of co-stimulatory molecules and cytokines. Therefore, we will elaborate on those studies that describe the mechanisms of cross-presentation induced by adjuvants, including the involvement of the cytosolic and vacuolar pathway of cross-presentation in DCs. In addition, we will focus on clinically relevant adjuvants, including aluminum-based nanoparticles, saponin-based adjuvants (including ISCOMs), and TLR ligands.

Aluminum-Based Nanoparticles

Aluminum salts are the most widely applied adjuvants in human vaccines and it is firmly established that they are safe and well-tolerated. Aluminum oxyhydroxide [AlO(OH)] is a positively charged vaccine carrier that strongly absorbs negatively charged antigens (80, 81). Its mechanisms of action include antigen retention and local inflammation via activation of the NLRP3. Either direct phagocytosis of the adjuvant or phagocytosis of stressed or dying cells that contain the aluminum salts and subsequent release of damage associated molecular patterns are able to activate the NLRP3 inflammasome (82). Aluminum adjuvants induce the production of IL-1β and IL-18 by DCs and a strong default Th2 differentiation promoting the production of antibodies (83). Therefore, current aluminum-based adjuvants exhibit a very limited potency to induce a cellular Th1 immune response as compared to other adjuvants (84).

Interestingly, Jiang et al. transformed the micrometer-sized aggregates of AlO(OH) adjuvant into nano-sized vaccine carriers by shielding its positive charge with a polyethylene glycol (PEG)-containing polymer (80). The resulting nanoparticles could be readily co-loaded with both antigen and the TLR ligand CpG without affecting size or Zeta-potential of the particles and these particles were effectively internalized by murine APCs. Using endocytic pathway inhibitors, they showed that internalization is highly dependent on scavenger receptor A-mediated endocytosis (Illustrated in **Figure 1**). Confocal microscopy revealed localization of the nanoparticles within the lysosomes as well as in the cytosol, indicating lysosomal escape. The cytosolic delivery of the nanoparticles is possibly caused by AlO(OH) induced destabilization of lysosomes as described previously by others (88). Most importantly, Jiang et al. showed that cytosolic delivery of the nanoparticles containing OVA protein effectively promotes cross-presentation by DCs compared to free OVA protein, as measured by a monoclonal antibody specifically detecting MHCI/OVA peptide complexes. Strikingly, the presence of CpG in the nanoparticle further enhanced the level of antigen cross-presentation by DCs. Further analysis revealed that brefeldin A, which inhibits protein transport from the ER to Golgi, and MG-132, which inhibits the proteasome, reduced DC cross-presentation, while the cysteine protease inhibitor leupeptin did not. These data are thus consistent with the cytosolic route being the dominant cross-presentation pathway activated by the nanoparticle. Interestingly, while the size and positive charge at neutral pH of AlO(OH) in the traditional vaccine prevented its targeting to lymph nodes, AlO(OH) packed into nanoparticles of <90 nm in diameter efficiently reached lymph node APCs *in vivo*. Especially, nanoparticles loaded with CpG were able to expand and mature DCs in the lymph nodes and induced production of TNF-α and IL-12p70. Moreover, the presence of CpG in the AlO(OH) nanoparticles was necessary for the effective induction of both IgG1 and IgG2 responses as well as strong $CD8^+$ T cell response and delayed growth of B16 melanoma tumors. Control vaccination with CpG and OVA antigen without the AlO(OH) nanoparticles was much less effective. In conclusion, AlO(OH) nanoparticles in combination with CpG is a very potent and promising adjuvant combination for the induction of cellular immune responses.

Two other studies using AlO(OH) adjuvant packed into nanoparticles confirm this is a promising strategy to promote cross-presentation and/or cross-priming. Dong et al. synthesized AlO(OH) nanoparticles containing a polyethyleneimine (PEI) modification to increase antigen loading capacity (89). Particles were successfully loaded with tumor autophagosome derived proteins that are potentially enriched for tumor associated antigens. Zhao et al. created Al_2O_3 nanoparticles containing the Vx3 ubiquitin binding protein to enrich for ubiquitinated proteins present in tumor lysates, also to potentially enrich for tumor associated antigens (90).

Thus, the application of aluminum-based adjuvants showed that the use of aluminum salts can be improved by using nano-sized particles, especially in combination with TLR ligands, and that cross-presentation by DCs can be enhanced. The AS04 adjuvant is clinically approved, and is a combination of MPL and aluminum salt (**Table 1**). AS04 has shown to be very potent and the aluminum hydroxide is able to prolong the MPL induced cytokine response. The fact that this vaccine is successfully used in the clinic demonstrates that aluminum can be a useful carrier of other immunostimulatory molecules and that combining adjuvants is a promising strategy for the induction of strong cellular immune responses.

Saponin-Based Adjuvants

Saponins are triterpene glycosides derived from the bark of the South American soapbark tree, *Quillaja saponaria*. Dalsgaard has obtained a heterogeneous mixture of soluble *Quillaja*-derived saponins, Quil-A®, which has been commercialized and used in veterinary studies showing humoral and cellular immunity (91, 92). Further, purification of this mixture led to the identification of 10 fractions containing adjuvant activity, including QS-21 (93). Since QS-21 showed the least hemolytic effect compared to the other fractions, it was extensively investigated as an adjuvant. QS-21 can induce a robust antibody and cell-mediated immune response activating both Th1 and $CD8^+$ T cells (94). QS-21 has been proposed to exert its immunomodulatory effects by acting on different cell types *in vivo* [reviewed in (95)]. One study has shown that QS-21 can activate NLRP3 inflammasomes to induce IL-1β and IL-18 production in murine DCs (96).

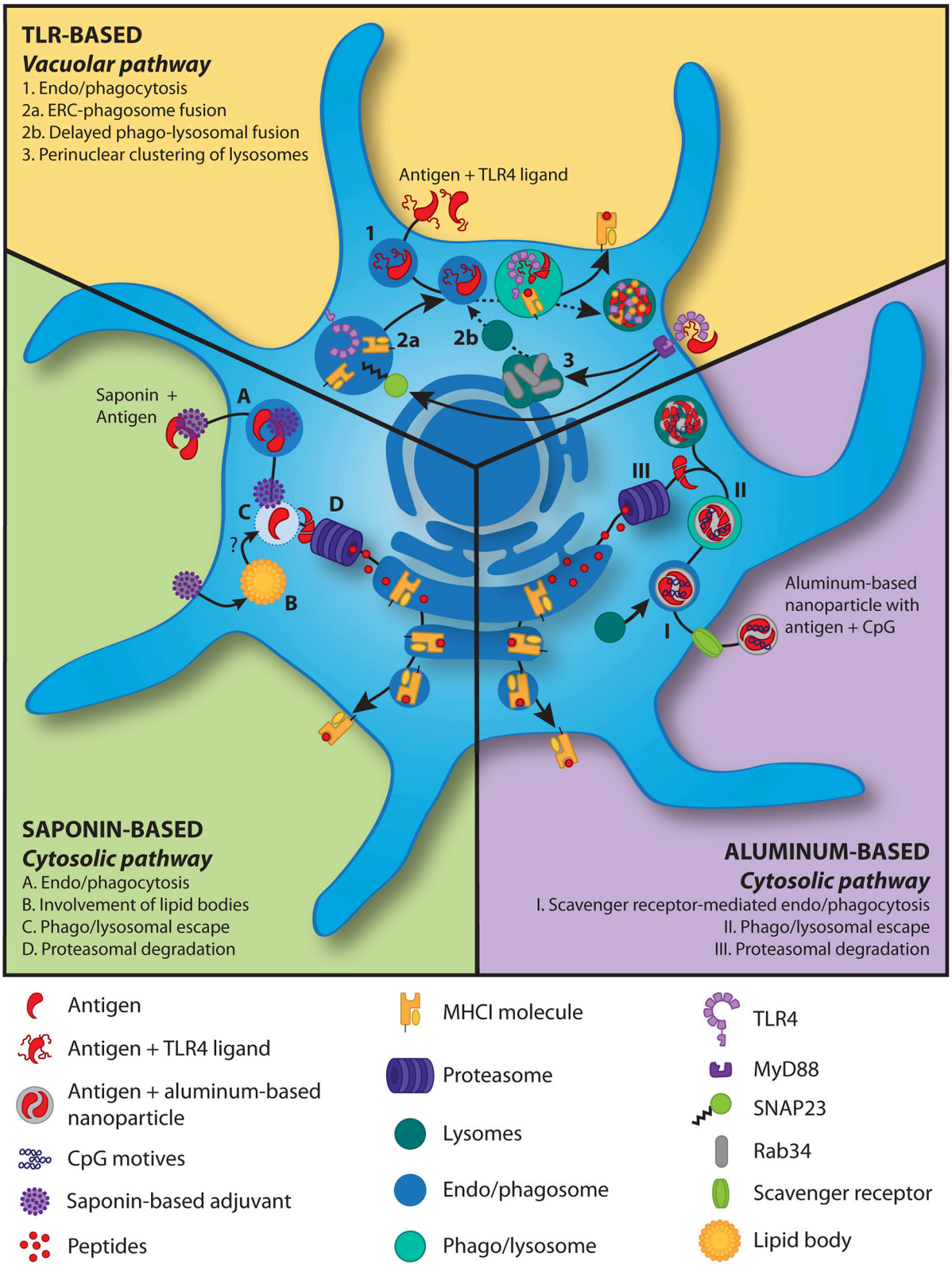

FIGURE 1 | Models for antigen cross-presentation mechanisms induced by adjuvants in DCs. *TLR-based adjuvants*: In the presence of TLR triggering, antigen is taken up by the DCs and delivered to phago/lysosomes (1). The MHCI molecules and TLR4 within the endosomal recycling compartment are shuttled into the phago/lysosome (2a) following TLR4 signaling induced phosphorylation of SNAP23 (85). TLR4 signaling further induces perinuclear clustering (3) of lysosomes in a

(Continued)

FIGURE 1 | Rab34-dependent manner (86), resulting in delayed (dashed line) phago-lysosomal fusion (2b). The latter slows down antigen degradation and thereby increases cross-presentation. *Saponin-based adjuvants*: Saponins, alone or in phospholipid and cholesterol particles, in combination with antigens are phagocytosed (A). The saponins induce lipid bodies (B) and increase cytosolic translocation of the antigen (C) and subsequent proteasome-dependent cross-presentation (D) (65, 87) via the cytosolic pathway. Lipid bodies play an unknown but crucial role in this process (B) (65). *Aluminum-based nanoparticles*: An aluminum-based nanoparticle loaded with antigen and the TLR9 ligand CpG is taken up via endocytosis, which is largely mediated through the scavenger receptor A (I) (80). After lysosomal fusion with the endosome, nanoparticle-mediated rupture of the vesicular membrane gains antigens access to the cytosol (II) and after proteasomal degradation (III) are cross-presented via the cytosolic pathway.

However, NLRP3-deficient mice showed higher levels of Th1 and Th2 antigen-specific T cell responses and increased IgG1 and IgG2c in the presence of QS-21, thus suggesting a more complex regulatory role for NLRP3. In human moDCs QS-21 has been reported to facilitate non-receptor-mediated uptake of exogenous antigen in a cholesterol-dependent manner (87). After endocytosis of antigen and QS-21, both are transported to the lysosomes where QS-21 causes lysosomal destabilization, followed by antigen release in the cytosol for further processing and cross-presentation (Illustrated in **Figure 1**). Moreover, they showed that cell activation depends on the activity of Syk kinase and cathepsin B, since Syk knockdown blocked NF-κB activation and cytokine production (IL-6 and TNF) in moDCs and shRNA-mediated knockdown of cathepsin B strongly decreased the expression of both TNF and IL-6 mRNAs. Moreover, cathepsin B-deficient mice showed lower cytokine (IL-2, TNF, and IFN-γ)-producing antigen-specific T cells. Neither for human nor for murine DCs has the mode of action of QS-21 on DC cross-presentation efficiency been investigated in detail.

When Quillaia saponins are admixed with cholesterol and phospholipid they spontaneously form open cage particles with a diameter of ∼40 nm, termed immune stimulating complexes (ISCOMs) (97). Due to the interaction of saponin with cholesterol, saponin is thought to be protected from hydrolysis and thereby stabilizing the adjuvant (98). Moreover, toxic side effects are greatly reduced since saponin interaction with membranes is decreased (99), while induction of antigen-specific T cell responses, prolonged antibody responses, and a balanced Th1/Th2 immunity are equal or even more potent (100, 101). In this review we will address the different saponin formulations as saponin-based adjuvants (SBAs).

Duewell et al. showed that SBA vaccines injected subcutaneously in mice resulted in the recruitment and activation of innate and adaptive immune cells in vaccine site-draining lymph nodes. They showed efficient uptake of antigen in DCs, induction of DC maturation, and IL-12 production *in vivo* (102). Moreover, they showed enhanced antigen cross-priming by CD8α$^+$ murine DCs relative to antigen alone, measured by induction of T cell proliferation, as well as protective anti-tumor immunity. The SBA vaccine induced activation and MHCI cross-priming by DCs in murine draining lymph nodes in a TLR-signaling adapter MyD88-independent manner (64). On the contrary, CD8$^+$ T cell-priming, NK cell activation, and potent antitumor activity in a prophylactic tumor challenge model *in vivo* were MyD88-dependent, suggesting a more downstream role of MyD88. They further showed that SBA induced efficient cross-priming by both CD8α$^-$ CD205$^+$ DCs as well as CD8α$^+$ CD205$^+$ DCs in draining lymph nodes 24 hours after vaccination. Surprisingly, murine splenic CD4$^+$ DCs were more efficient than CD8α$^+$ DCs at cross-priming soluble antigen formulated with SBA. Studies using another SBA formulation called Matrix-M^{TM}, which consists of two individually formed particles, Matrix-A and Matrix-C, together with cholesterol and phospholipid, also showed an increase in CD8$^+$ and CD4$^+$ T cell responses and 100% protection in a lethal viral challenge murine model (103). However, the precise mechanism how T cell induction was achieved was not investigated.

Two recent papers provide more insight in the mechanism of SBA induced cross-presentation by DCs. They demonstrated that saponin fraction C alone or formulated as an SBA can both induce an unprecedented level of DC cross-presentation in murine GM-CSF generated DCs *in vitro*, as shown by activation of the co-stimulation independent B3Z reporter T-cell line (47, 65). Moreover, SBA encounter did not change levels of CD80 or CD86 on *in vitro* cultured murine DCs. They further demonstrated that SBA predominantly act by inducing cross-presentation in the monocytic CD11b$^+$ DC subset *in vitro* and *in vivo*, a population distinct from the well-described CD8α$^+$ cross-presenting DCs. The presence of SBA increased cytosolic translocation of antigen, resulting in proteasome-dependent cross-presentation. Strikingly, specifically in this monocytic CD11b$^+$ DC subset, SBA enhanced DC cross-presentation by lipid body induction. Both pharmaceutical and genetic interference with lipid body formation inhibited the SBA-induced cross-presentation in these DCs *in vitro* and *in vivo* (Illustrated in **Figure 1**).

Human moDC studies have shown that SBA induced efficient cross-presentation of the cancer testis antigen NY-ESO-1 based on IFN-γ production by CD8$^+$ T cells (101). Interestingly, NY-ESO-1/SBA cross-presentation was studied for three distinct HLA-restricted epitopes. Independent of whether NY-ESO-1 is delivered in combination with SBA as two separate entities or formulated into one particle (ISCOMATRIX), the generation of two epitopes (HLA-A2, HLA-Cw3) was proteasome independent while the generation of the third epitope was highly proteasome dependent, as was the processing of the melanoma-differentiation antigen Melan-A when combined with SBA. Further analysis uncovered that cytosolic tripeptidyl peptidase II (TPPII) was involved in the generation of the HLA-A2, HLA-Cw3 epitopes of the NY-ESO-1/SBA vaccine. In line with this finding, they showed rapid antigen translocation from lysosomes into the cytosol in the presence of SBA. Thus, SBA vaccines are compatible with both cytosolic TPPII and the proteasome to generate immunogenic epitopes for MHCI antigen cross-presentation. In a follow-up study they showed that *in vitro* generated moDCs and freshly isolated CD1c$^+$ DCs from blood

could both cross-present NY-ESO-1 and Melan-A epitopes (104). However, when the antigen was limited, moDCs were more efficient than CD1c$^+$ DCs in cross-presentation *in vitro*. In addition, under these conditions physically incorporating the antigen into SBA (ISCOMATRIX) was superior compared to separate administration of antigen and adjuvant to CD1c$^+$ DCs. In conclusion, also in human DCs, SBAs can efficiently induce DC cross-presentation and different epitopes from the same protein can be processed by different pathways in DCs.

Currently, only the saponin QS-21 is approved for use in formulation with MPL as AS01 adjuvant in a human vaccine against malaria (**Table 1**). Furthermore, QS-21 has been added as adjuvant to a recombinant retroviral subunit vaccine against feline leukemia virus (105) in cats. In the human setting, SBAs in combination with NY-ESO-1 protein have now also been used in human clinical trials in patients with NY-ESO-1$^+$ tumors, generating high-titer antibody responses, and strong CD8$^+$ and CD4$^+$ T cell responses (106). To further extend the clinical application of SBAs, it will be important to fully understand the mode of actions of the adjuvant on cross-presentation by different DC subsets, including the role of lipid body induction. In addition, defining saponin adjuvant antigen formulations showing limited side effects while inducing maximal antigen cross-presentation capacity should further pave the way for their clinical application.

TLR Ligands

TLR ligands are well-known for their ability to induce DC maturation resulting in expression of co-stimulatory molecules and pro-inflammatory cytokines. The capacity to induce potent cellular immunity makes them a powerful addition to the armamentarium for cancer vaccinations. Interestingly, recent studies show that TLR ligands can also have direct effects on cross-presentation by DCs, making TLR ligands even more attractive for use in cancer vaccines. Upon TLR4-induced DC maturation, cross-presentation is first enhanced and followed by down-modulation of antigen internalization and cytosolic delivery (107). The two following studies focus on the first hours following TLR4 activation, in which the cross-presentation capacity is increased (85, 86).

Nair-Gupta et al. described a new pathway, in which TLR signaling, especially TLR4 triggering, can lead to increased cross-presentation by murine DCs (85). They showed that Escherichia coli expressing OVA protein (*E. coli*-OVA) is able to induce cross-priming of CD8$^+$ T cells by wildtype DCs, but not by Trif$^{-/-}$MyD88$^{-/-}$ DCs. Trif$^{-/-}$MyD88$^{-/-}$ DCs could induce CD8$^+$ T cell priming when provided with the pre-processed SIINFEKL epitope, thereby excluding a general inability to activate T cells. Confocal microscopy analysis showed the selective accumulation of MHCI molecules within the LAMP1$^+$ phagosomes also carrying the TLR4 ligand. These MHCI molecules were shown to be derived from the perinuclear Rab11a$^+$ vesicle-associated membrane protein (VAMP)3/cellubrevin$^+$ and VAMP8/endobrevin$^+$ endosomal recycling compartment (ERC) which contains large amounts of MHCI. Silencing Rab11a dissolved the existence of the perinuclear reserves of MHCI and diminished TLR-mediated cross-presentation. Of note, these Rab11a$^+$ MHCI$^+$ pools are predominantly found in the CD8α$^+$ DCs, suggesting that the existence of MHCI pools contributes to their strong cross-presentation capacity. Trafficking of MHCI from the ERC to the phagosome is, however, Rab11a independent but controlled by TLR4 induced IKK2-dependent phosphorylation of SNAP23. In conclusion, TLR signaling, especially via TLR4 leads to phosphorylation of SNAP23 and SNAP23-mediated trafficking of the perinuclear MHCI pools from the ERC to the LAMP1$^+$ TLR ligand$^+$ phagosomes (Illustrated in **Figure 1**). Alloatti et al. uncovered another mechanism how LPS treatment of DCs results in improved cross-presentation of both soluble and bead-bound OVA protein as well as proliferation and activation of antigen specific CD8$^+$ T cells *in vitro* and *in vivo* (86). By single organelle-based flow cytometry they showed that upon LPS stimulation, phagosomes contained more OVA protein and expressed less LAMP1, indicating less antigen degradation and lower levels of phago-lysosomal fusion, respectively. This effect was completely dependent on TLR4. Liquid chromatography-tandem mass spectrometry analysis of phagosomal proteins of both resting DCs and LPS stimulated DCs showed that phagosomes of resting DCs were highly enriched for the majority of lysosomal hydrolases, consistent with the LPS induced reduction in phago-lysosomal fusion. Moreover, LPS induced perinuclear clustering of LAMP1$^+$ lysosomes in maturing DCs, while broad peripheral distribution was observed in unstimulated DCs. This same perinuclear clustering was previously seen by Nair-Gupta et al. upon TLR stimulation (85). The perinuclear accumulation of lysosomes delayed phagosome maturation and phago-lysosomal fusion, resulting in improved cross-presentation, which was controlled by the GTPase Rab34 (Illustrated in **Figure 1**). Rab34 has been previously linked to cross-presentation efficiency (108). Interestingly, TLR7 and TLR9 activating ligands were able to show similar effects, but to a lower extent. Since antigen degradation is not mediated through the proteasome and loading of MHCI molecules with antigen does not happen in the ER but in the phago/lysosome, we believe the vacuolar pathway is followed.

TLR9 ligand CpG has potent immunostimulatory adjuvant activity and preferentially induces Th1 responses and tumor-specific CD8$^+$ T cells (109, 110). As TLR9 is located intracellularly, CpG needs to be internalized to exert its immunomodulatory effect. Consistent with the aforementioned findings, the cross-priming ability of murine DCs was shown to be dependent on the colocalization of antigen and TLR9 ligand in the same endocytic compartment within DCs (111, 112). Indeed, the failure or success of CpG as an adjuvant in the tumor setting was dependent on the timing of CpG relative to the release of tumor antigen following ablation (111). Similarly, combining TLR ligand and antigen in the same vaccine particle is more potent compared to separate administration (112). Thus, addition of a TLR ligand as an adjuvant to a vaccine is a promising treatment strategy to induce both enhanced cross-presentation and cross-priming by DCs.

In summary, since their discovery a lot of knowledge has been acquired regarding the mode of action of TLRs and their ligands, including their role in antigen cross-presentation. Many TLR

ligands have now also been tested as adjuvants for therapeutic cancer vaccines in clinical trials. However, only MPL has been approved as a purified TLR ligand for clinical use in several adjuvants (**Table 1**) (113). It will be interesting to test MPL as well as other TLR ligands in clinical development for their capacity to induce antigen cross-presenting in human DC subsets for future clinical application.

FUTURE PERSPECTIVES

For vaccines aiming to induce cell-mediated immunity such as cancer vaccines, it is important they stimulate both antigen cross-presentation by DCs and DC maturation to initiate an optimal $CD8^+$ T cells response. The "ideal" adjuvant thus combines both these characteristics and is able to prolong antigen exposure to the immune system. SBAs stand out to enhance DC cross-presentation, but are relatively poor in immune activation. Therefore, additional DC activation by e.g., TLR ligands is crucial. Moreover, combination of multiple PRR agonists can induce synergistic effects on DC activation (114). Furthermore, activating both the vacuolar and cytosolic pathway might be beneficial to enhance DC cross-presentation. To achieve prolonged antigen exposure another type of adjuvant formulation might be required. Based on pre-clinical as well clinical data, a picture is emerging that an optimal vaccine adjuvant may actually require a combination of adjuvants rather than a single adjuvant entity. The clinically approved vaccines adjuvants AS01, AS02, and AS04 show that a combination of different adjuvants, especially TLR ligands combined with other adjuvant(s) such as saponins or alum, can be both potent and safe to use in the clinic.

An important aspect to consider when choosing an adjuvant is that different DC subsets show differential cross-presentation efficiencies, which makes it important to study the response in subsets and potentially even to specifically target the most effective subsets. Targeting antigens directly to DCs using antibodies is explored for better antigen uptake, DC activation and thereby T cell-mediated immunity. Moreover, directly targeting specific DC subsets or receptors that allow strong cross-presentation can further enhance immune responses. Many studies targeting C-type lectin receptors on DCs including DEC205, DC-SIGN, and DNGR1 (Clec9A) showed efficient antigen-specific $CD8^+$ T cell responses (115). A potential drawback of (too) specific DC targeting is that *in vivo* the different DC subsets are known to work in concert and that antigen presentation by different DC subsets during the course of an immune response may be important to unleash a powerful immune response. Also vaccines with a different design, that are beyond the scope of this review, showed promising results, including the work of Sahin et al. (116, 117). Vaccines consisting of RNA encoding tumor antigen derived epitopes and containing immunostimulatory motifs were delivered by nano-sized lipoplexes that preferentially target and activate DCs in the spleen and have already been tested in a few patients. It is important to realize that so far, most of the studies looking into the potency and mode of action of adjuvants use murine DCs and hardly differentiate between different DC subsets. Extrapolation of the murine data on adjuvants to human DCs and preferentially also DC subsets will be important for future clinical application. It may be especially rewarding to test adjuvants in clinical development for their capacity to induce antigen cross-presenting by human DCs to select for adjuvants inducing T cell-mediated immunity. In conclusion, many aspects, from choosing the antigen, targeting specific DC subsets, activating DCs via PRR signaling, to stimulating efficient DC cross-presentation, need to be considered when choosing a vaccine and adjuvant. Understanding the underlying mechanisms will boost the development of next generation vaccines for clinical application.

AUTHOR CONTRIBUTIONS

NH, LH, and GA wrote the manuscript. TR reviewed the manuscript and made the figure illustration.

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Therapeutic Cancer Vaccines—T Cell Responses and Epigenetic Modulation

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There is great interest in developing efficient therapeutic cancer vaccines, as this type of therapy allows targeted killing of tumor cells as well as long-lasting immune protection. High levels of tumor-infiltrating $CD8^+$ T cells are associated with better prognosis in many cancers, and it is expected that new generation vaccines will induce effective production of these cells. Epigenetic mechanisms can promote changes in host immune responses, as well as mediate immune evasion by cancer cells. Here, we focus on epigenetic modifications involved in both vaccine-adjuvant-generated T cell immunity and cancer immune escape mechanisms. We propose that vaccine-adjuvant systems may be utilized to induce beneficial epigenetic modifications and discuss how epigenetic interventions could improve vaccine-based therapies. Additionally, we speculate on how, given the unique nature of individual epigenetic landscapes, epigenetic mapping of cancer progression and specific subsequent immune responses, could be harnessed to tailor therapeutic vaccines to each patient.

Keywords: cancer vaccine-adjuvants, T cells, epigenetics, DNA methylation, histone modifications, microRNAs, long non-coding RNAs, biomarkers

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INTRODUCTION

To address the possibility of designing therapeutic cancer vaccines to work optimally in patients whose immune system may have been epigenetically modified, either by cancer cell-driven immunomodulation or by other external cues such as previous chemotherapy, it is first necessary to understand the different types of epigenetic imprinting that may be induced by vaccine therapy. Herein, we will firstly introduce fundamental concepts, and then review in depth: (1) the epigenetic mechanisms involved in vaccine-induced T cell mediated immunity, (2) T cell responses and epigenetic modulations induced by adjuvant systems to promote an anti-cancer environment, and (3) the epigenetic mechanisms involved in cancer immune escape, and possible ways to counteract them. On this basis, the potential use of the knowledge in epigenetic mechanisms to improve vaccine-based therapy will be discussed. Additionally, given epigenetics are both heritable and flexible following environmental cues (1), the epigenetic profile of each individual is unique. Based on this fact we also discuss the potential use of epigenetic biomarkers to diagnose cancer and predict an individual's immune response to therapeutic cancer vaccines.

Vaccines Can Induce Effective Tumor-Specific T Cell-Mediated Immunity

Tremendous scientific advances have been made in the last decade in therapeutic cancer vaccine development, with many entering phase II and phase III clinical trials (2). Most cancer vaccines in development aim to promote tumor-associated antigens to be presented by antigen presenting cells (APCs) to generate long-lasting T cell immunity against cancer (3). Because dendritic cells (DCs) are the most efficient APCs, effective presentation of tumor antigens by DCs is considered a key determinant for cancer vaccine development (4).

Usually, the immune system identifies and destroys neoplastically-transformed cells. This immune surveillance mechanism functions as the body's primary defense against cancer. $CD8^+$ T lymphocytes are the primary player in the recognition and destruction of cancer cells (5, 6). Following stimulation through tumor antigen recognition presented by DCs, naive $CD8^+$ T cells are stimulated to proliferate and differentiate into effector cells, namely cytotoxic T lymphocytes (CTLs). Following recognition of major histocompatibility complex (MHC) class I-antigen complexes on tumor cell surface, activated CTLs induce tumor cell lysis by secreting perforin, granulysin and granzyme, as well as producing the death ligands including Fas Ligand (FasL) and tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) (7). A subset of antigen-specific T cells will differentiate into memory cells for long-lived anti-tumor protection. DCs also activate $CD4^+$ T helper (Th) cells, that are critical for $CD8^+$ T cell activation (8). This cross-priming is required to produce effective and durable CTL responses by breaking cross-tolerance and providing protection of CTLs from activation-induced cell death (AICD) (8, 9). Additionally, Th cells are also capable of eradicating tumor cells following activation (10, 11).

Several conditions, however, result in the failure of the immune system to destroy malignant cells (**Figure 1**). These include having a low number of tumor-specific T cells, suppression of T cell infiltration into tumor microenvironments, and T cell dysfunction/exhaustion (5, 6, 12–14). A low number of tumor-specific T cells results in a reduced number of cells capable of recognizing and killing neoplastic cells, hence tumor immune escape (6). Both failure in tumor antigen presentation and the development of immune tolerance contribute to this condition (5, 6). As tumor cells develop into a solid tumor mass, they create an immunosuppressive local microenvironment by secretion of specific factors that may restrict T cell infiltration, inactivate CTLs, and induce T cell apoptosis (13), further hampering cancer elimination. Due to chronic antigen exposure, T cells can also become dysfunctional and exhausted (12, 14). These T cells exhibit loss of the effector functions and upregulation of their immune checkpoint receptors such as PD1 and LAG3, the receptors that promote tolerance induction that subsequently prevents T cell activation upon stimulation.

To create neoplastic immunity, patients need to increase both the number and functionality of their cancer-specific T cells. This currently can be achieved by *de novo* generation of T cell-mediated immunity (15–18), through presentation by DCs (19, 20). One strategy utilizes a patient's own DCs as the therapeutic vaccine. DCs are maturated *ex vivo* using stimulatory cytokines and toll-like receptor (TLR) agonists, such as a combination of interferon (IFN)γ and lipopolysaccharide (LPS), and then loaded with patient-specific tumor antigens or proteins (21). The cells are then intradermally injected back into the patient together with adjuvants with the aim of generating a prolonged host immune response (22). In 2010, this strategy resulted in the first US Food and Drug Administration (FDA)-approved cancer vaccine, called Sipuleucel-T for prostate cancer patients (23). Increased survival in patients who received this personalized DC vaccine was achieved, suggesting successful long-lasting T cell immunity (24). Whilst this strategy has been successful in some patients, it has generally been inefficient. This is because the *ex vivo* DC vaccine preparation alters DC viability and functionality, is laborious and the output is of variable quality (19, 20). Moreover, the autologous DC generated from the patient's peripheral blood DC precursors, may have already been the subject of epigenetic imprinting by chemotherapy, radiation, immunotherapy or immune dysregulation by cancer cells, as such therapies have been shown to induce phenotypic alterations in immune cells (25). Understanding and modifying the epigenetic imprint of DC *ex vivo* (26), for example by the use of epigenetic modulators during tumor antigen loading, offers an intriguing avenue for future therapeutic exploration. Another strategy that currently holds promise in cancer vaccine development includes

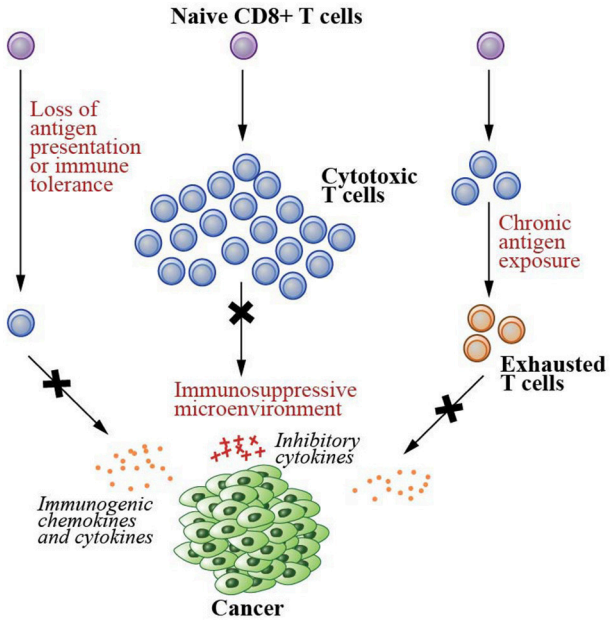

FIGURE 1 | Failed immunity conditions that can be rescued by therapeutic cancer vaccines. Therapeutic cancer vaccines generate *de novo* T cell immunity that can repair the conditions that cause the failure of T cell-mediated immunity. These conditions include (1) having a low number of tumor specific T cells due to the lack of tumor antigen presentation and development of immune tolerance, (2) suppression of T cell infiltration into the solid tumor mass due to immunosuppressive microenvironments created by the cancer cells, and (3) T cell dysfunction/exhaustion due to chronic antigen exposure.

the injection of antigenic peptides or genetic material encoding for these peptides, in combination with adjuvants, to target DCs *in vivo*. However, despite appropriate antigen and adjuvant selections, many therapeutic cancer vaccines still fail to provide sustained T cell immunity, due to the many immune escape mechanisms available to neoplastic cells.

Examining Epigenetic Involvement in T Cell Immunity Against Cancer

Recently several studies, as discussed in (27–31) show that epigenetic mechanisms drive phenotypic changes in both immune and cancer cells during their interactions. Epigenetics examines chemical modifications to a cell's deoxyribonucleic acid (DNA) that alters gene expression and thus the properties and behavior of cells, without changing their DNA sequence. These modifications include DNA methylation, histone modifications and ribonucleic acid (RNA)-associated mechanisms, via microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) which mediate alterations in chromatin accessibility at regulatory regions that determine cell fate (32–35). For example, DNA methylation results in a closed conformation of the chromatin, inhibiting binding of the transcription machinery and thus preventing gene expression (32). Various histone modifications, on the other hand, regulate cellular gene expression by modifying the polarity of the nucleosome particle, and/or by recruiting protein complexes, to result in either a closed or open chromatin conformation (33). Similarly, lncRNAs regulate gene expression by direct binding to chromatin remodeling complexes and targeting them to specific genomic loci to alter DNA methylation or histone marks (35). Additionally, miRNAs are able to regulate gene expression post-transcriptionally (34). In the following section we will discuss epigenetic changes in both immune and cancer cells that may be induced by cancer vaccine therapy.

EPIGENETIC MECHANISMS INVOLVED IN VACCINE-INDUCED T CELL IMMUNITY

Epigenetic Mechanisms Involved in Vaccine-Induced CD8$^+$ T Cell Differentiation Into Effector Cells

Therapeutic cancer vaccines commonly utilize tumor-associated antigens presented by DCs to expand naive CD8$^+$ T cells and drive their differentiation into both effector and memory cells. Activation of CTLs requires three signals (**Figure 2**): the first originates from the engagement of the T cell receptors (TCRs) with antigens as complexes with the MHC class I molecules on the surface of DCs; the second is the interactions of costimulatory molecules of DCs with cognate receptors of T cells including interactions between CD80/CD86 and CD28, CD70 and CD27, 41IBBL and 41IBB, OX40L and OX40, as well as GITRL and GITR (8, 36); and the third derives from cytokines including interleukin (IL)2 and IL12 secreted by DCs (37). Additionally, the tumor specific DCs activate Th cells through the interactions between TCRs and MHC class II-antigen complexes as well as the binding between their costimulatory molecules, such as the binding between CD80/CD86 and CD28. The activated Th cells

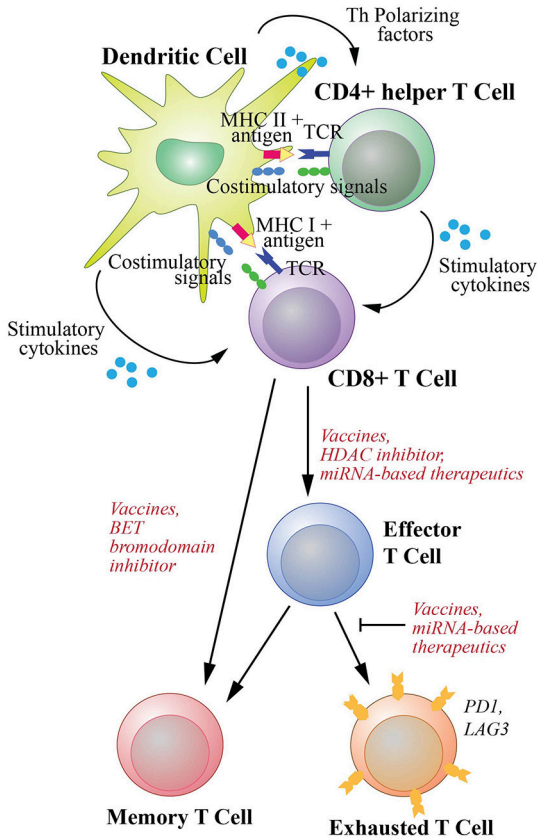

FIGURE 2 | T cell activation and differentiation into effector cells and subsequent memory and exhaustion phenotypic changes. Differentiation of naive CD8$^+$ T cells to cytotoxic T cells (CTLs) requires three signal interactions with dendritic cells (DCs). These include (1) the engagement of the T cell receptors (TCRs) with antigens as complexes with MHC class I molecules, (2) the interaction of DC costimulatory molecules with their receptors on CD8$^+$ T cells, (3) stimulatory cytokines secreted by DCs to activate T cells. Additionally, co-stimulation of CD8$^+$ T cells by T helper cells activated by DCs through MHC class II-antigen-TCR and costimulatory molecule complexes are required to promote efficient and durable CTL responses. The differentiation and activation of CD8$^+$ T cells could potentially be enhanced by an HDAC inhibitor and miRNA-based therapeutics. Differentiation of naive cells into memory T cells is required for long-lasting protection and can be enhanced by a BET bromodomain inhibitor. Furthermore, upon chronic exposure to antigen, T cells can develop exhaustion phenotype. However, this exhaustion can be counteracted by cancer vaccines that generate *de novo* T cell immunity. miRNA-based therapeutics could potentially be used to help rejuvenate exhausted T cells.

in turn license DCs by upregulating their CD40L and LTαβ to interact with CD40 and LTαβR on DCs, respectively (36). The licensed DCs then produce polarizing factors such as IL12 to further differentiate CD4$^+$ helper cells. The licensed DCs also increase the expression of CD80, CD70, OX40L, 41BBL, and GITRL, and secrete stimulatory cytokines such as IL2, IL12 and IFNγ, to generate CTLs with prolonged life-span with more effective effector function as reviewed in (8, 9, 36) (**Figure 2**).

Existing effector memory T cells can rapidly expand upon effective vaccination and differentiate into effector T cells

to further mediate specific tumor destruction (15, 16). The vaccine-induced generation of antigen-specific T cells with distinct cellular phenotypes from genetically identical naive cells is mostly mediated by global epigenetic reprogramming. Recent work shows that epigenetic mechanisms control gene expression during $CD8^+$ T cell differentiation following activation (27, 31). Epigenetic profiles also provide heritable maintenance of the phenotype of the differentiated T cells, following signal withdrawal (27, 31, 38, 39).

DNA methylation plays a significant role in $CD8^+$ T cell differentiation into both effector and memory cells. In mammals, DNA methylation occurs mostly on CG dinucleotides (CpG). DNA methylation in CpG islands, short regions in the genome with high frequency of CpGs, is associated with transcriptional repression (32). During $CD8^+$ differentiation, CpG islands become highly methylated at the promoters of silenced genes, and demethylated at the promoters of expressed genes (40–42). This alteration in methylation pattern dictates lineage-specific changes during differentiation following antigen-induced activation (43).

Like DNA methylation, promoters and other regulatory regions in the genome also undergo histone modifications during $CD8^+$ T cell differentiation. Multiple studies show that in effector cells at the gene loci that are reduced in expression such as the memory cell-associated genes, activating histone marks including acetylation at lysine 9 on the histone 3 tail (H3K9Ac) and trimethylation at lysine 4 on the histone 3 tail (H3K4me3) are lost (41, 44–52). At the same gene loci, repressive marks including DNA methylation and trimethylation at lysine 27 on the histone 3 tail (H3K27me3) are gained. On the other hand, in the same cells, the effector cell-associated genes are upregulated and demonstrate decreased repressive and increased activating epigenetic marks (41, 44–52).

Importantly, in the absence of antigen presentation, memory cell subsets maintain their epigenetic patterns in order to retain their cellular phenotype (53). DNA methylation patterns of memory cells for example are preserved after antigen is withdrawn. This indicates involvement of epigenetic regulation in the maintenance of cellular phenotype to promote long-lasting vaccine-induced immunity. Similarly, di-acetylated histone H3 (diAcH3) is highly present in the expressed gene loci of activated effector CTLs, and this epigenetic mark remains present in the acquired memory cells (54). Additionally, several gene loci in naive and memory T cells remain poised in a resting state by the presence of bivalent epigenetic marks; the activating H3K4me3 and the repressive H3K27me3. This bivalency has been shown to be a crucial mechanism in regulating T cell faith, since following antigen stimulation, the activated gene loci are readily resolved into a monovalent H3K4me3 state subsequently allowing rapid differentiation into effector cells (45).

Recently, epigenetic enhancers have been shown to regulate $CD8^+$ T cell differentiation in response to antigen presentation. The activation of the enhancers during differentiation was mapped based on genome-wide analysis of several epigenetic marks including H3K4me3, H3K27ac, H3K4me1 and the binding of histone acetyltransferase p300 (49, 55). These regions display striking epigenetic specificity in naive, effector, and memory T cells. Distinct transcription factors have also been shown to bind specifically to the enhancers of different subsets of $CD8^+$ T cells (40). Similarly, chromatin accessibility profiles indicate unique regulatory regions in different $CD8^+$ T cell subsets that also correspond to the expression of subset-specific genes (56, 57).

Furthermore, the levels of epigenetic modifier and transcription factor expression are distinct amongst T cell subsets. These may influence the capacity of T cells to react upon antigen stimulation. Indeed, the lack of DNA methyltransferase 3A (DNMT3A), a *de novo* methylating enzyme, promotes bias toward memory cell differentiation (58). Absence of the epigenetic modifier methyl-CpG-binding domain protein 2 (MBD2) causes impaired T cell differentiation into the effector phenotype (59). The epigenetic modifier BMI1, a reader of H3K27me3, and EZH2, a writer of H3K27me3 are both highly expressed upon T cell stimulation and differentiation into effector cells (60, 61). Histone deacetylases, SIRT1 (50) and HDAC7 (54) as well as BRD4, a reader of acetylated lysines (62) epigenetically repress gene expression and have been shown essential in directing differentiation of $CD8^+$ T cells to gain their effector function.

In effector T cells, transcription factor PRDM1/Blimp1 (63), TBX21/Tbet (64, 65), and ID2 (66) are highly expressed to control CTL function via epigenetic regulations. PRDM1 for example, has been shown to recruit the repressive epigenetic modifier G9A and HDAC2 to both *IL2RA* and *CD27* loci, promoting differentiation of $CD8^+$ T cells into effector cells (51). TBX21 is necessary to induce the expression of IFNγ, granzyme B, and perforin, by inducing rapid DNA demethylation and histone acetylation at the promoters of these gene loci (67–69). Furthermore, in both naive and memory cells, EOMES (65, 70), TCF1 (71), and FOXO1 (72–75) are highly expressed and have been shown to readily promote differentiation of these cells into CTLs, although their mode of action in regulating epigenetic changes in T cells remains unexplored.

Epigenetic Modifications in T Cell Exhaustion

Another benefit of therapeutic cancer vaccines is their potential to revitalize exhausted T cells, by promoting *de novo* generation of T cell-mediated immunity (15–18). Exhausted T cells are a hallmark of cancer and the result of persistent antigen stimulation (76). They exhibit defective proliferation capacities, impaired stimulatory cytokine secretion, increased checkpoint receptor expression, and impaired effector functions (76). Recent studies show direct involvement of epigenetic mechanisms in T cell exhaustion. For example, compared to functional T cells, exhausted T cells exhibit reorganization of chromatin accessibility and activation of the exhaustion-specific enhancers (77, 78). Exhausted T cells also exhibit lower levels of diacetylated histone H3 (diAcH3) in comparison to functional T cells (79).

Both DNA methylating enzymes, DNMT1 and DNMT3B are upregulated in exhausted T cells (80), whilst DNMT3A has been demonstrated to functionally establish *de novo* exhaustion-specific DNA methylation patterns (81). Indeed, by blocking *de novo* DNA methylation, exhausted T cells retained their effector function (81). In exhausted T cells however, the expression

levels of checkpoint/coinhibitory receptors, including PD1 and LAG3 were highly elevated (78, 82), which correlated with demethylation (83) and binding of transcription factor GATA3, BLIMP1, IRF4, BATF, and NFATc1 to the gene loci (51, 82, 84).

Additionally, lncRNAs including lncRNA-CD244 and lncRNA-Tim3 (85, 86) and miRNAs including miR-720, miR-31, miR-92a-3p, miR-21-5p, miR-16-5p, miR-126, and miR-182-5p (87–89) are capable of inducing exhaustion phenotypic changes by targeting specific pathways that impair T cell effector function. Therapies targeting these regulatory RNAs therefore may help restore T cell anti-tumor functions.

Potential Epigenetic Interventions to Improve Vaccines

Therapeutic cancer vaccines are able to direct the proliferation and differentiation of naive and memory $CD8^+$ T cells into CTLs through epigenetic modifications. As previously discussed, the involvement of epigenetic modifiers and transcription factors have been observed in directing T cell differentiation. This knowledge could potentially be used to improve the efficacy of therapeutic cancer vaccines.

For instance, BRD4 and SIRT1 are known to regulate differentiation of naive T cells into CTLs (50, 62). The absence of these two epigenetic modifiers promotes T cell differentiation into memory cells. Inhibition of these two epigenetic modifiers using the pharmacological inhibitor JQ1, results in the differentiation of naive $CD8^+$ T cells into memory T cells that are long-lived, self-renewing and provide maintenance of acquired functional immunity, indicating that this pharmacological agent can be used to help create long-lasting immune response (62).

Another example is histone acetylation in Tbet-mediated IFNγ expression in CTLs. An HDAC inhibitor, trichostatin-A (TSA), can bypass the control of Tbet in inducing IFNγ expression (90). As IFNγ is critical for CTLs to exert their tumor killing activities, this pharmacological epigenetic modifier could potentially be used to enhance the efficacy of cancer vaccines.

Recently generation of CTLs was shown to depend on T cell receptor-mediated let-7 miRNAs downregulation. Decrease of let-7 miRNAs is necessary for the acquisition of effector function through derepression of the let-7 targets (91). On the other hand, miR-155 is necessary to generate effector $CD8^+$ T cells (92). Therefore, it has been suggested since that modulation of let-7 miRNAs or miR-155 can be used to potentiate immunotherapies for cancer.

Furthermore, as previously mentioned, therapeutic vaccines can reverse systemic exhaustion by promoting *de novo* generation of functional T cells. This T cell exhaustion phenomenon is dependent on the host DNA methylation profile. Recently, in mice, T cell exhaustion was successfully reversed by inhibition of *de novo* methylation using Decitabine, an FDA-approved DNA demethylating agent (81). Moreover, as exhausted T cells overexpress checkpoint receptors that prevent them from killing tumor cells, the use of checkpoint inhibitors has proven useful to remove such molecular breaks. Thus, these pharmacological agents could potentially be used in combination with therapeutic cancer vaccines to rejuvenate exhausted T cells, whilst effectively promoting new T cell-mediated immunity.

The magnitude of T cell activation and the accompanying epigenetic modulations dictate the efficacy of a vaccine being developed. The strength of the immune response elicited by vaccines is also highly dependent on the chosen antigens. Several strategies have been recently implemented to optimize this selection. These include personalized peptide vaccines that utilize multiple cancer peptides to complement pre existing host immunity (93). Another strategy is using neoantigens, that is, antigens that arise because of mutations in tumor cell DNA. Once identified, patient's T cells are used to screen which neoantigens harness the potential for effective antitumor responses. Vaccines are then developed based on these screening results. Recently, cancer-specific epigenetic marks have been explored to be used as therapeutic target antigens in vaccines. For instance, several miRNAs have been used in cancer vaccine development (94). Such strategies may provide significant additional resources for individualized cancer treatment.

T CELL RESPONSES AND EPIGENETIC MODULATIONS INDUCED BY ADJUVANT SYSTEMS TO PROMOTE AN ANTI-CANCER ENVIRONMENT

Adjuvants have long been an integral component of vaccines to elicit a strong antigen-specific T cell-mediated immune response. Classically, adjuvants allow gradual antigen release or increase antigen recognition by innate cells to create a prolonged immune response elicited by the vaccine. Alternatively, delivery systems may be used to efficiently deliver a specific antigen to APCs. Nowadays, adjuvants in therapeutic cancer vaccines are not only used to improve anti-tumor immunity, but they are also selected based on their properties that directly promote tumor cell killing and induce an anti-tumor microenvironment. Additionally, adjuvants and delivery systems that promote $CD8^+$ T cells are optimal for cancer vaccine development, though historically many adjuvants have been poor inducers of a $CD8^+$ T cell response. Here, we describe key adjuvants and delivery systems that have progressed to investigation in human clinical trials in cancer patients. Subsequently, we discuss the epigenetic modulations induced by adjuvants, and how such modifications may facilitate vaccine-based therapies in cancer patients.

Vaccine Adjuvants

In most cancer vaccines, adjuvants and immunostimulants are chosen to facilitate generation of $CD8^+$ T cell responses to MHC class I-presented tumor antigens. For this reason, the adjuvant should activate APCs such as DCs, promote antigen presentation and subsequent presentation to induce secretion of stimulatory cytokines, such as IFNγ, IL12, and IL2 (**Figure 2**). Adjuvants that promote cytokine production and Th1 differentiation (95) are desired as Th1 cells costimulate native $CD8^+$ T cells to differentiate into CTLs (8) (**Figure 2**). Moreover, following the stimulation, Th1 cells produce IFNγ that in turn increase antigen presentation on cancer cells (10), to enhance direct killing

of tumor cells (11) as well as create an immunogenic tumor microenvironment (96), thus further helping tumor control. Adjuvants additionally can be selected based on their ability to induce specific innate cells such as natural killer (NK) cell-mediated tumor killing. NK cells are the effector cells of the innate immune system that upon stimulation can directly lyse tumor cells via perforin and granzyme (7). They also have a main role as rapid and potent cytokine producing cells, such as IFNγ and TNFα, that stimulate killing through the death receptor pathways (7, 96). Moreover, NK cells induce DC maturation and amplify T cell anti-tumor responses (97).

One of the main antigen recognition and activation pathways utilized by APCs are TLRs. TLRs are receptors expressed by APCs that can recognize conserved structures derived from pathogens, namely MAMPs (microbe-associated molecular patterns). The same receptors can also recognize DAMPs (damage-associated molecular patterns) that are expressed by cells under conditions of stress. TLR ligands/agonists are widely used to stimulate innate immune responses. TLR agonists, especially those targeting endosomal TLRs, have been shown to generate anti-tumor immunity (98). Thus, cancer vaccines targeting TLR activation could result in the generation of a range of cytokines that stimulate a Th1 bias, as well as promote CTL induction and NK cell-mediated killing that can then be utilized for directed tumor treatment strategies (99).

TLR3, TLR7, TLR8, and TLR9 are predominantly endosomal. It is known that different subsets of DCs have been shown to express distinct arrays of TLRs (100). TLR3 for example is predominantly expressed in conventional DCs (101). This subset of DCs are especially efficient in activating $CD8^+$ T cells and inducing adaptive immune responses against tumor cells (100). Additionally, several cancer cells have been shown to express TLR3 at various levels, including hepatocellular carcinoma (102), breast cancer (103), and neuroblastoma (104). Polyinosinic:polycytidylic acid (Poly I:C) and polyadenylic:polyuridylic acid (Poly A:U) are synthetic analogs of viral dsRNAs which are recognized by TLR3 (105) that have been extensively used as an adjuvant in many clinical trials for cancer vaccines (106). The agonists of TLR3 are capable of activating APCs and cancer cells to induce secretion of inflammatory cytokines including type1 interferons that in turn activate T cell responses against cancer cells (107, 108). Poly I:C is also capable of reversing the pro-cancer innate immune response to anti-cancer immunity, especially within the tumor microenvironment (109). In clinical trials, albeit with limited numbers of patients, both Poly ICLC and Poly I:C12C, modified versions of Poly I:C, were shown to boost anti-tumor activity by inducing potent tumor-specific CTL and NK responses (110, 111).

TLR8 is expressed by conventional DCs and monocytes, whereas TLR7 is expressed predominantly in plasmacytoid DCs (101). Plasmacytoid DCs are a major producer of stimulatory cytokines in response to many viral infections (100). The ligands of TLR7 and TLR8 have been exploited as adjuvants. Their receptors are similar in structure but promote secretion of distinct sets of proinflammatory cytokines by APCs. TLR7 induces the secretion of type I interferons such as IFNα, while TLR8 promotes secretion of TNFα and IL12 (112). Both receptors are endosomal and recognize viral ssRNA (105) and also bind their synthetic analogs, including imiquimod and resiquimod (113, 114). In clinical studies, TLR7/8 agonists enhanced $CD8^+$ T cell responses of a vaccine to prostate-specific peptide and NY-ESO-1, an tumor-specific antigen (115). Additionally, imiquimod has been approved for the treatment of basal cell carcinoma by the FDA (116).

TLR9 agonists are also potent adjuvants. TLR9 itself is predominantly endosomal, and present abundantly in DCs, especially plasmacytoid DCs. It binds microbial DNA, recognizing in particular the unmethylated CpG motifs in viral and bacterial genomes (105). The synthetic TLR9 ligand, CpG oligodeoxynucleotide (CpG-ODN), a short unmethylated ssDNA, activates DCs to secrete type I interferons, and promotes a strong CTL response (117). When used in combination with DC-based cancer vaccines, CpG-ODN enhances $CD8^+$ T cell activity. In combination with tumor-specific-peptide-based vaccines, such as NY-ESO-1 and MART1, CpG-ODN resulted in elevated $CD8^+$ T cell responses, however tumor eradication was rarely achieved (115).

Unlike their endosomal counterpars, TLRs expressed on the cell surface typically recognize extracellular foreign microbes. TLR4, one of the surface TLRs, recognizes LPS molecules of gram-negative bacteria (105). In humans, LPS can cause septic shock syndrome, due to its potent immune stimulatory activity (118). A derivative of LPS, monophosphoryl lipid A (MPL) in combination with the classical adjuvant alum, was licensed by the FDA for use as part of the human papillomavirus vaccine in 2009 (119). In clinical trials, MPL has also been used as an adjuvant for cancer vaccines to promote Th1-specific immune responses (120, 121).

Other adjuvants that have been used to induce T cell responses have included classic formulations/emulsions including oil or saponin. QS-21 is a potent saponin-based adjuvant that is isolated from *Quillaja Saponaria* (122). Although its mechanism of action is largely unknown, QS-21 has been shown to activate the secretion of IL2 and IFNγ, stimulate the proliferation of CTLs and induce Th1 bias (123). Formulations of QS-21 has been tested in human clinical trials for various cancer vaccines (124, 125). Another strong adjuvant that has been trialed for cancer vaccines is Montanide. The aim of this classical adjuvants is to allow sustained antigen release from the immunization site. This strategy is used to create a prolonged and higher amplitude of CTL-mediated immune response. Montanide-based adjuvants are water-in-oil emulsions that promote slow release of antigens and thus prolong antigen presentation to the immune system (126). In clinical trials, montanide ISA720 and ISA51 promote Th1 immune responses and significant CTL activation (127, 128).

Delivery Systems

Several delivery systems, including virosomes, liposomes, viral-like proteins (VLPs), and immune-stimulating complexes (ISCOMs) have been developed and used in clinical trials to improve the efficacy of cancer vaccines. Virosomes are empty viral particles that can carry tumor-specific antigens as vaccines (129). In metastatic breast cancer patients, the

modified influenza virosomes containing the breast cancer peptide (Her/neu$^+$) are well tolerated and not only promote secretion of proinflammatory cytokines, including IL2, TNFα and IFNγ but also promote T cell immunity (130). Liposomes are synthetic phospholipid vesicles that work similarly to virosomes. They are often used to deliver messenger RNA (mRNA) encoding for a specific antigen (131). They have shown promise in delivering mRNA to APCs in clinical trials for non-small cell lung cancer, prostate cancer and follicular lymphoma patients (132, 133), and inducing antigen-specific CD8$^+$ T cell responses. Liposomes that carry DNA have also been developed to stimulate TLR9, activate DCs and subsequently CTLs (134). VLPs are multimeric structures of viral proteins devoid of viral genetic material. Similar to native viruses, specific epitopes on VLPs can be recognized and presented by APCs to promote immune responses as reviewed in Ong et al. (135). VLPs have been developed for use in vaccines for various forms of cancer, including liver, cervix, lung, skin, breast, and prostate, as they not only promote antigen-specific immunity, but also counteract the immunosuppressive microenvironment created by a tumor mass (135). Finally, ISCOMs are composed of saponin, cholesterol and phospholipid. They are regularly used as a vaccine delivery system, however saponin can also stimulate the immune system by activating DCs and inducing robust antigen-specific T cell responses (136). Furthermore, ISCOMATRIX® has been used with the recombinant NY-ESO-1 protein in cancer patients to induce T cell immune responses (137, 138), however this vaccine failed to promote an adequate immune response in advanced melanoma patients (139).

Epigenetic Modulations Induced by Adjuvants and Their Potential to Improve Cancer Vaccines

Whereas a number of whole-pathogen-based vaccines against infectious diseases have been shown to modulate the epigenetic landscape of immune cells, much less is known about the adjuvants and carriers used in cancer vaccines and patients. For example, vaccination with the bacillus Calmette-Guérin (BCG) vaccine for tuberculosis, has been shown to specifically alter epigenetic profiles of monocytes and broadly enhance protection against multiple infectious pathogens (beyond tuberculosis) in humans (140). This suggests that vaccines could leave stable epigenetic marks in certain immune cell populations and alter how the immune system reacts toward subsequent diverse challenges after vaccination, perhaps including cancer. In fact, the non-specific beneficial effects of the BCG vaccine are used in the clinic to treat bladder cancer (141). Modulation of T cell immunity using vaccines in combination with specific adjuvants may provoke changes in epigenetic profiles of immune cells and improve anti-tumor immunity. These beneficial epigenetic profiles may be further potentiated by the use of epigenetic modulating drugs. Indeed, epigenetic potentiation of the NY-ESO-1 protein vaccine with montanide-based adjuvant using decitabine, a DNMT inhibitor, has been successful in treating epithelial ovarian cancer (28).

Several adjuvants currently used in cancer vaccines are indeed capable of altering immune cell interactions with cancer cells by inducing stable epigenetic modifications in both host immune and cancer cells. These adjuvants could therefore be harnessed as promising candidates to promote beneficial epigenetic modulations in vaccine-based therapies. The use of TLR-ligand adjuvants could indeed be promising, as studies have shown that epigenetic reprogramming can be achieved via TLR stimulation. For instance, stimulating TLR3 with Poly I:C activates the epigenetic machinery causing a global change in the expression of epigenetic modifiers that in turn promotes chromatin remodeling and nuclear reprogramming (142). In addition, TLR3 receptor combined with Poly I:C directly promoted global DNA methylation in peripheral blood mononuclear cells (PBMCs) in pigs (143). Poly I:C promotes the expression of pro-inflammatory cytokines IL23 and IL33 by direct modification of the epigenetic marks on the promoters of these gene loci (144, 145). Furthermore, it reactivates the expression of several silenced miRNAs in tumor cells that subsequently leads to its direct anti-tumor activity (146). Such direct epigenetic modifications by Poly I:C are highly beneficial to improve the efficacy of therapeutic cancer vaccines.

Another advantageous cancer vaccine adjuvant candidate could be CpG-ODN, a ligand for TLR-9. Although there is less data available regarding the effects of CpG-ODN on global epigenetic reprogramming, it has been shown to promote chromatin changes in specific gene loci. For example CpG DNA induced production of IL12 due to its ability to elicit epigenetic modifications on the IL12p40 promotor, including histone acetylation and nucleosomal remodeling, which leads to gene activation (147). In cancer cells, CpG-ODN has been shown to directly exert its anticancer potential. For instance, in chronic lymphocytic leukemia (CLL), CpG-ODN promotes epigenetic changes associated with active transcription, namely, H3K9/K14 acetylation and H3K4 trimethylation at the promoter of *PRDM1* (148). PRDM1 expression promotes terminal differentiation of CLLs (149, 150), which is established as a potent therapy for CLL.

Epigenetic-modulating activites of TLR4 ligand adjuvants may mimic those exerted by LPS. This classical TLR4 ligand promotes innate immune responses by reprogramming monocytes to accumulate active histone marks such as H4Ac, at promoters of genes involved in inflammation and phagocytic pathways (151). However, further stimulation of innate immune cells by LPS can promote tolerance, by removal of H4Ac at promoters of inflammatory gene loci, such as *IL6 and TNFα* (152, 153). It was further identified that Trichostatin A, a deacetylase inhibitor could reverse the repression of *IL6* and restore H4Ac (152).

Additionally, adjuvants that deliver genetic materials can also potentially be used to promote beneficial epigenetic modulations during vaccine-based cancer therapies. RNA-LPX, a liposome-based adjuvant for example, has been shown to efficiently target DCs and promote strong antigen-specific T cell responses in melanoma patients (131). Since the expression of many miRNAs are altered in various cancer cells, such form of adjuvant could potentially be used to target miRNAs to both alter epigenetic imprinting in the cancer cells and promote cancer elimination.

Despite progression in the knowledge of adjuvants for cancer therapy, their mode of action and the precise epigenetic mechanisms involved are still largely unmapped. As discussed earlier, all types of adjuvants may exert direct and indirect effects, which might result in epigenetic modifications in the cells of the immune system and the associated cancer cells. The accumulating evidence highlighted above provides a rationale to investigate more broadly the potential use of epigenetic modifications by vaccine-adjuvants in the context of cancer therapy.

EPIGENETIC MECHANISMS INVOLVED IN CANCER IMMUNE ESCAPE AND WAYS TO COUNTERACT SUCH MECHANISMS

Disruption of epigenetic regulatory mechanisms is prevalent in cancerous cells leading to altered gene expression, perturbed functionality and malignant transformation. Due to the reversible nature of epigenetic modifications and their involvement in cancer, several epigenetic-modifying drugs have now been approved by the FDA for cancer treatment (154). Several mechanisms including downregulation of antigen presentation machinery, upregulation of coinhibitory/checkpoint ligands and establishment of a pro-cancer environment are involved in immune evasion by cancer cells (**Figure 3**). In this section, we will discuss epigenetic mechanisms involved in cancer escape from T cell-mediated immunity, and epigenetic drugs that may be able to counteract such mechanisms.

To escape from CTL-mediated killing, cancer cells commonly downregulate the expression of their antigens. This is achieved by epigenetically modifying their DNA, through methylation, commonly observed for MHC class I antigens, and via histone deacetylation, often seen for MHC class II antigens (155, 156). *In vitro,* the HDAC inhibitor mocetinostat increases antigen presentation by MHC class II molecules (157). Other available epigenetic drugs that may modulate the level of expression of antigens in cancer cells include histone methyltransferase (HMT) and demethylase (HDM) inhibitors (158, 159) (**Figure 3**). In patients, reduced expression of antigens and the components of antigen presentation machinery, such as MHC class I molecule has been shown to correlate with malignancy (160–162). Epigenetic-modifying drugs, such as DNMT and HDAC inhibitors have been widely used to reverse this downregulation of tumor antigens (154). DNMT inhibitors for example, including 5-azacytidine (5-AC) and 5-aza-2'-deoxycytidine (DAC) have been approved by the FDA for the pre-leukemic disorder myelodysplasia (MDS) (163).

The components of the cellular antigen presentation machinery including MHC class I, TAP1, TAP2, LMP2, and LMP7 are epigenetically downregulated in many cancers (164–166). Similarly, tumor cell downregulation of costimulatory molecules including CD40, CD80, CD86, and ICAM1 have been observed and associated with the rapid progression of various cancers as reviewed in (167). Additionally, cancer cell escapes from CTL-induced apoptosis by downregulating the expression of their death receptors, such as TRAIL-R and Fas (168). In *in vitro* and animal models, both DNMT and HDAC inhibitors have been shown to induce the expression of the antigen presentation molecules (164–166), surface costimulatory molecules and death receptors (166, 169–174), which then increases the sensitivity of tumor cells to immune-mediated cell killing.

Another known mechanism of immune evasion by cancer cells is to increase their expression of checkpoint ligands, such as PD-L1, CD80, and CD86 (**Figure 3**) and promote T cell tolerance. The use of DNMT and HDAC inhibitors in such cancer cells may synergistically upregulate the expression of the checkpoint ligands on the surface of cancer cells (175). This is however argued to be beneficial since these epigenetic-modifying agents will sensitize tumor cells for checkpoint inhibitor therapy and allow CTL-mediated killing (176, 177). On the other hand, a BET bromodomain inhibitor (JQ1), has been shown to directly downregulate the expression of checkpoint receptors on cancer cells, rendering them sensitive to CTL-mediated cell death (178, 179).

Many cancer cells suppress certain miRNA expression, in order to increase the expression of checkpoint ligands on their cell surface. These miRNAs include miR-34 (180), miR-29 (181), and miR-200 (182) in lung cancers, miR-138 in glioma (183), miR-187 in renal cell carcinoma (184) and miR424(322) in ovarian carcinoma (185). Based on this knowledge, therapeutic miRNAs could be developed to repress checkpoint ligand expression on the surface of cancer cells. However, their use as therapeutic treatment agents will require rigorous clinical testing as miRNAs may not be specific and thus pose significant concerns regarding non-specific adverse effects in patients.

Another recently identified mechanism of tumor immune escape is the repression of chemokine expression. Chemokines are required for T cell infiltration into the tumor microenvironment (**Figure 3**). For example, in ovarian cancer, tumor cell production of chemokines CXCL9 and CXCL10 are epigenetically repressed by EZH2-mediated H3K27me3 and DNMT1-mediated DNA methylation (186). Inhibition of EZH2 methytransferase increases chemokine production and improves T cell infiltration in patients with ovarian cancers (186). Similarly HDAC inhibitors have been used to increase chemokine expression and T cell infiltration in lung cancer patients (187).

Although epigenetic drugs are mainly used to target cancer cells, they may also exert their effects on host immune cells. For example, HDAC inhibitors can increase histone acetylation on several gene promoters in NK cells, including the death-induced receptors Fas and TRAIL-R2, which potentiate NK cell-mediated immune surveillance against cancer cells (173, 174, 188, 189). However, the global modulating effects of these drugs on T cells and other cells than cancer, are currently unknown.

Extensive clinical research has been carried out that has resulted in FDA approval for the use of seven epigenetic drugs for cancer treatment (154), though the role of such epigenetic inhibitors or modulators in altering the epigenetic landscapes of cells other than cancer cells is currently largely unknown. This is an important issue since epigenetic-modifying drugs as well as miRNA therapies, may not be specific, and thus may cause

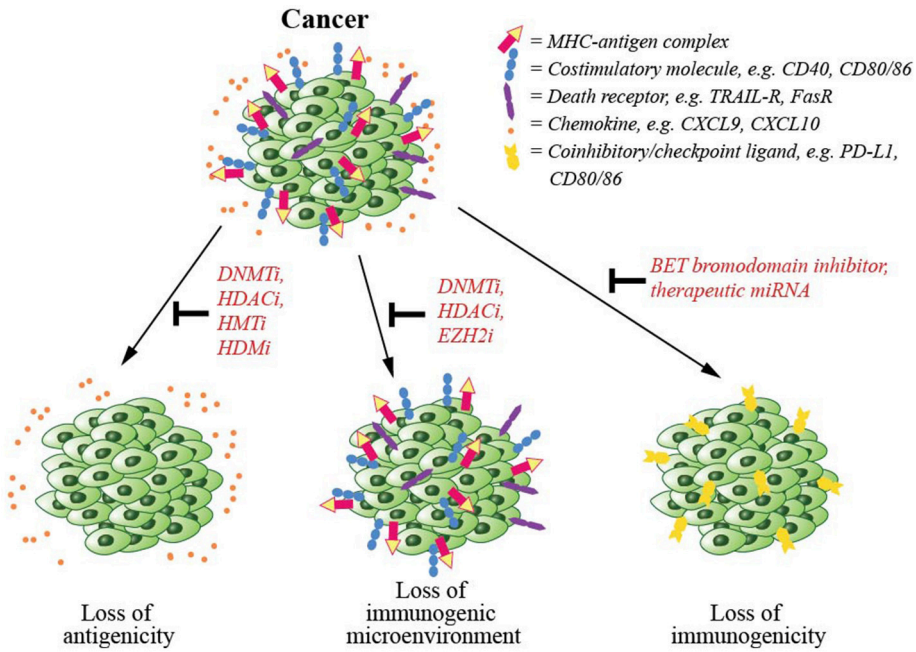

FIGURE 3 | Cancer immune evasion and the epigenetic modifications counteracting such mechanisms. To escape from immune-mediated killing, cancer cells exploit several evasion strategies. These are (1) downregulation of antigen presentation and costimulatory molecules, which could be counteracted by the inhibitors of epigenetic regulators including DNA methyltransferase (DNMT), histone deacetylase (HDAC), histone acetyltransferase (HAT), or histone demethylase (HDM), (2) downregulation of chemokines that signal T cells to infiltrate tumor mass, again that could be inhibited by inhibitors of DNMT, HDAC, or EZH2, and (3) upregulation of coinhibitory/checkpoint ligands, which could also be blocked by a BET bromodomain inhibitor.

multiple unknown effects in patients. However, further clinical studies are certainly warranted to fully investigate potential treatment side-effects, especially when the epigenetic therapy is used in combination with immunotherapy, such as in cancer vaccines.

EPIGENETICS AS CANCER BIOMARKERS IN VACCINE IMMUNOTHERAPY

Epigenetic marks including DNA methylation, histone modification, and RNA-associated mechanisms, such as miRNAs and lncRNAs are found to be heritable mitotically from cell to cell and meiotically from generation to generation. Epigenetics has explained how gene activity can be modulated by external environmental factors, such as lifestyle and diet. Due to this unique characteristic, epigenetic marks gained from external environmental cues that shape the parent's DNA are heritable, thus allowing the transfer of experiences from the parents to offspring (190). A person's own lifestyle also shapes that individual's epigenetic profiles. As these profiles dictate cell identity and function, they also dictate individual susceptibility to diseases including cancer (191) and the capacity of their immune system to respond to different challenges. Such profiles can thus be exploited as non-invasive markers for cancer susceptibility, diagnosis and prognosis (192) and possibly predicting the effectiveness of vaccine therapy.

Epigenetic alterations can be readily detected as circulating biomarkers and may prove useful in clinical cases where surgery is contraindicated and biopsy results are inconclusive, such as in gliomas (193). Many circulating epigenetic biomarkers have been developed based on specific DNA methylation pattern of the cancerous cells, as reviewed in (194, 195). For example, in prostate cancer, methylated *MCAM* detects early stage of cancers with 66% sensitivity and 73% specificity, which is an improvement from PSA with only 42.8% sensitivity and 41.1% specificity (196). Circulating nucleosomes and histone modifications may also serve as markers to increase specificity and sensitivity of current diagnostic and prognostic tests as reviewed in (197, 198). Other attractive circulating epigenetic biomarkers in cancer are the circulating miRNAs, as reviewed in (199). For example, in pancreatic ductal carcinoma, miR-155-5p in plasma is a marker for cancer presence, and increased expression levels in cancerous tissues are associated with a more advanced tumor stage and poorer prognosis (200–202).

Importantly, such non-invasive biomarkers would also be effective tools for both choosing and monitoring the effectiveness of cancer vaccines for each individual case. For example, in gastric cancer, an increased plasma miR-222 level is significantly correlated with a more advanced tumor stage and a lower overall survival (203). This marker can thus be used to predict the outcome of the disease and in combination with T cell functional markers such as IL2, TNFα, and IFNγ could predict patient's response to specific cancer vaccine. Certainly, epigenetic marks identified in a person's immune cells, such as the levels of

specific miRNAs involved in T cell effector function and T cell exhaustion, may be used as functional biomarkers to predict T cell activity following vaccine therapy and additionally to help create an effective combination therapy for that particular person.

THE FUTURE OF THERAPEUTIC CANCER VACCINES AS IMMUNOTHERAPY

As therapeutic cancer vaccines evolve and additional knowledge of their mode of action is established, more effective personalized treatment strategies will be developed. Combination therapies for cancer using complementary vaccine-based therapy with epigenetic inhibitors and/or checkpoint inhibitors will also become more widely used. As the nature of both cancer cell and the associated host immune response is dependent on host epigenetic profiles, additional detailed knowledge of the epigenetic modulations involved in vaccine-generated T cell immunity against cancer cells could prove instrumental to the development of effective vaccine-based immunotherapy. Whilst the epigenetic landscape of cells is unique amongst individuals, specific epigenetic profiles of cancerous cells, as well as of immune cells may be harnessed as biomarkers for early detection of tumors, and also to guide the selection of a targeted therapy.

AUTHOR CONTRIBUTIONS

AK and MP contributed to the conception and design of the review study. AK wrote the first draft of the manuscript. AK, MDP, MC, KW, JB, JC and MP wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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REVIEW
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Diamonds in the Rough: Harnessing Tumor-Associated Myeloid Cells for Cancer Therapy

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Therapeutic approaches that engage immune cells to treat cancer are becoming increasingly utilized in the clinics and demonstrated durable clinical benefit in several solid tumor types. Most of the current immunotherapies focus on manipulating T cells, however, the tumor microenvironment (TME) is abundantly infiltrated by a heterogeneous population of tumor-associated myeloid cells, including tumor-associated macrophages (TAMs), tumor-associated dendritic cells (TADCs), tumor-associated neutrophils (TANs), and myeloid-derived suppressor cells (MDSCs). Educated by signals perceived in the TME, these cells often acquire tumor-promoting properties ultimately favoring disease progression. Upon appropriate stimuli, myeloid cells can exhibit cytoxic, phagocytic, and antigen-presenting activities thereby bolstering antitumor immune responses. Thus, depletion, reprogramming or reactivation of myeloid cells to either directly eradicate malignant cells or promote antitumor T-cell responses is an emerging field of interest. In this review, we briefly discuss the tumor-promoting and tumor-suppressive roles of myeloid cells in the TME, and describe potential therapeutic strategies in preclinical and clinical development that aim to target them to further expand the range of current treatment options.

Keywords: tumor-associated dendritic cells, tumor-associated macrophages, myeloid-derived suppressor cells, tumor-associated neutrophils, cancer immunotherapy, tumor microenvironment

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INTRODUCTION

For a long time, tumors were thought to consist mainly of malignant cells, however this view changed in the past decades and tumors are now considered to behave as organ-like structures that contain besides cancer cells a large array of stromal cells. These tumor-infiltrating stromal cells comprise among others, immune cells, fibroblasts, pericytes, and endothelial cells, which closely interact with the cancer cells, forming the tumor microenvironment (TME) (1).

The interactions between the cancer cells and the immune system are initially hostile, resulting in many occasions in a successful eradication of the malignant cells (2). However, due to their rapid evolution, cancer cells can develop immune evasion mechanisms enabling them to avoid immune destruction (1). Furthermore, chronic inflammation caused by the tumor associated immune cells, secreting growth factors, cytokines, chemokines and reactive oxygen species, ultimately leads to an increased survival, growth and heightened rate of mutations in the DNA of the cancer cells (3). The presence of these tumor-promoting immune cells is often associated with an increased resistance

to cancer therapies (4–8). Nevertheless, some of these tumor-associated immune cells still retain their anti-tumoral properties, the latter being suppressed by several factors produced in the TME (6, 9–12).

Deploying the immune system in anti-cancer therapies enables the specific targeting of (metastatic) cancer cell in the body expressing the specific tumor-associated antigens (TAAs). Most current immunotherapeutic approaches focus on lymphoid cells, particularly on the reactivation of pre-existing anti-tumoral T cells or adoptive transfer of tumor-specific T cells. In this respect, several immunotherapeutic strategies already made it to the clinic, such as CAR T-cell therapy or immune checkpoint inhibitors against PD-1, PD-L1, or CTLA-4, which are capable of re-invigorating T-cell responses in the TME (13–16). However, despite their success, *de novo* or acquired resistance against these therapies is widespread among patients (17), urging for the development of new immune therapies.

Targeting of tumor-associated myeloid cells, which abundantly infiltrate most solid tumors, might provide novel therapeutic approaches for cancer patients and is an emerging field of interest.

In this review, we briefly describe the role of several distinct tumor-associated myeloid cell subsets, i.e., macrophages, dendritic cells, neutrophils and MDSCs, with emphasis on their tumor-promoting and/or tumor-suppressive roles. Subsequently, the potential of myeloid cells in future cancer immunotherapy will be addressed.

MACROPHAGES

Referred to as "big eaters," macrophages are one of the largest types of leukocytes, specialized in the phagocytosis of dead cells and pathogens. Besides their role in immune surveillance, macrophages are key players in tissue homeostasis maintenance and tissue repair (18). Macrophages are present in all tissues and originate from yolk sac macrophages, fetal liver monocytes and circulating monocytes that colonize the tissues in sequential waves (19, 20).

In tumors, macrophages can comprise up to 50% of the total hematopoietic compartment, negatively correlate with tumor progression and/or clinical outcome in many cancer types (21), with the majority of TAMs originating from circulating monocytes (22). However, certain studies, using orthotopic tumor models, showed that a fraction of the TAMs arises from the tissue-resident macrophages surrounding the tumor (23, 24). Recent evidence in several murine brain tumor models pointed out that the tissue-resident TAMs (microglia in this case) retained some of their tissue-specific traits, resulting in differential transcriptional profiles and activation states between microglia and monocyte-derived macrophages in the TME (23).

Importantly, multiple studies in mice showed that the TME was infiltrated with a heterogeneous monocyte-derived compartment and encompassed at least two molecular and functionally distinct TAM subsets, which populate different tumor microenvironments, namely a M1-like TAM subset, characterized by a more pronounced pro-inflammatory profile and higher expression of MHC-II and co-stimulatory molecules and a pro-angiogenic and immunosuppresive M2-like TAM subset (**Figure 1**) (10, 25, 26). The characteristics and emergence of these subsets are discussed elsewhere (7, 22, 27, 28).

It is, however, important to note that this M1/M2 dichotomy is an oversimplified representation of the vast range of activation states macrophages can adopt *in vivo* (29). Furthermore, recent studies in human tumors question the existence of distinct M1- and M2-like TAM subsets (30–32), indicating the need for a revised TAM nomenclature, which could be based on activation states, such as functional or metabolic programming, or by respecting a graded scale rather than separate entities, in line with the spectrum model of macrophage activity.

Two main TAM-based therapeutic strategies have recently gained interest in the fight against cancer: (i) depletion of TAMs through elimination of resident macrophages or inhibition of monocyte/macrophage recruitment to the TME and (ii) repolarization of immunosuppressive M2-like TAMs into anti-tumor M1-like TAMs. The first strategy is not the major focus of this review and is therefore only discussed briefly.

Depleting TAMs Through Elimination of Resident Macrophages and/or Inhibition of Monocyte/Macrophage Recruitment

Several molecules were shown to efficiently deplete TAMs from the TME. The tunicate-derived chemotherapeutic molecule trabectedin demonstrates a cytotoxic activity against circulating monocytes and TAMs by activating the apoptotic pathway via TRAIL, which was successfully tested in several murine tumors models. This ultimately resulted in a decreased number of mononuclear phagocytes and an increased infiltration of anti-tumoral effector T cells in the TME (33, 34). Another group of drugs selectively targeting myeloid cells are bisphosphonates, such as clodronate-liposomes (35, 36) which induce the apoptotic pathway in TAMs as well. After liposome uptake, clodronate is released intracellularly and converted to a non-hydrolizable ATP analog, ultimately leading to the formation of pore openings in the mitochondrial inner membrane, eventually resulting in apoptosis. Finally, the conventional chemotherapeutic drug doxorubicin, which inhibits topoisomerase II, has been shown to significantly deplete TAMs in mice with orthotopic MMTV-Wnt1 triple-negative breast carcinoma, when encapsulated in nanoparticles specifically targeting TAMs, i.e., DOX-AS-M-PLGA-nanoparticles (37).

In the aforementioned treatment strategies, all TAMs are targeted, hence also depleting M1-like TAMs with potential anti-tumoral characteristics. Therefore, selectively depleting M2-like macrophages has gained interest. The identification of MMR as a marker for M2-like TAMs, residing in hypoxic tumor areas (10, 25), enables the visualization of these pro-tumoral macrophages for diagnostic purposes using anti-MMR Nanobodies *in vivo* (38, 39) and could potentially be coupled to toxic moieties for selective depletion of M2-like TAMs (40).

In order to prevent monocytes from maturing to tumor-promoting TAMs, the inhibition of monocyte recruitment to the TME can also be envisaged. One approach is to interfere with the

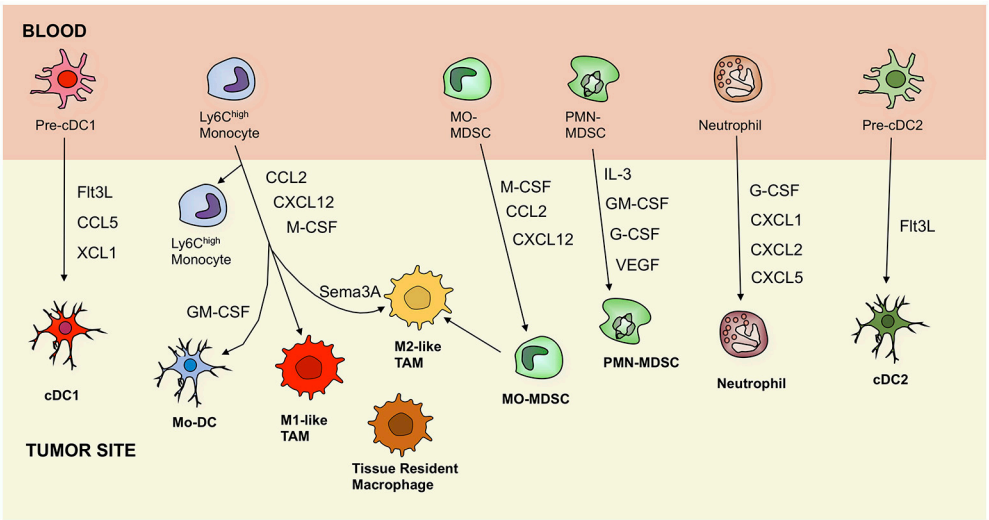

FIGURE 1 | Ontogeny of tumor-associated myeloid cells, including dendritic cells, macrophages, monocytes, myeloid-derived suppressor cells, and neutrophils. Black arrows indicate recruitment pathways that are driven by secreted factors. cDC, conventional dendritic cell; Mo-DC, monocyte-derived dendritic cell; TAM, tumor-associated macrophage; MO-MDSC, monocytic myeloid-derived suppressor cell; PMN-MDSC, polymorphonuclear myeloid-derived suppressor cell; Flt3L, Fms-related tyrosine kinase 3 ligand; CCL5, C-C motif chemokine ligand 5; XCL1, lymphotactin; GM-CSF, granulocyte-macrophage colony-stimulating factor; CXCL12, C-X-C motif chemokine 12; M-CSF, macrophage colony-stimulating factor; Sema3A, semaphorin 3A; IL-3, interleukin 3; GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; VEGF, vascular endothelial growth factor.

CCL2/CCR2 axis, using an anti-CCL2 antibody (41) or bindarit, which inhibits CCL2 synthesis (42). Another important regulator of monocyte recruitment toward the TME is the CSF-1 receptor, whose inhibition leads to macrophage depletion in several murine and human tumors (43–45). Moreover, CSF-1R blockade using monoclonal antibodies or small molecule inhibitors not only leads to a reduced attraction of monocytes to the tumor, but also to the preferential differentiation of monocytes toward M1 TAMs, resulting in a higher intratumoral M1/M2 ratio in mice (46, 47). In addition, inhibition of either CCR2 or CSF-1R has been shown to decrease the chemotherapy-resistance of pancreatic tumors and to increase the T-cell mediated anti-tumor immune response in mice (48).

Reprogramming of the TAM Phenotype

Enforcing M1 programming of TAMs may reduce their tumor-promoting functions and help stimulate anti-tumor immunity, opening a new field in immunotherapy aiming at the repolarization of the M2-like TAMs to M1-like TAMs (**Figure 2**) (22, 49).

Inhibition of Intracellular Signaling Pathways

A promising candidate for the repolarization of TAMs is the selective inactivation of phosphatidylinositol-3-kinase γ (PI3Kγ). This intracellular kinase has been shown to induce a transcriptional program via Akt and mTOR signaling ultimately leading to immune suppression in the TME (50). Inhibiting PI3Kγ genetically or via small molecules (TG100–115 or IPI-549) resulted in decreased tumor growth and prolonged survival in several murine tumor models, including head and neck squamous cell carcinoma, lung carcinoma and spontaneous breast carcinoma models. TAMs from mice lacking PI3Kγ showed increased levels of MHC-II and pro-inflammatory cytokines and were less immunosuppressive, which resulted in a restored $CD8^+$ T-cell activation and cytotoxicity (50). In 4T1 breast carcinoma and B16-GM-CSF melanoma models, the inhibition of PI3Kγ by the small molecule inhibitor IPI-549, significantly improved the T-cell function and reduced immune suppression by increasing the M1/M2 ratio. Furthermore, in combination with PD-1 and CTLA-4, IPI-549 resulted in complete remission in 30% of the 4T1-bearing and 80% of B16-GM-CSF-bearing mice (51). Another key regulator of human M2 TAM gene expression is hematopoietic cell kinase (HCK), a member of the Src family kinases. Poh et al. showed that high HCK expression and activation correlated with a reduced survival of colorectal cancer patients and the preferential accumulation of M2-like TAMs respectively. Pharmacological inhibition or genetic reduction of HCK activity suppressed M2-like TAM activation and the growth of colon cancer xenografts, making HCK a promising target for cancer therapy (52). Finally, the inhibition of a group of histone deacetylases, HDAC class IIa, by a specific inhibitor, TMP195, reduced tumor burden and pulmonary metastasis by modulating the TAM phenotype in the murine MMTV-PyMT breast cancer model, and enhanced chemo-and T-cell checkpoint blockade therapy (53).

Toll-Like Receptor Agonists

Toll-like receptor (TLR) agonists have been shown to be capable of stimulating the repolarization of M2-like TAMs toward M1-like TAMs, and therefore entail a promising future therapy. An example of such a ligand is the TLR7/8 agonist, 3M-052,

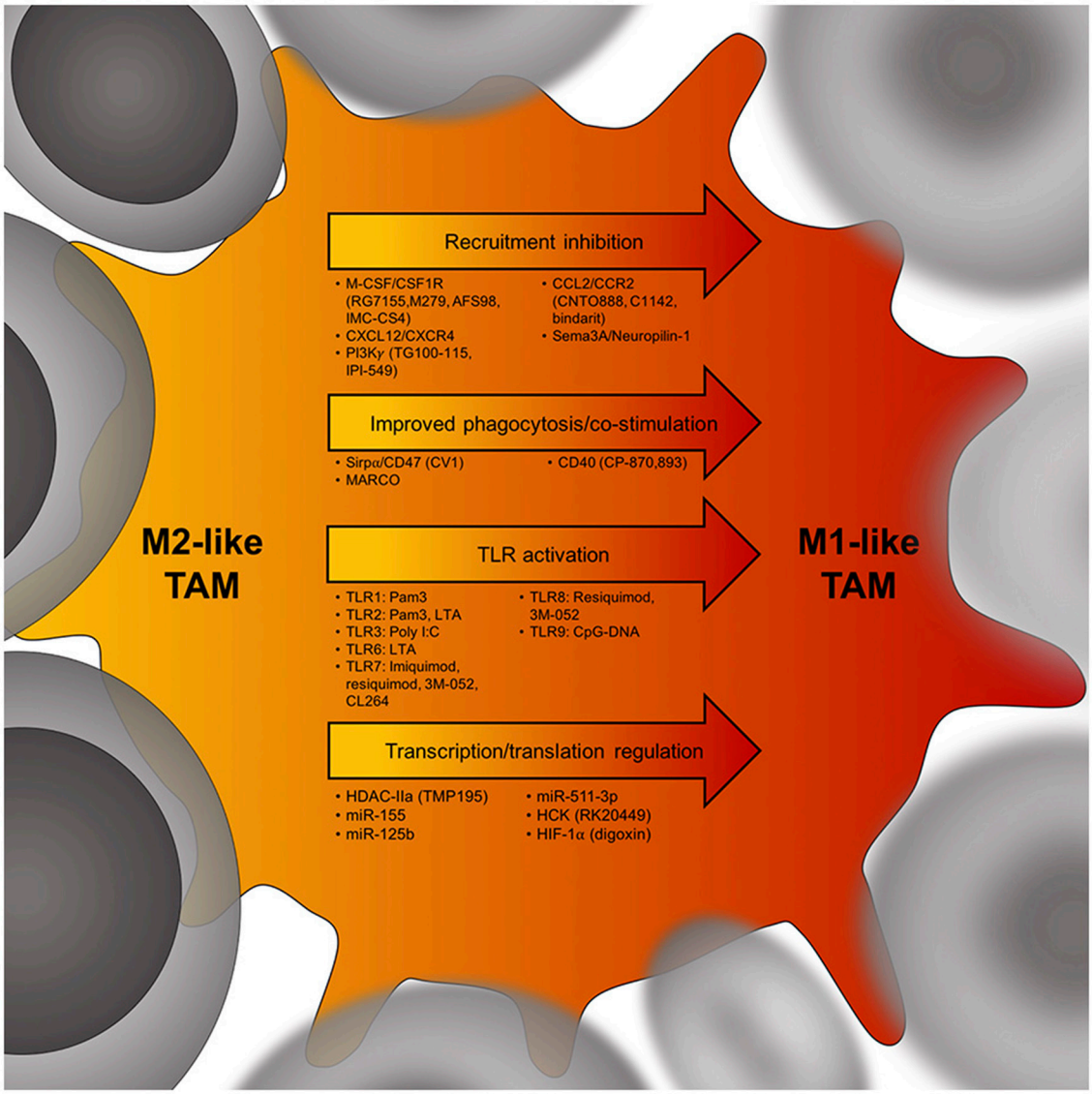

FIGURE 2 | Potential targets to skew the TAM phenotype from an immunosuppressive M2-like TAM (yellow) to an anti-tumor M1-like TAM (red). Cancer cells are in gray, arrows indicate potential targets to induce a TAM phenotype shift within tumors. Below each arrow are specific targets that could influence M2-like TAM phenotypes. M-CSF, macrophage colony-stimulating factor; CSF1R, colony stimulating factor 1 receptor; CXCL12, C-X-C chemokine ligand 12; CXCR4, C-X-C chemokine receptor 4; PI3Kγ, phosphatidylinositol-3-kinase γ; CCL2, C-C chemokine ligand 2; CCR2, C-C chemokine receptor 2; Sema3A, semaphorin 3A; Sirpα, signal regulatory protein alpha; MARCO, Macrophage receptor MARCO; CD40, cluster of differentiation 40; TLR, toll-like receptor; HDAC-IIa, histone deacetylase IIa; miR155, microRNA 155; HCK, proto-oncogene HCK; HIF-1α, hypoxia-inducible factor 1-alpha.

which stimulated M2 to M1 polarization upon intratumoral injection. This approach resulted in a significant decrease of murine B16-F10 melanoma tumor growth through an elevated M1 phenotype-shifted macrophage infiltration with additional activation of $CD8^+$ T cells, B cells, and pDCs. When used in combination with anti-PD-L1 and anti-CTLA-4 antibodies, cytotoxicity of TAMs and $CD8^+$ T cells in the same melanoma model was potentiated (54). One of the TLR7 ligands, imiquimod, has been approved by the US Food and Drug Administration to topically treat early skin cancers. The use of imiquimod not only resulted in an inhibition of tumor growth, but also in complete regression of murine TSA mammary tumors, when used in combination with radiotherapy or low dose of cyclophosphamide (55). Another agonist of TLR7 and TLR8, namely R848 or resiquimod, loaded into β-cyclodextrin nanoparticles induced a functional re-orientation of the TME, in which the M2-like TAMs shifted toward a M1-like TAM phenotype, reducing tumor growth in multiple murine tumor models (56).

The use of a dsRNA analog, poly I:C, which is a potent TLR3 agonist, resulted in lewis lung carcinoma (LLC) regression in mice through the increased presence of tumor-suppressive

M1-like TAMs (57). Strikingly, already 1 h after intraperitoneal injection, TNF-α levels increased, leading to the subsequent decrease of LLC tumor growth (57). The TLR9 agonist CpG-DNA, was able to induce reprogramming of TAM from a M2-like to a M1-like phenotype, alone or in combination with an anti-IL-10R Ab when injected intratumorally in 4T1 breast tumor-bearing mice (58). In addition to the repolarization of TAMs, this molecule was able to stimulate a cytotoxic T-cell response in the murine EG7-OVA lymphoma model (59).

Aside from the aforementioned strategies, combination therapies using both TLR agonists and immune checkpoint inhibitors have also been shown to be beneficial. Intratumoral injections of TLR7 and TLR9 agonists [1V270 and SD-101(CpG), respectively] alongside with systemic administration of anti-PD-1 mAbs successfully suppressed tumor growth in murine models of head and neck squamous cell carcinoma (60). Regression was not only observed at the primary tumor site, but distant tumors were suppressed as well, with a clear increased ratio of M1-like to M2-like TAMs (60). In addition, the efficacy of anti-PD-1 treatment in athymic nude mice implanted with human osteosarcoma relied on the presence of macrophages in the tumor. As such, anti-PD-1 treatment led to a higher activation of M1 macrophages due to repolarization from M2 TAMs, likely due to STAT3 signaling blockade (36).

Müller et al. tested a whole panel of TLR agonists with or without co-administration of IFNγ in an *in vitro* cancer cell growth inhibition assay using bone marrow-derived macrophages. Their results pointed out that IFNγ and the TLR agonists [LPS, poly(I:C), TLR1/2 agonist Pam3, TLR2/6 agonist LTA, TLR7 agonist CL264, and TLR9 agonist CpG] acted in synergy to induce macrophage tumoricidal activity and production of both NO and pro-inflammatory cytokines. These results suggest that IFNγ secretion in the TME may be an important factor that determines the effectiveness of TLR agonists (61).

Analogous to the activation of TLRs, bacterial species can be inoculated in the TME, resulting in acute inflammation and M1-like TAM activation. Bacteria mediated tumor therapy has been extensively reviewed elsewhere (62, 63).

TAM Repolarization and miRNAs

One of the post-transcriptional regulators that mediate differentiation of monocytes into either M1-like or M2-like TAMs are miRNAs, small non-coding pieces of RNA of approximately 20–25 nucleotides. While their exact functions in macrophage polarization are yet to be fully elucidated, some have already gained interest for future therapies.

A gain of function study showed that overexpressing miR-155 in M2-activated macrophages led to repolarization of these cells into proinflammatory M1-like macrophages (64). Through the regulation of FGF2 expression, miR-155 was able to decrease tumor progression, making it a potential target in future immunotherapy (65). Overexpression of another miRNA, namely miR125b, using a viral vector, proved to promote the M1-like activation, leading to an increased cytotoxic activity against EL4 cancer cells *in vitro* and *in vivo* (66). Transfecting miR125b using CD44 targeting nanoparticles led to a 6 fold increase in the M1/M2 ratio in a mouse model of non-small cell lung cancer (67). Another strategy involved the enforced expression of miR-511-3p, which is encoded by MRC1 genes, in TAMs, resulting in a decreased protumoral gene signature of MCR1 $(MMR)^+$ TAMs and inhibited murine LLC tumor growth (68).

Finally, the importance of miRNAs in the differentiation of macrophages in the TME was demonstrated by Baer et al. in mice, where the inactivation of the miRNA-processing enzyme DICER in TAMs promoted the intratumoral expansion of M1-like TAMs, with a pronounced IFN-γ/STAT1 transcriptional signature and the concurrent demise of M2-like TAMs. The TAM's phenotype switch was associated with enhanced tumor infiltration by cytotoxic T-cells (CTLs) and IFN-γ production, MC38 tumor inhibition and, importantly, increased tumor responsiveness to PD1 checkpoint blockade (69).

Tumor Vascularization and TAM Repolarization

The high consumption of nutrients and oxygen by the cancer cell mass demands a constant and sufficient intratumoral blood flow. To that end, angiogenesis is promoted in the TME through excessive secretion of pro-angiogenic factors, such as vascular endothelial growth factors (VEGFs). However, this uncontrolled tumor vascularization leads to imperfect and leaky blood vessels, promoting metastatic dissemination and intratumoral hypoxia (70). For a long time, the preferred strategy was to further disrupt the vessel composition in order to starve cancer cells. However, this resulted in a more aggressive tumor and often increased metastatic outgrowth. These findings suggest that the opposite strategy, i.e., improving the functionality of the tumor vasculature (also termed vessel normalization), might be more beneficial to the patient (71). Both aforementioned strategies also have their impact on the TAM composition in the TME.

Although intratumoral vessel disruption strategies lead to more aggressive cancer progression and metastasis, their use has also been shown to elicit macrophage phenotype skewing, demonstrating potential tumor-suppressive functions. An example of this strategy is the vascular disrupting agent 5,6-di-methylxanthenone-4-acetic acid, DMXAA, which was shown to induce the repolarization of M2-like TAMs to an M1-like phenotype in a mouse model of non-small cell lung cancer (72). However, vascular disruption also resulted in increased hypoxia, leading to the subsequent activation of HIF-1α, resulting in a more aggressive cancer phenotype. Accordingly, inhibition of HIF-1α using digoxin was synergistic with DMXAA and led to stronger inhibition of tumor growth and metastasis of murine B16-F10 melanoma than DMXAA or digoxin alone (73). However, the direct effect of the treatment on M1-like TAMs remains to be elucidated. Another vascular disruption agent which showed such characteristics, is Z-GP-DAVLBH, which induced the secretion of GM-CSF and the skewing of M2-like to M1-like TAMs in hepatocellular carcinoma and breast cancer xenografts, leading to higher rates of cancer cell apoptosis (74).

Vessel normalization strategies, such as the inhibition of ANG2 and VEGF, also have the potential to induce repolarization of TAMs. In murine and human glioblastoma models, a bispecific antibody against ANG2/VEGF was shown to induce prolonged survival through reprogramming of TAMs from a

M2 to a M1 phenotype (75). Similar observations were made by other research groups when using peptibodies inhibiting both the ANG2 and VEGF receptors or a bispecific antibody inhibiting ANG2 and VEGF themselves (76–78). Finally, another factor capable of promoting TAM repolarization and vessel normalization is histidine-rich glycoprotein (HRG), which is generally only expressed in low levels in the TME. A gain-of-function experiment, transducing *HRG* in T241 fibrosarcoma, Panc02 pancreatic carcinoma and 4T1 breast carcinoma models, showed reduced growth mediated by an increased presence of M1-like TAMs (79).

Alternative Strategies Increasing M1/M2 Ratios

The use of antibodies in the reprogramming of TAM ratios has also proven successful when agonistic anti-CD40 antibodies were administered in combination with gemcitabine, resulting in tumor regression in both mice and human patients with pancreatic ductal carcinoma (80). In this study, tumor regression did not seem to depend on gemcitabine or T cells, but on the presence of activated macrophages (80). Interestingly, CD40 agonist antibodies have been shown to induce tumoricidal properties in macrophages and to promote the maturation of antigen presenting cells, making them an ideal choice for combination therapies with immune checkpoint inhibitors (81, 82).

Similarly, antibody-mediated targeting of other surface receptors such as the pattern recognition receptor MARCO on TAMs resulted in altered macrophage polarization and a reduction in tumor growth and metastasis in a mouse model of breast cancer (83).

Moreover, the intratumoral localization of TAMs within the TME can also be targeted, as hypoxia or increased lactate levels, induces a proangiogenic, immunosuppressive TAM phenotype (25, 84). Therefore, retaining the TAMs in normoxic regions in order to prevent M2-like TAM differentiation could prove to be a valuable strategy. Blunting the Sema3A/Neuropilin-1 pathway through genetic deletion of neuropilin-1 in mice demonstrated decreased migration of TAMs to the hypoxic regions, resulting in a strengthened immune response (85).

A strategy which does not involve direct reprogramming of the macrophages, comprises the blockade of the "don't eat me" signal CD47, which is overexpressed by most cancer cells, or its corresponding receptor on macrophages, signal regulatory protein α (SIRPα). SIRPα interacts with CD47, leading to the downregulation of phagocytotic programs. Hence, inhibition of CD47 signaling increases phagocytosis by TAMs (86). These observations prompted clinical trials with anti-CD47 antibodies, which are currently ongoing (87). Alternatively, the administration of a CD47 antagonist, namely the engineered SIRPα variant CV1, in combination with other molecules inducing phagocytosis, such as IgG4, significantly increased the phagocytic activity of macrophages and suppressed tumor growth of xenografts in mice (88).

In the search for molecules that could prolong survival of cancer patients, the anti-malaria drug chloroquine was tested. As a small molecule with a long clinical record which is affordable for clinical use, it was proven to induce repolarization of M2 macrophages toward the tumoricidal phenotype in the murine B16 melanoma model, showing promising results for future clinical trials (89). Another experimental treatment involved the use of a copper chelate to trigger activation of mitogen-activated protein (MAP) kinases via ROS generation. This led to the upregulation of IL-12 and IFNγ production and subsequent repolarization of the tumor-promoting M2 TAMs in the Ehrlich ascites carcinoma model (90).

Overall, repolarization of TAMs appears to be a viable approach based on a large number of preclinical studies using a wide range of therapeutic agents, however, the safety and clinical efficacy of most therapies still remain to be investigated.

DENDRITIC CELLS

The bridge between the adaptive and the innate immune system is formed by antigen presenting cells (APC) such as dendritic cells (DCs). DCs are specialized in the processing of foreign antigens and their subsequent presentation, alongside relevant costimulatory molecules, to effector cells of the adaptive immune system in secondary lymphoid organs, such as the lymph nodes. Eventually, these effector cells, being cytotoxic $CD8^+$ T cells, helper $CD4^+$ T cells and B cells, will differentiate and engage in the elimination of those cells expressing the foreign antigen.

DC Identity

DCs can be subdivided into two distinct specialized lineages, being the conventional/myeloid DCs (cDCs) and the plasmacytoid DCs (pDCs) (**Figure 1**). Both in mice and in humans, the existence of two cDC populations was demonstrated: $CD8\alpha^+$ or $CD103^+$ cDC1s and $CD11b^+$ cDC2s in mice and $CD141^+$ (or $BDCA3^+$) cDC1s and $CD1c^+$ (or $BDCA1^+$) cDC2s in humans (91–93). Finally, a population of monocyte-derived DCs (Mo-DCs) is also distinguished both in mice and in humans, as part of the myeloid DC lineage (94, 95). Based on single-cell RNA sequencing data, six populations were distinguished in human peripheral blood during steady-state. Two populations were identified as two cDC2 $CD1c^+$ subpopulations and one was appointed as a new unidentified population of $AXL^+SIGLEC6^+$ cells (95). The latter was shown to stimulate both $CD8^+$ and $CD4^+$ T-cell proliferation in a way similar to cDCs, while they express several pDC markers as well. Other populations resembled the $CLEC9A^+$ cDC1, the $CD1c^-CD141^-CD11c^+$ monocyte-derived DCs (mo-DCs) and pDCs (95).

The cDC1s were shown to interact mainly with $CD8^+$ T cells to induce potent CTL responses, while cDC2s can induce Th2 or Th17 responses, through presentation of tumor associated antigens (TAAs) on their MHC-II complexes (12, 94, 96). Plasmacytoid DCs engage in the secretion of type-I IFN, IL-6, and TNF-α and in this way interact with cDCs, T cells and B cells in order to counteract infections (97). Mo-DCs arise from monocytes during inflammation, and could hence be seen as an activated type of macrophages, and have been shown to express immunosuppressive properties (94, 98).

Within the TME, DCs were originally described as immunosuppressive cells, characterized by an immature

differentiation state, marked by a high antigen uptake and inadequate antigen presentation (99). These DCs are thought to enable further tumor growth and are therefore referred to as tolerogenic or regulatory DCs (9). The factors, responsible for the shift and maintenance of the immunosuppressive TADC phenotype are described in Conejo-Garcia et al. (100), while the mode of regulation by which these TADC exhibit immune suppression is reviewed in Keirsse et al. (9). Interestingly, the coexistence of distinct cDC subsets with anti-tumoral properties was recently shown in several murine models and patient biopsies (94, 101, 102). In this review, we focus on the anti-tumoral properties of TADCs and the strategies deploying TADCs for immune therapy.

DC Vaccination Strategies

DCs display a high potential for the development of immunotherapy, considering their ability to induce a potent anti-tumoral immune response involving the activation of anti-tumoral T cells ($CD8^+$ and $CD4^+$). These anti-tumoral T cells are not only capable of fighting the primary tumor but also their metastatic lesions and potential recurrence. The development of DC-based immunotherapy led to the emergence of DC-based vaccines, whereby DCs are activated through: (i) *ex vivo* incubation with a maturation cocktail containing cytokines and/or TLR agonists, (ii) the administration of TAAs *ex vivo* or *in vivo,* or (iii) intra-tumoral administration of immuno-stimulatory molecules that activate TADCs. These DC-based vaccines can be categorized into distinct generations based on when they were first applied in the clinic (103), and are intensively studied in (pre-)clinical trials for their application in future cancer immunotherapy (104).

First generation DC-vaccines involved Mo-DCs that were isolated from the blood of the patient or that were generated *ex vivo* (105). However, these DCs were not matured any further using maturation cocktails, but were incubated *ex vivo* with synthetic TAAs or tumor lysates. The fact that these cells remained largely immature explains their inability to elicit a strong and durable anti-tumoral response (105). Therefore, during development of the second generation of DC vaccines, Mo-DCs were maturated using a maturation cocktail containing both cytokines and TAAs, successfully activating the APC properties of the dendritic cells (106). The first DC-based vaccination strategy that received FDA approval, being Sipuleucel-T in 2010, which specifically acts against metastatic castration-resistant prostate cancer (CRPC) is an example of a second-generation DC vaccines. In this strategy, immature dendritic cells were isolated from the blood and incubated with a fusion protein PA2024, which contains GM-CSF, a prostate antigen and prostate acid phosphatase (107).

The delivery of antigens to DCs can be performed *in vivo* or *ex vivo* through several strategies listed by Garg et al. (104). The genetic modification of dendritic cells for more efficient vaccine activity using mRNA and siRNA but also viral transfection and fusion with malignant cells has been reviewed in Abraham et al. The application of this approach is generally to improve cancer cell-targeting, however it also helps in reducing the effect of tumor-mediated immunosuppression on the reinjected DCs (108).

Recent developed strategies aim for the *in vivo* loading of TAAs, without the need for additional *in vitro* maturation or treatment. This involves the *in vivo* injection and targeting of TAAs to dendritic cells (109). However, recent research in mice demonstrated the potential of using TADCs (cDC1 and cDC2) isolated directly from the primary tumor (94). The reinjection of these TADCs, which took up the TAAs *in vivo*, led to the onset of immunological memory. Prophylactic vaccination with tumor-derived cDC1s elicited an anti-tumor CTL response in B16-OVA melanomas, whereas cDC2 vaccination reduced LLC-OVA tumor growth through a Th17 response (94). It remains to be elucidated, whether tumor-derived DCs can induce an efficient memory response against tumor antigens in cancer patients.

The antigen-loading can also be induced by immunogenic cell death (ICD), in which cancer cell apoptosis is induced, resulting in the release of antigens (110). As such, photodynamic therapy, which generates ROS-mediated ER stress, induced immunogenic apoptosis in cancer cells characterized by phenotypic maturation and functional stimulation of dendritic cells as well as induction of a protective antitumor immune response (111). This strategy has been shown to increase the survival of high grade glioma-bearing mice when activated DCs were administered as a prophylactic vaccine (110). In combination with conventional chemotherapy (temozolomide), the ICD-based DC vaccines enabled an increased survival and complete tumor rejection (110). Similarly, the treatment of cancer cells with high hydrostatic pressure enhanced the *in vitro* uptake and presentation of TAA. This DC-based vaccine inhibited tumor growth of TC1 tumors in mice when combined with docetaxel chemotherapy (112).

Combining DC-Vaccination With Co-stimulatory Molecules

Success rates of DC-based vaccination strategies can be improved through co-injections of stimulatory molecules, like TLR agonists or CD40 agonists, which can enhance the antigen presenting function of TADCs (109). *In vivo* TAA presentation by TADCs can be induced through the intratumoral injection of TriMix mRNA, containing mRNA coding for the CD70 costimulatory molecule, the activation stimulus CD40L, and constitutively active TLR4 (113). Administration of DCs electroporated with TriMix mRNA and a melanoma antigen (gp100, tyrosinase, MAGE-A3 or MAGE-C2 fused to DC.LAMP) demonstrated durable clinical benefit in clinical trials involving patients with advanced melanoma when combined with the CTLA-4 inhibitor ipilimumab (114, 115). CD40 signaling induces important changes in DCs, including the induction of antigen presentation and upregulation of MHC- II and co-stimulatory molecules CD80 and CD86 (116). The use of an agonistic anti-CD40 antibody proved to successfully activate cDC populations (117), making it an interesting adjuvant for DC vaccination. Moreover, CD40 and TLR agonists act synergistically and the combination of these immunostimulants can significantly suppress B16-F10 tumor growth in mice

(118). Aside from CD40L, Fms-like tyrosine kinase 3 receptor ligand (Flt3L), a potent growth factor typically associated with DC development (119), was also suggested as an interesting candidate for the maturation of the TADCs. In this respect, co-administration of an adenoviral vector encoding Flt3L (pAd-Flt3L) and cell lysate of the colon cancer model CT26 into the footpad of the mouse prior to subcutaneous injection at the same location with CT26 resulted in the successful priming of both cDCs and pDCs, enabling tumor regression (120).

Other promising candidates are the TLR7/8 agonist FSME, which stimulates pDCs, and GM-CSF, which promotes myeloid-derived DC maturation. Administration of FSME or GM-CSF prior to DC vaccination in melanoma patients resulted in the induction of potent anti-tumor immune responses (121, 122). Also, intratumoral injection of GM-CSF secreting whole cell tumor cell vector (GVAX) formulated with the TLR4 agonist LPS showed potent induction of DC maturation and therapeutic efficacy in CDT26-tumor bearing mice (123).

Interestingly, Salmon et al. observed significant activation of $CD103^+$ DC progenitors (cDC1s) in the TME of the B16-OVA breast cancer model in mice after systemic administration of Flt3L, alongside intratumoral injection of the TLR3 agonist poly I:C (124). This therapy also enhanced the response to anti-PD-L1 therapy and BRAF inhibition (124), opening up possibilities for combination therapy with both immune checkpoint inhibitors and DC vaccination. The TLR3 agonist poly I:C was also employed in the development of a nanovaccine which was loaded with poly I:C, together with small interfering RNA (siRNA) against STAT3 and the ovalbumin antigen. The use of this carrier induced a significant tumor regression of B16-OVA tumors in mice with an increase of TADCs and decrease of immunosuppressive cells in the tumor draining lymph nodes (125). Similarly, a poly(lactic-co-glycolic acid) nanoparticle loaded with poly I:C and coated with a CD40 agonist antibody was directed toward CD40 expressing $CD11c^+CD11b^+F4/80^-$ DCs *in vivo*, resulting in prolonged survival of B16-OVA-tumor bearing mice (126). While the use of nanocarriers, which facilitate the *in vivo* delivery of antigens to dendritic cells, represents a promising strategy, it still requires validation through clinical trials in human patients.

The immune system in cancer patients is not only suppressed in the TME, but is altered systemically, whereby activation of immune cells in the draining lymph nodes is also counteracted (127). Intradermal injection of combined CpG-B/GM-CSF administration resulted in enhanced *in vivo* maturation and frequencies of cDCs in the lymph nodes of patients with stage I-II melanoma and these cDCs displayed increased cross-presentation capacities after *ex vivo* culture (128), suggesting the potential of CpG-B/GM-CSF as a possible new combination partner for DC-based immunotherapies against metastatic spread. Given the existence of systemic immune suppression, tumor-specific $CD8^+$ T-cell responses mediated by DC-vaccinations can be maximized using a multi-site injection strategy. This approach has been applied using a replication-deficient adenovirus serotype 5-vectored cancer vaccine. This vaccine specifically targeted the dopachrome tautomerase antigen in melanoma and led to an increase in systemic TAA-specific T-cells. Hence, the use of multi-site injections could also show potential in future DC vaccination strategies (129). Since systemic activation of the immune system in cancer is considered as beneficial for the efficacy of immunotherapy (130), systemic activation of DCs leading to an anti-tumoral immune response is another field of investigation. With the administration of RNA-lipoplexes, lipid carriers containing RNA encoding antigens (ovalbumin, gp70), efficient systemic uptake by DCs led to maturation and induction of effector/memory T-cell responses resulting in IFNα-mediated tumor inhibition (131).

Other DC-Based Strategies

The amount of cDCs that can be recovered from the circulation or tumors can be critical for enabling DC-based vaccination strategies. The accumulation of cDC1s appears to depend, besides Flt3L signaling, also on natural killer (NK) cells that secrete CCL5 and XCL1, which are potent cDC1 chemoattractants. Böttcher et al. proved in mice that the production of PGE_2 by the tumor impaired NK cell chemokine secretion and cDC1 chemokine receptor expression, leading to a decreased recruitment and anti-tumoral action of cDC1s in the tumor (132). The discovery of the CCL5-XCL1 mediated attraction of cDC1s into the TME, opens possibilities for future cancer immunotherapy, employing injection of these chemokines intratumorally alongside intranodal injection of TAA-loaded cDC1s. Efficient cross-presentation of tumor antigens to $CD8^+$ T cells by cDC1s is a major determinant of antitumor immune responses, thus therapeutic enhancement of this activity in the TME and the lymph nodes is of great interest (133).

A recent strategy shown to induce a cytotoxic T-cell response and NK cell activation, comprises the use of DC-derived exosomes, which contain functional MHC complexes (both MHC-I and-II) including costimulatory molecules (134) and demonstrated to successfully slow down tumor growth and increase a anti-tumoral immune cell infiltration when injected intravenously in a murine hepatocellular carcinoma model (135).

Lastly, low-dose administration of chemotherapeutic agents such as cyclophosphamide or paclitaxel was shown to enhance DC maturation, migration and function (136). Administration of immature DCs in the peritumoral environment of head and neck cancer patients together with low-dose cyclophosphamide and docetaxel as well as a multi-cytokine inducer OK-432, reduced immunosuppression and enhanced T-cell immunity, as a consequence of DC maturation (137). Combination therapy with low-dose cyclophosphamide and DC vaccination also demonstrated to reduce the tumor-induced immune suppression in patients with mesothelioma (138).

NEUTROPHILS

Neutrophils are highly phagocytic innate immune cells that make up 50–70% of all circulating leukocytes and live 5 to 8 h in the blood (139). In the steady-state, neutrophils are retained

in the bone marrow through the secretion of CXCL12 by osteoblasts. Upon infection and tissue damage, endothelial cells secrete CXCL1 and CXCL2, the major chemokines involved in the recruitment of the neutrophils, which are both recognized by CXCR2 (140). Another important player, counteracting retention of the neutrophils in the bone marrow is G-CSF (141). This growth factor does not only play an important role in the activation of neutrophils, but is also a major actor in the infiltration of neutrophils into the TME (142). When neutrophils migrate to the site of threat, they become activated and recruit other types of immune cells, leading to acute inflammation. When encountering harmful microorganisms, neutrophils will engage in three ways: (1) phagocytosis, (2) degranulation, and (3) release of neutrophil extracellular traps (NETs) (3).

Being the largest group of circulating white blood cells in the body, neutrophils play a substantial role in the interaction with malignant cell growth. Neutrophils in the TME, also called tumor associated neutrophils (TANs), tend to live longer (up to 17 h) under the influence of different signals present in the tumor, such as G-CSF and hypoxia (143). In humans, neutrophils are identified through their expression of the cell surface markers CD66b, CD15, CD16, and CD10 (144). Additionally, the lectin-type oxidized low-density lipoprotein receptor-1 (LOX1) is a potent marker which can be used to separate them from polymorphonuclear-MDSCs (PMN-MDSCs) (145), which can be described as immature neutrophils and are $LOX1^+$ (see section Myeloid-Derived Suppressor Cells). Besides these surface markers, it is also possible to identify TANs based on high expression of typical neutrophil-associated enzymes such as the serine protease neutrophil elastase (NE) (146) and myeloperoxidase (MPO) (147).

Peripheral blood neutrophil to lymphocyte ratio can be used in a clinical context as a prognostic biomarker and is associated with a poor overall survival in many solid tumors (148–150). TAN infiltration is mediated via the known neutrophil recruiting chemokines, being CXCL1, CXCL2, and CXCL5, secreted by cancer cells (**Figure 1**) (139, 151). Strikingly, it has also been shown that some malignancies can stimulate osteoblasts to upregulate the production and recruitment of tumor-promoting neutrophils (152). When neutrophils are initially recruited to the tumor, they appear to exhibit anti-tumoral properties and only over time become tumor-promoting, through the action of several factors secreted in the TME (147, 153). The initial tumor killing capacity of neutrophils is illustrated by an *in vitro* study, where Yan et al. demonstrated that neutrophils derived from the peripheral blood of healthy individuals were able to kill four different human cancer cell lines (154). Neutrophils, whose phenotype has switched toward tumor promotion facilitate metastasis (155), angiogenesis via secretion of proangiogenic factors, such as MMP9 and VEGF (156, 157) and immunosuppression either directly or through the recruitment of regulatory T cells (Tregs) (153).

TAN Repolarization

The tumor-suppressive properties of TANs appear to be reversible, based on mouse studies, leading to an anti-tumor neutrophil phenotype often termed N1 as opposed to the pro-tumor N2 phenotype, analogous to the M1/M2 concept used to describe the extremes of macrophage polarization. One of the central signals in the TME that induces the pro-tumor TAN phenotype appears to be TGFβ, which induces the expression of CXCL1, VEGF, and MMP9, which are all factors leading to a more persistent tumor growth (158). Accordingly, using a TGFβ receptor inhibitor SM16 led to a suppression of tumor growth by the anti-tumor N1-like TANs in mice, which expressed TNFα, MIP1α, H_2O_2, and NO, ultimately being cytotoxic to cancer cells (159). Other molecules, such as type I IFNs can also induce the shift toward an anti-tumor TAN phenotype (157, 160, 161). Therefore, it might be interesting to further explore the generation of N1-like TANs as a potential new immunotherapy approach.

Increasing Anti-tumoral TAN Infiltration

The creation of an acute inflammatory response instead of the wound-healing and tissue-repair response characteristic for the TME (162), could also prove to be a promising strategy. The ample evidence pointing toward the potential of neutrophils to serve as anti-tumor effectors was reviewed by Souto et al. (163). One of the approaches to enhance anti-tumor neutrophil infiltration could be radiotherapy. Infiltration of neutrophils producing large amounts of reactive oxygen species following radiotherapy were reported to exhibit a potent anti-tumor effect by inducing oxidative damage and apoptosis in cancer cells in several mouse tumor models (142). Therapies aiming to induce systemic neutrophil expansion (e.g., G-CSF) in combination with agents that promote the generation of anti-tumor neutrophils (e.g., TGFβ targeting) might act synergistically, and induce greater cytotoxicity in the tumor. It remains to be investigated, whether such combination therapies could be beneficial considering the largely negative effect of G-CSF administration on disease outcome. Until now, G-CSF has been administered to induce neutrophil expansion in order to help patients recover from chemotherapy-induced neutropenia (141). However, many studies have shown negative effects of this growth factor on disease outcome (141, 164, 165) and suggest G-CSF neutralization as a target for immunotherapy (166, 167). Accordingly, although administration of G-CSF in mice expanded neutrophils, it failed to induce a cytotoxic neutrophil response (168). Furthermore, in mice, G-CSF has also been shown to inhibit neutrophil migration through inhibition of CXCR2 (169). Therefore, other signaling molecules, such as intratumoral delivery of IL-8 could be used to stimulate neutrophil infiltration in order to induce acute inflammation and consequential inhibition of tumor growth (170, 171). A wide range of chemokines haven been shown to induce neutrophil cytotoxicity *in vitro*, including CCL2, CCL3, CCL5, CXCL1, CXCL12, and CXCL16, therefore approaches that increase the secretion of these factors in the TME might also prove to be beneficial (168). Inhibition of certain receptor tyrosine kinases (cMET, VEGFR2, RET, KIT, AXl, and FLT3) using a promiscuous small molecule inhibitor, cabozantinib, has also led to higher neutrophil infiltration into the tumor. Ultimately,

these neutrophils induced a highly effective eradication of murine prostate cancer (172). The precise mechanism behind the higher infiltration is not entirely clear, as (1) the exact RTK targeted is not yet identified (172) and (2) the application of cabozantinib inhibited tumor infiltration of immature neutrophils in another study on a more aggressive type of prostate cancer (173).

Inhibiting Immunosuppressive TAN Infiltration

In contrast to inducing an acute form of inflammation via an increased neutrophil infiltration, in the last decade, many researchers have focused on developing strategies to inhibit neutrophil recruitment to the TME. This is due to the finding that neutrophils often acquire an immunosuppressive phenotype upon infiltration of the TME. One strategy in preclinical studies was the inhibition of the general neutrophil recruitment pathway, involving the blockade of the IL-8/CXCR1/CXCR2 axis (140) with CXCR2 antagonists (174) or anti-IL8 antibodies (156). Moreover, there are indications in mice that the inhibition of RTK MET can also result in decreased tumor infiltration of immunosuppressive neutrophils in response to adoptive T-cell therapy leading to enhanced anti-tumoral T-cell function (175). However, in certain murine tumor types, inhibition of MET has been reported to diminish infiltration of antitumor neutrophils, resulting in increased tumor growth and metastasis (176).

Another possible strategy could be the induction of reverse migration or retrotaxis of TANs out of the TME in the bloodstream, lowering the abundance of TANs in the tumor microenvironment. These reverse migrated TANs could then possibly induce a more systemic anti-tumor response by antigen presentation or direct T-cell stimulation (177, 178). Therapeutic induction of neutrophil reverse migration has only been witnessed in case of wound-induced inflammation, however the development of reverse migration-inducing drugs might potentially open up opportunities for future cancer therapies (179). Two signaling pathways involved in reverse migration have already been discovered, namely the redox-regulated Src family kinase signaling (180) and the leukotriene B4-neutrophil elastase axis (181).

Other TAN-Based Strategies

Other strategies that have been investigated to target neutrophils in the TME involve inhibition of enzymes and mediators known to induce pro-tumorigenic properties, namely NE (182), a2 isoform V-ATPase (146), arachidonate 5-lipoxygenase (155), IL-23 (139), and IL-17 (183). Again, the latter can also promote anti-tumor activities (158), illustrating that the role of TANs appears to be highly context-dependent, determined by the histological origin and stage of the tumor as well as the therapies applied in the treatment.

MYELOID-DERIVED SUPPRESSOR CELLS

Myeloid-derived suppressor cells (MDSCs) comprise a heterogeneous group of immature myeloid cells characterized by their co-expression of CD11b and GR1 (184). In mice, two large populations can be distinguished, called polymorphonuclear (PMN)-MDSCs and monocytic (MO)-MDSCs (**Figure 1**). PMN-MDSC can be defined as $CD11b^{+}Ly6G^{+}Ly6C^{int}$ cells with high production of ROS, while MO-MDSC on the other hand are defined as $CD11b^{+}Ly6G^{-}Ly6C^{high}$ cells with high NO production (185, 186). In humans, MDSCs comprise three populations, a PMN-MDSC population identified by a $CD14^{-}CD11b^{+}CD15^{+}$ (or $CD66^{+}$) profile, a MO-MDSC population defined by a $CD14^{+}CD11b^{+}HLA\text{-}DR^{low/-}CD15^{-}$ phenotype and a population of "early stage MDSCs" or eMDSCs identified through the $HLA\text{-}DR^{-}/CD33^{+}Lin^{-}$ profile (with Lin being CD3/14/15/19/56) (184). The presence of MDSCs is not restricted to cancer, but can occur in every form of chronic inflammation, including pathogenic infection (187), autoimmune diseases (188), and Alzheimer's disease (189). Their main role during inflammation is to temper the immune response in order to protect the body from tissue damage that can be caused by a prolonged and uncontrolled immune response (6, 190).

Tumor-associated MDSCs arise in the TME as the result of two groups of overlapping signals. On one hand, the presence of factors, such as GM-CSF, G-CSF, and M-CSF causes expansion of immature myeloid cells. On the other hand, a wide range of pro-inflammatory factors, e.g., PGE_2, TNF, IL-1β, IL-6, S100A8, S100A9, IFNγ, IL-4, IL-10, and IL-13 secreted by cancer cells and leukocytes residing in the tumor inhibit the differentiation of myeloid progenitors and enhance their suppressive capacity (191). During cancer progression, MDSC levels do not only rise in the TME, but also increase in the spleen (192) and bone marrow (193), where they exert inhibitory functions on the immune system. However, the MDSCs in the TME were shown to exhibit higher immunosuppressive capacities than the peripheral MDSCs from the spleen (194) or bone marrow (193). In the TME of most cancer types, the PMN-MDSC fraction makes up around 80% of the total MDSC (6), with most of the MO-MDSC rapidly differentiating into TAMs (47).

In the TME, MDSCs exhibit different tumor-promoting and immunosuppressive functions and hence correlate with poor prognosis in cancer patients (195). The tumor-promoting functions comprise (i) remodeling of the TME (196), (ii) induction of (lymph)angiogenesis (196), (iii) promotion of metastasis (197), (iv) inhibition of cellular senescence (198), (v) suppression of T-cell function and migration (199, 200) and (vi) resistance to chemo-and immunotherapy (201–203). It is important to note that the immunosuppressive activity of MDSCs is not limited to a single mechanism, with MDSCs engaging several mechanisms throughout the progression of the tumor (6, 204–206), including; (i) expansion of Tregs (207), (ii) expression of galectin-9 on the MDSC surface, resulting in T-cell apoptosis (208), (iii) inhibition of NK cells through membrane-bound TGFβ1 (209), (iv) the secretion of ROS [O_2^-, H_2O_2 and peroxynitrite ($OONO^-$)](210, 211), (v) expression of enzymes involved in amino acid catabolism, like Arginase-I and IDO, collectively inhibiting T-cell proliferation (212, 213), and (vi) secretion of S100A8 and S100A9, resulting in the recruitment of more MDSCs and inhibition of dendritic cell maturation (214, 215).

Treatments targeting MDSCs in the TME aim to (i) reduce the number of MDSCs via their elimination or inhibition of recruitment or (ii) induce "re-education" or differentiation of these cells into anti-tumoral cells.

Elimination of MDSCs or Inhibition of MDSC Recruitment

In order to counteract the immunosuppressive actions of MDSCs, many depletion strategies have been applied (**Table 1**). The use of the chemotherapeutic agents gemcitabine, 5-fluorouracil and cisplatin, is able to eliminate MDSCs in murine tumors by inducing their apoptosis (216–218). As mentioned above, S100A9 is one of the central inflammatory mediators promoting MDSC recruitment. Accordingly, peptibodies against S100A9 led to reduced MDSC recruitment in tumor-bearing mice (219). Tyrosine kinase inhibitors, such as ibrutinib and sunitinib, respectively in mice and in humans, have also been shown to decrease tumor growth and decrease the numbers of MDSCs present in the TME (221, 225). Interestingly, the antidiabetic drug phenformin has been recently shown to selectively deplete PMN-MDSCs in the TME in mouse models of melanoma through the activation of AMPK (226). Activation of TRAIL receptor 2 (TRAIL-R2, also known as DR5) using an agonist antibody provides a more selective approach to induce MDSC apoptosis due to high expression of TRAIL-R2 on MDSCs (231). The TRAIL-R2-targeting antibody has already progressed to a phase I clinical trial, which demonstrated efficient depletion of MDSCs (particularly PMN-MDSCs) in the blood of patients with various solid tumor types (224). Interestingly, however, only a subset of patients showed a decrease of MDSCs in the tumor microenvironment (224).

Since both MO-MDCSs and TAMs derive from monocytic precursors, many inhibitors described to reduce the abundance of TAMs (cfr partim Macrophages) can be used to inhibit MO-MDSC recruitment as well (**Table 1**). For instance, in mice the use of the CSF-1R inhibitors GW2850 and PLX3397, led to a reduced recruitment of MO-MDSCs in the TME (227). Aside from CSF-1R inhibitors, the inhibition of $PI_3K\gamma$ or integrin α_4 prevented the accumulation of MDSCs as well as the expression of immunosuppressive molecules in the TME of LLC tumors (223). Analogously, genetic deletion of integrin-αM (also known as CD11b) in mice resulted in decreased recruitment of PMN-MDSCs to colorectal carcinomas and led to reduced tumor burden and improved survival, establishing integrin-αM as an additional therapeutic target (228). Similar findings were observed after inhibition of the IL-6/STAT3 pathways, leading to a significant inhibition of MDSC expansion and tumor growth of the murine TC1 tumor model (222). Also in mice, SAR131675, an inhibitor of VEGFR-3, led to a reduction in the frequency of MDSCs in the tumor and in the spleen (220). In patients, the inhibition of phosphodiesterase 5 using tadalafil reduced peripheral MDSC numbers which was associated with an enhanced proliferative capacity of patient-derived T cells in head and neck squamous cell carcinoma (230). Epigenetic modulators are generally thought to primarily affect cancer cells through inducing reexpression of silenced genes often involved in antigen presentation, potentially leading to enhanced antitumor immunity. However, administration of 5-azacytidine and entinostat to inhibit DNA methyltransferases and class I HDAC enzymes, respectively, has been shown reduce circulating and tumor-infiltrating PMN-MDSC levels which led to improved responses to immune checkpoint blockade therapy in mice (229). Interestingly, entinostat but not 5-azacytidine markedly reduced the viability of MDSCs (229). Nevertheless, the exact mechanism by which epigenetic regulators exert their inhibitory function on MDSCs remains to be elucidated.

The interplay of MDSCs with mast cells has also been considered an interesting future target. While mast cells have been associated with allergic reactions, they have also been reported to play either an immunostimulatory or an immunosuppressive role in the TME, depending on the tumor type (232). In the tumor-promoting context, mast cells do not only secrete immunosuppressive cytokines, but are also involved in the recruitment of MDSCs (233). Therefore, targeting the recruitment/function of tumor infiltrating mast cells could lead to diminished recruitment of MDSCs to the TME. Only few depletion strategies have been employed, which are reviewed in Varricchi et al. (232). Hence, further research on mast cells as a potential target in cancer immunotherapy is still needed.

Although the inhibition of MDSC recruitment to the TME provides a promising strategy, it can also be of interest to promote the differentiation of MDSCs toward either mature myeloid cells with antigen-presenting and/or cytotoxic activity.

Differentiation of MDSCs Into Anti-tumoral Myeloid Cells

A method to convert immunosuppressive MDSC to anti-tumoral myeloid cells might rely on TLR activation. For instance, the administration of a TLR7/8 agonist, resiquimod, led to the differentiation of bone marrow-derived MO-MDSC into $F4/80^+$ macrophages and $CD11c^+$ dendritic cells *in vitro* (234, 235). A recent study by Shayan et al. also demonstrated that the use of a TLR8 agonist in combination with the EGFR inhibitor cetuximab led to repolarization of monocytes toward an M1-like TAM phenotype and resulted in less MDSC-mediated suppression of T-cell activity *in vitro*. Furthermore, administration of the combination treatment was associated with a more immune-permissive TME in patients with head and neck squamous cell carcinoma (236). This however raises the question whether the differentiated monocytes were in fact MO-MDSCs that differentiated toward an anti-tumoral M1 TAM, as proposed in Wang et al. [2015] or whether the differentiation of monocytes toward M1-like TAMs overruled the suppressive actions of the MDSCs present in the TME (237).

Conversely, TLRs can also be involved in sustaining MDSC-mediated immune suppression. For instance, in pancreatic cancer in mice, TLR9 activation has been shown to induce MDSC proliferation *in vivo* and activate pancreatic stellate cells to display protumorigenic effects *in vitro* (238). Accordingly, activating TLR2 signaling in the murine EG7 lymphoma model via the Pam2CSK4 lipopeptide, leads to an increased immunosuppressive activity of MO-MDSCs as they further

TABLE 1 | Myeloid-derived suppressor cell depletion or recruitment inhibition strategies in murine cancer models and patients.

Tumor model	Treatment	Target	Amount/type of MDSC	Outcome	Reference
Mouse Lymphoma/melanoma	Gemcitabine-loaded nanopatricles	DNA synthesis	MO-MDSCs depletion	Attenuated immune suppression	(216)
Mouse Melanoma	5-Fluorouracil	DNA-synthesis	MDSCs depletion	Induced $CD8^+$ T-cell response	(217)
Mouse Melanoma	Cisplatin	DNA-synthesis	MDSCs depletion	Partially abrogated immune suppression	(218)
Mouse Thymoma	Pep-H6 Pep-G3	S100A9	MDSC depletion	Retardation tumor growth	(219)
Mouse Breast carcinoma	SAR131675	VEGFR	Prevents MDSC accumulation + M1-like TAM differentiation	Reduced tumor growth and metastasis	(220)
Mouse Melanoma	Ibrutinib	Bruton's tyrosine kinase	MDSC reduced	Enhanced the efficacy of anti-PD-L1	(221)
Mouse HPV-expressing TC-1 cells	Anti-IL6R mAb	IL6	MDSC reduced	Reduced tumor growth	(222)
Mouse HPV-expressing TC-1 cells	S31	STAT3	MDSC reduced	Reduced tumor growth	(222)
Mouse Lung carcinoma	Anti-PI3Ky/Integrin α_4 mAb or KO mice for both	PI3Ky Integrin α_4	Prevents MDSC accumulation	Reduced tumor growth	(223)
Mouse Melanoma	Phenformin (+anti-PD1)	Mitochondrial complex 1 of the respiratory chain (+ PD1)	PMN-MDSC depletion in spleen	Reduced tumor growth	(226)
Mouse Sarcoma	GW2850 PLX3397	CSF1R	Prevents MO-MDSC accumulation	Reduced tumor growth	(227)
Mouse Colorectal carcinoma	CD11b KO	CD11b	Decreased MDSC accumulation	Reduced tumor growth	(228)
Mouse Breast carcinoma Colon carcinoma	Entinostat (+ anti-PDL1 + anti-CTLA4)	Class I HDAC	MDSC inhibition	Reduced tumor growth	(229)
Human Head and neck squamous carcinoma	Tadalafil	PDE-5	Decreased MDSC circulating	Reversed immune suppression	(230)
Human Multiple cancers	DS-8273a (TRAILR2 agonist)	TRAILR2	MDSC depletion	NA	(224)
Human Renal cell carcinoma	Sunitinib	Multitargeted tyrosine kinase inhibitor	MDSC reduced	Improved tumor-infiltrating lymphocytes	(225)

differentiate into protumoral macrophages (239). However, the administration of N6-(1-Iminoethyl)-L-lysine (L-NIL), an iNOS inhibitor, decreased the immunosuppressive effect, showing the therapeutic potential of Pam2CSK4 when used in combination with other therapeutic agents (239). Another ligand for TLR2, Hsp72, has also proven to activate and increase the suppressive capacities of MDSCs in murine lymphoma, mammary carcinoma and colon carcinoma models, and showed relevance in humans as the human tumor cell line TDE triggered the suppressive function of MDSCs in a Hsp72 dependent manner (240). Also Hsp90, a regulator of TLR4 signaling, showed to be involved in the induction of the suppressive capacities of MDSCs *in vitro* (241). Therefore, the use of TLRs in MDSC-based immunotherapy remains to be further investigated.

Interestingly, oral administration of yeast-derived whole β-glucan particles (WGP) activated the dectin-1 receptor, leading to reduced amounts of PMN-MDSC in the spleens and tumors of LLC and E0771 tumor-bearing mice and decreased their immunosuppressive properties *in vitro*. In an *in vitro* assay, the presence of WGP induced the differentiation of MO-MDSC into $F4/80^+$ $CD11c^+$ myeloid cells, serving as potent APCs and when injected intratumorally, WGP-treated MO-MDSCs were capable of inhibiting tumor growth in subcutaneously inoculated LLC (242).

Using the antibody 2aG4 against another therapeutic target, phosphatidylserine, also showed repolarization from M2-like TAMs to the M1-like phenotype, together with differentiation of MO-MDSCs into M1-like TAMs and dendritic cells *in vitro* (243). Interestingly, curcumin-based chemotherapy (docetaxel) showed to selectively eliminate the PMN-MDSCs, while sparing the MO-MDSC which then repolarized toward M1-like TAMs in a murine 4T1 mammary carcinoma model (244).

A study performed on *in vitro* generated MDSCs co-cultured with the human A375 melanoma cell line demonstrated a shift of the MDSC phenotype toward a profile associated with immunostimulatory dendritic cells, through the inhibition of macrophage migration inhibitory factor (MIF) with 4-iodo-6-phenylpyrimidine (245). However, these results remain to

be confirmed *in vivo* before MIF inhibition can be further explored in a therapeutic setting. Shen et al. also witnessed a similar shift of the immunosuppressive MDSCs toward a more immunostimulatory myeloid cell type in response to tasquinimod, a quinoline−3-carboxyamide analog with anti-angiogenic properties when administered to mice injected with either castration-resistant prostate cancer or melanoma cells (246).

Moreover, the administration of axitinib, a small molecule tyrosine kinase inhibitor of VEGFR-1/2/3, reduced the immunosuppressive activity of splenic and tumor-infiltrating MO-MDSCs besides its anti-angiogenic effect. Moreover, MO-MDSCs from axitinib-treated tumors in mice were able to stimulate T-cell activation, suggesting a phenotype switch from immunosuppressive to antigen-presenting activity (247).

CONCLUDING REMARKS

The use of tumor-associated immune cells unlocks an interesting field of potential therapies in the fight against cancer. Severe side effects inflicted by conventional therapies are overcome as the body's own immune system engages in specific anti-tumoral immune responses. Moreover, the genomic stability of tumor-associated immune cells as opposed to the high genetic plasticity and heterogeneity of cancer cells, decreases the risk of developing resistance against immunotherapies.

Still many hurdles are to be overcome in order to completely rely on the immune system to ensure specific and long-term immune responses against tumors. The observation that the abundance of myeloid cell (sub)population can differ substantially between tumor types (248, 249), urges for the verification of their therapeutic potential in distinct tumor models. Additionally, high variability in the frequency of distinct myeloid cell subsets is also witnessed between patients with the same tumor type (30–32). As highlighted in this review, clinical translation of some of the therapeutic strategies targeting myeloid cells is ongoing. The observations above have two crucial implications for future translational efforts. Firstly, murine models will likely fail to predict therapeutic responses to myeloid cell-based therapies in patients with cancer, as tumor models in mice, particularly transplantable ones, show rapid progression and low variability in their immune microenvironment. Thus, there is an urgent need for the development and application of more advanced pre-clinical models that recapitulate the patient-to-patient heterogeneity of the tumor immune microenvironment. Secondly, similar to ICIs, likely not all patients will benefit from myeloid cell-targeted therapies. Thus, it will be essential to investigate the differences between the responder and non-responder populations in order to identify biomarkers predicting therapy response. Due to the highly patient-specific nature of tumor antigens and the tumor immune microenvironment, the future myeloid-cell targeted therapies will have to be integrated in combination therapies tailored to each patient, in which adoptive T-cell transfer, ICIs, co-stimulatory molecules, low-dose chemo-or radiotherapy are combined with the (re)activation of tumor-associated myeloid cells.

AUTHOR CONTRIBUTIONS

EC wrote the manuscript draft. AM designed the figures HV revised the manuscript draft. MK and DL supervised and wrote the final manuscript with input from all authors.

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Combination of Synthetic Long Peptides and XCL1 Fusion Proteins Results in Superior Tumor Control

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Cross-presenting Xcr1$^+$CD8α DCs are attractive APCs to target for therapeutic cancer vaccines, as they are able to take up and process antigen from dying tumor cells for their MHCI-restricted presentation to CD8 T cells. To this aim, we developed fusion proteins made of the Xcr1 ligand Xcl1 fused to an OVA synthetic long peptide (SLP) and IgG1 Fc fragment. We demonstrated the specific binding and uptake of the Xcl1 fusion proteins by Xcr1$^+$ DCs. Most importantly, their potent adjuvant effect on the H-2Kb/OVA specific T cell response was associated with a sustained tumor control even against the poorly immunogenic B16-OVA melanoma tumor. The increased tumor protection correlated with higher tumor infiltration of antigen-specific CD8+ T cells, increased IFNγ production and degranulation potential. Altogether, these results demonstrate that therapeutic cancer vaccines may be greatly improved by the combination of SLP antigen and Xcl1 fusion proteins.

Keywords: therapeutic cancer vaccine, antigen cross-presentation, Xcr1$^+$ DC, Xcl1, synthetic long peptides

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INTRODUCTION

One of the key requirements for successful therapeutic cancer vaccinations relies on the ability to target antigen to cross-presenting dendritic cells (DCs), a subtype of DCs which have the capacity to shunt a proportion of internalized antigens from the endosomal compartments to the cytosol, where they are processed for loading onto MHC class I molecules, resulting in efficient CD8$^+$ T cell responses (1). The chemokine receptor Xcr1 was shown to be the main marker characterizing murine (2) as well as human cross-presenting DCs (3–5), and their superior cross-presentation capacities of soluble and cell-associated antigens has been demonstrated in both mice (2, 6, 7) and humans (3, 8). The Xcr1 chemokine receptor is co-expressed with CLEC9A (DNGR1) and the ontogeny of Xcr1-positive DCs is strictly dependent on the transcription factor Batf3 (2, 9). In mice, Xcr1 is expressed in ∼80% of lymphoid organ-resident CD8α$^+$ DCs as well as in ∼80% of migratory dermal CD103$^+$ DCs (6). In humans, XCR1 is expressed in the majority of CD141$^+$ CD11c$^+$ blood DCs (3) and CD141^{hi} tissue-residents DCs in dermis, liver, and lung (4, 5). Of note, Xcr1 is co-expressed with DEC205 and CADM1 (5), which suggests the strong functional role of Xcr1$^+$ DCs in the cross-presentation of antigens derived from necrotic cells (10). Xcr1-expressing DCs migrate toward the chemokine Xcl1 secreted by activated CTLs, NK and NKT cells involved in the cytotoxic response (3, 11). In contrast to many chemokine ligands that bind to several receptors, Xcl1 binds exclusively to the Xcr1 receptor and is often co-secreted with Th1 profile cytokines, such as IFNγ, MIP-1α, MIP-1β, and RANTES by activated murine NK cells, Th1 cells, and CD8$^+$ T lymphocytes (12).

Vaccinations involving synthetic long peptides (SLPs) have given successful results in clinical studies with cancer patients (13, 14), and are thought to avoid immunological tolerance induced by exact length MHC class I-restricted peptides. Indeed, unlike short synthetic peptides (SSP), SLPs require cellular processing and cross-presentation, which avoids suboptimal presentation by non-professional antigen presenting cells and hence efficiently induce specific CTL responses (15, 16). SLPs are generally 20–30 amino acids long and may harbor both MHC class I and class II-restricted epitopes, resulting in enhanced CTL expansion by triggering concomitant T helper responses. In addition, antigens in the form of SLPs have been compared against whole protein antigens in DC cross-presentation studies and have been shown to be better processed resulting in improved cross-priming of $CD8^+$ T cell responses (17). Indeed, while whole protein traffics only to endosomal compartments which primarily promotes the priming of $CD4^+$ T lymphocytes, SLPs traffic not only to endosomes, but also to cytosol, allowing the priming of both $CD4^+$ and $CD8^+$ T cell responses (18).

Antitumor immunity relies greatly on antigen cross-presentation to allow debris from a dying tumor cell to be processed and presented to CTLs. Nevertheless, cross-presenting DCs are present at very low frequencies in human tissues, and specific DC targeting strategies represent an important step in optimizing cancer vaccines. Strategies recently used for targeting antigen to DCs have included recombinant proteins resulting from the genetic fusion of the antigen to mAbs that target DC markers, such as DEC-205 (19) and CLEC9A (20–22), or to chemokines (23).

In this context, we aimed to target to Xcr1+ DCs tumor antigens in the form of SLP genetically fused or not to the Xcl1 chemokine. In therapeutic tumor vaccination settings, vaccination with the OVA SLP fused or not to Xcl1-Fc fusion proteins enhanced $CD8^+$ T cell responses and delayed B16.OVA tumor growth. These results correlated with higher tumor infiltration of antigen-specific CTLs as well as their increased IFNγ production. These results demonstrate that therapeutic cancer vaccines may be greatly improved by Xcl1-antigen fusion proteins.

MATERIALS AND METHODS

Mice

Age and gender-matched C57BL/6 mice were purchased from Envigo Laboratories (France). Batf3 knock out (KO) mice were bred in our facilities under specific pathogen-free conditions. All animal experimentation was performed according to ethical approval from the Canton de Vaud authorities, Switzerland. Veterinary authorization number VD2273.

Production of Xcl1-SLP muIgG1 Fc Fusion Proteins

DNA sequences were inserted into the expression vector pMP-PB (Excellgene) by In-Fusion technique (Clontech). DNA sequences are shown in **Supplementary Figure 1**. Positive clones were verified by DNA sequencing (Microsynth). Middle scale protein production was performed in Chinese Hamster Ovary (CHO) cells at the Laboratory of Cellular Biotechnology of EPFL, Lausanne, Switzerland. Xcl1 fusion proteins were purified from the supernatants of 7-day CHO cultures. Purification was performed by affinity chromatography using Protein A resin (GE Healthcare, cat no 17-1281-02). Proteins were eluted with Glycine 0.1 M pH 3.0 and dialyzed against PBS overnight. After confirming their size and purity by SDS-PAGE, recombinant proteins were passed through a Mustang Q membrane (PALL Corporation) for endotoxin removal. Commercial Xcl1 was purchased from Hölzel Diagnostika Handels GmbH, Germany (item n°50677-M08B).

In vitro Binding of Fusion Proteins to DCs

Spleens from naïve WT (C57BL/6) and $Batf3^{-/-}$ mice were enriched for $CD11c^+$ cells using CD11c (N418) microbeads (cat number 130-052-001, Miltenyi Biotec). DC-enriched suspensions from spleens of WT or $Batf3^{-/-}$ mice were incubated with purified Xcl1-(OVA SLP)-Fc and Xcl1-Fc fusion proteins at 37°C for 35 min. Cells were washed and binding of fusion proteins was assessed using PE-conjugated anti-mouse IgG1 antibody.

Chemotaxis Assay

Spleens from naïve WT (C57BL/6) mice were enriched for $CD11c^+$ cells using CD11c (N418) microbeads (cat number 130-052-001, Miltenyi Biotec). 1 x 10^6 cells ($CD11c^+$ DC purity of ∼50%) were resuspended in 0.1 mL of chemotaxis medium (RPMI1640, 1% BSA, 50 µM ß-ME, 100 µg/mL penicillin/streptomycin) and added to the upper chamber of a 24-transwell plate (with 8 µm pore, Corning). In the lower chamber, 0.5 mL of chemotaxis medium was added, containing either 250 ng/mL of commercial Xcl1, or 1,000 ng/mL of Xcl1-(OVA SLP)-Fc or Xcl1-Fc fusion protein to have an equimolar concentration of Xcl1 of 25 nM. After incubation for 2 h at 37°C (5% CO_2), bottom chambers were flushed with ice-cold PBS containing 10 mM EDTA and DCs were analyzed by FACS. Cells were incubated for 5 min on ice with 2.4 G2 to block Fc receptors, $Xcr1^+$ DCs were detected via incubation with Xcl1-Fc protein (19 nM) for 30 min at 37°C, followed by washing and staining with PE-conjugated anti-mouse IgG1 on ice for 30 min. Afterwards, surface markers antibodies were added in a mix, on ice, for 30 min. DCs were identified by first excluding $CD3^+$ $B220^+$ and $CD11b^+$ cells and gating on $CD11c^+$ $CD8\alpha^+$ cells.

In vivo Uptake of Alexa-488-Labeled Xcl1 Fusion Proteins

Alexa-488 dye (DY-490-NHS-Ester, from Dyomics, product number 490-01) was resuspended in DMSO (the molar ratio between 1 mg of dye and 1 mg of the Xcl1 fusion proteins is 40.2, hence 40.2 µL of DMSO were added). The dye and the 10x reaction buffer (1 M Na Phosphate, 1.5 M NaCl, pH 7.1) were added to the fusion proteins at a volume ratio of 1:10, and mix was incubated at room temperature for 1.5 h in rotation and protected from light. Desalting columns (Zeba Spin desalting column, Thermo Scientific, product number 89,890) were washed with PBS by spinning 1,000 g for 2 min. The labeled proteins were added to the column and spun down. This step

was repeated with the flow-through and final fusion proteins concentrations were measured by BCA.

WT and Batf3 KO mice were injected intradermally in the footpad with a mix of 50 μg of CpG and 6 μg of Alexa 488-labeled Xcl1-(OVA SLP)-Fc or Xcl1-Fc fusion proteins. Inguinal LNs were harvested 16 h post injection for measurement of uptake in different cell populations.

Peptide Solubilization

OVA SLP was solubilized with 10% sterile DMSO and 90% sterile PBS. The OVA SLP amino acid sequence is K*ISQAVHAAHAEINEAG*R**SIINFEKL**TEWT, which includes the MHC class I-restricted epitope (in bold) and the MHC class II-restricted epitope (in italic).

Immunizations

Vaccine formulations were prepared sterile, immediately before injections. Mice were immunized with a volume of 30 μL intradermally in the hind paw, on the ipsilateral side of the tumors.

Tumor Engraftment

Mice were engrafted subcutaneously in the left flank either with 1 x 10^6 EG7 or 2 x 10^5 B16.OVA cells, or 1 x 10^5 B16.WT. Tumor volumes were monitored every 2 days and were calculated using the following formula: (length × width × thickness)/2.

Tumor Digestion: Tumors were harvested and digested using the tumor dissociation kit from Miltenyi Biotec (cat number 130-096-730), according to manufacturer's instructions. Cells were then stained for flow cytometry.

Intradermal Vaccination

Mice received equimolar amounts of Xcl1 and OVA SLP antigen injected intra-dermally in the footpad. Doses were the following: 20 μg of Xcl1-(OVA SLP)-Fc; or 17.6 μg of Xcl1-Fc + 1.3 μg of free OVA SLP; or 1.3 μg of free OVA SLP + 5.9 μg free Xcl1; or 1.3 μg of free OVA SLP. All mice received 50 μg CpG-B (ODN 1826, U133-L01A; Trilink Biotechnologies).

Isolation of TILs

Tumors were digested as described above. Samples were then diluted in 7 mL of complete DMEM and added to 5 mL of Lymphoprep (cat number 1114547, Axis-Shield), followed by a centrifugation of 1,800 rpm for 20 min. Cells at the interphase were collected, washed once, and plated in a 96-well plate for *in vitro* peptide restimulation.

In vitro peptide restimulation and Intracellular Cytokine Staining: TILs were incubated at 37°C for 1 h with 10 μM SIINFEKL and anti-mouse CD107a (LAMP1) antibody-FITC was also added (1/100) to wells. After 1 h, 1 μg/mL GolgiPlug and GolgiStop (BD biosciences) were added to the wells and TILs were then incubated for a further 4 h at 37°C before intracellular cytokine staining. Cells were permeabilized and stained using the Cytofix/Cytoperm kit (BD Biosciences), according to manufacturer's instructions and stained for intracellular IFNγ and TNFα.

Calculation of the CD8/Tregs ratio: TILs were counted under the microscope before surface/intracellular staining and FACS acquisition. CD8/Treg ratio were calculated using the FACS percentages of tetramer$^+$ CTLs and CD4$^+$ CD25$^+$ FoxP3$^+$, and total TIL numbers.

Flow Cytometry

Blood and spleen samples were treated with Red Blood Cell Lysis Solution (Qiagen) for 15 min at 37°C and 3 min at room temperature, respectively, before staining. LIVE/DEAD Aqua fluorescent stain (Invitrogen) was used to discriminate between live and dead cells. For tetramer staining, samples were incubated with phycoerythrin (PE)-conjugated SIINFEKL-H-2k^b multimers (TC Metrix, Switzerland) for 35 min at room temperature. Samples were washed and incubated on ice for 30 min with CD8α-PerCp Cy5.5 (clone 53.6.7–eBioscience), CD3–PE Cy7 (clone 145.2C11–eBioscience), CD4–FITC (clone GK1.5–produced in house, Ludwig Cancer Research). For *in vitro* binding and chemotaxis assays the following antibodies were used: IgG1–PE (clone A85-1–BD biosciences), B220–Pacific blue (clone RA3-6B2 - LICR), CD8a–PerCp Cy5.5 (clone 53.6.7–eBioscience), CD3–PE Cy7 (clone 145.2C11–eBioscience), CD11c–eFLuor 660 (clone N4/18–eBioscience), CD11b–Alexa700 (clone M1/70–eBioscience), CD103–PE. Data were acquired on a LSRII or LSRII (SORP) and FACS analyses were done with Flow Jo software.

Statistical Tests

Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA). Normally distributed data were compared using one-way ANOVA or two-way ANOVA (**Figures 3A,B**, **5A**). Multiple comparisons were corrected using Tukey tests. Normality was tested with a Shapiro-Wilk test. On the graphs, data represent mean ± SE ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$).

RESULTS

Xcl1-(OVA SLP)-Fc Fusion Proteins Bind to CD11c$^+$ CD8α$^+$ DCs and Induce Chemotaxis of Xcr1$^+$ DCs

With the aim to optimize synthetic long peptide (SLP) vaccines by targeting the antigen to Xcr1$^+$ cross-presenting DCs, a recombinant fusion protein was produced with the ovalbumin (OVA) SLP antigen fused to the Xcl1 chemokine, followed by the murine IgG1 Fc for stability, dimerization and purification purposes (**Supplementary Figure 1**). We opted for an Fc part harboring the Asp to Ala mutation at amino acid position 265, which prevents its binding to Fc receptors (24). A recombinant protein lacking the OVA SLP antigen (Xcl1-Fc) was also produced to evaluate the potency of Xcl1-mediated antigen targeting (**Figure 1A**). The fusion proteins were tested for their capacity to bind to CD11c$^+$-microbeads purified CD8α$^+$ DCs from spleen (**Figure 1B**). CD11c$^+$-enriched DCs from naïve WT and Batf3$^{-/-}$ mice were incubated with the Xcl1-(OVA SLP)-Fc fusion proteins at 37°C, and specific binding was detected with a fluorescently-labeled anti-IgG1-Fc antibody. Significant binding of Xcl1 fusion proteins was seen in WT mice, when gating on CD11c$^+$ CD8α$^+$DCs, while some heterogenous non-specific binding was observed on the remaining CD8α$^+$ cells from Batf3$^{-/-}$ mice, which are deficient in Xcr1$^+$ DCs (25)

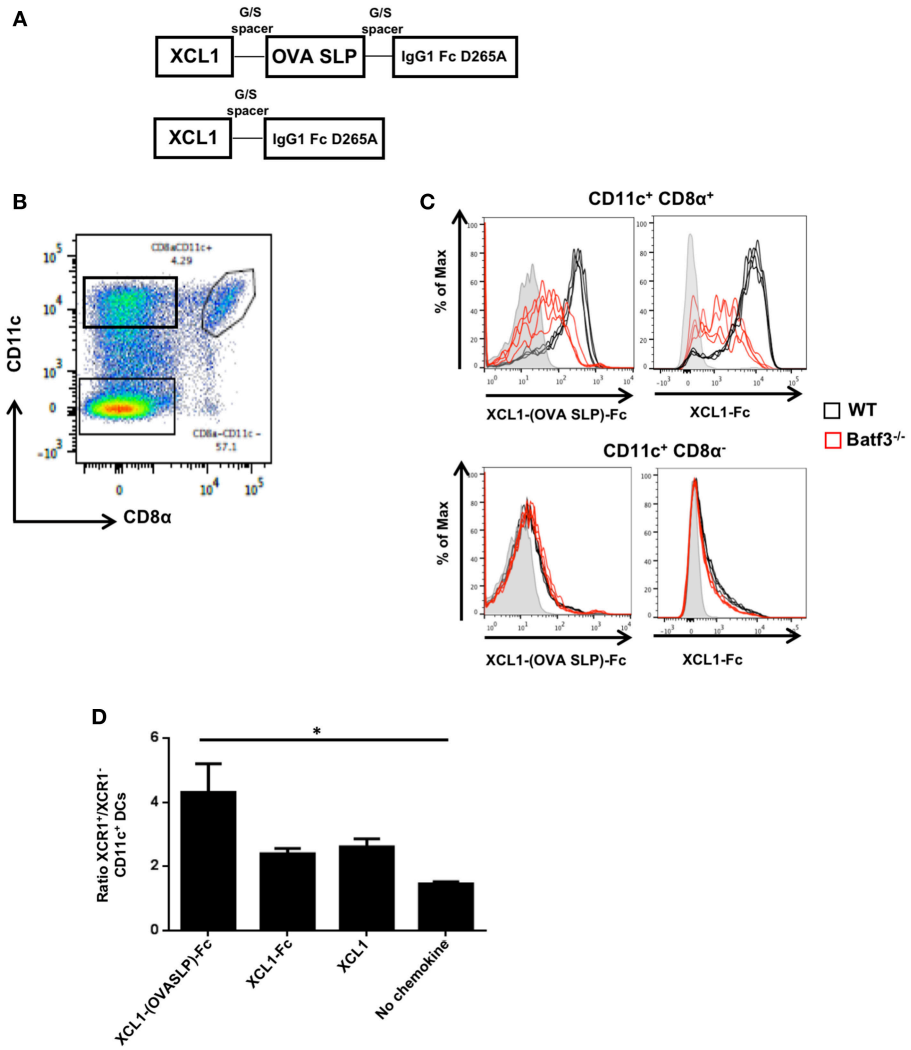

FIGURE 1 | *In vitro* characterization of Xcl1-(OVA SLP)-Fc fusion proteins. **(A)** Design of Xcl1-(OVA SLP)-Fc fusion proteins. The OVA SLP was fused to the C-terminus of the murine Xcl1 amino acid sequence via an uncharged glycine/serine linker. The C-terminus of OVA SLP was connected to the murine IgG1 Fc, carrying the D265A mutation. **(B)** Gating strategy to identify CD8α DCs. **(C)** CD11c-enriched DCs from splenocytes of naive WT (C57BL/6) (black line) or Batf3$^{-/-}$ (red line) mice were incubated with purified Xcl1-(OVA SLP)-Fc or Xcl1-Fc fusion proteins. Binding of XCL1 fusion proteins was assessed using a fluorescent anti-mouse IgG1 antibody. Gray histograms represent control wells without fusion proteins. Each line represents a replicate. Results are representative of two independent experiments. **(D)** *In vitro* chemotaxis assay performed with WT splenic DCs (enriched ~50% CD11c^{+}). Migration of DCs was assessed toward Xcl1-(OVA SLP)-Fc fusion proteins at 25 nM (calculated based on the content of Xcl1 in the reagents). Results are expressed as the ratio between the number of Xcr1^{+} and Xcr1^{-} CD11c^{+} CD11b^{-} DCs, which migrated toward Xcl1 fusion proteins or commercial Xcl1. Data are shown as mean ± SEM. Results are representative of three independent experiments. *$p < 0.05$.

(**Figure 1C**). Similarly, the Xcl1 fusion proteins did not bind to CD8α negative WT and Batf3 KO CD11c^{+} DCs (**Figure 1C**), supporting the binding specificity to CD11c^{+} CD8α^{+} DCs, 80% of which express Xcr1. To test whether the Xcl1-(OVA SLP)-Fc fusion protein was capable of inducing chemotaxis of Xcr1^{+} DCs, trans-well migration experiments were performed with 1 x 10^{6} CD11c^{+} enriched DCs in the upper chamber and medium containing 25 nM of Xcl1 fusion proteins or commercial Xcl1 in the bottom well. After a 2-h incubation at 37°C, analysis of the bottom well-showed that Xcr1^{+} DCs had migrated between 2 and 4-fold more than Xcr1^{-} DCs in all wells containing Xcl1 fusion proteins or free Xcl1 (**Figure 1D**). Overall, these data demonstrated that the Xcl1-(OVA SLP)-Fc and Xcl1-Fc proteins induced chemotaxis to a similar extent as the native chemokine Xcl1 (**Figure 1D**).

XCL1-(OVA SLP)-Fc Fusion Protein Bind *in vivo* to CD11c^{+} CD8α^{+} LN-Resident DCs

To investigate *in vivo* which population of DCs will preferentially bind the Xcl1-(OVA SLP)-Fc fusion proteins, Xcl1-Fc and Xcl1-(OVA SLP)-Fc were fluorescently-labeled with Alexa 488 and injected intradermally into WT or Batf3$^{-/-}$ mice. Skin draining

LNs were harvested 16 h post immunization and analyzed for the presence of the fusion protein in different subsets of $CD11c^+$ DCs (**Figure 2A**). In WT mice injected with 6 μg of labeled Xcl1-(OVA SLP)-Fc, about 10% of $CD8\alpha^+$ LN-resident were Alexa 488 positive, compared to only 2% in $Batf3^{-/-}$ mice (**Figure 2B**). Increased uptake of Alexa 488-labeled Xcl1-Fc by WT $CD8\alpha^+$ was also observed, as shown by 18% compared to 4.7% in the same DC population in $Batf3^{-/-}$ mice. With regards to $CD103^+$ DCs, there was a tendency for increased uptake of the fusion proteins by WT mice, although not significant due to a large dispersion. Importantly, B cells, which are negative for Xcr1 expression, did not bind the Xcl1 fusion proteins, while <5% of phagocytic $CD11b^+$ DCs, also negative for Xcr1, became Alexa 488 positive for the Xcl1 fusion proteins both in WT and $Batf3^{-/-}$, indicating a non-specific uptake (**Figure 2C**). Altogether, these results suggest that the Xcl1-(OVA SLP)-Fc fusion proteins were preferentially and specifically taken up by the $Xcr1^+$ expressing $CD8\alpha^+$. Representative profiles of *ex vivo* Alexa 488^+-labeled cells are shown in **Supplementary Figure 3**.

Therapeutic Vaccines Involving Xcl1 Fusion Proteins Lead to Regression of OVA-Expressing Tumors

Given that cancer vaccines are ultimately evaluated for their capacity to protect against tumors, the Xcl1 fusion proteins were tested in therapeutic settings against the OVA-expressing EL-4 lymphoma model (EG7). Gender and age-matched C57BL/6 mice were engrafted subcutaneously on day 0 with 1 x 10^6 EG7 cells (**Figure 3A**). On day 7, when tumors were established and measurable, mice received an adoptive cell transfer of 10^5 OT-I cells, followed on day 8 by intradermal vaccination with the Xcl1 fusion proteins or with free OVA SLP +/- Xcl1. Except for the untreated group, all mice received 50 μg of CpG-ODN. In both cohorts vaccinated with the Xcl1-(OVA SLP)-Fc fusion proteins, all tumors started to shrink 5 days post immunization. In contrast, in mice receiving free OVA SLP + free Xcl1, tumor volumes started to decrease only by day 15 but did not disappear, while in mice receiving only the OVA SLP and CpG, only a delay in tumor growth was obtained but no transient decrease of tumor volumes (**Figure 3A**).

In view of the potent antitumor activity of Xcl1 fusion proteins observed in the EG7 tumor model, we assessed the tumor protective immunity of the Xcl1-mediated tumor vaccine in the less immunogenic B16-OVA melanoma tumor model. Mice were grafted on day 0 with 2 x 10^5 B16.OVA cells and on day 7, when all tumors were reaching an average volume of 30 mm^3, mice received an adoptive cell transfer of 10^5 naïve OT-I cells, followed on day 8 by the intradermal vaccinations as described for the EG7 challenge (**Figure 3A**). A significant tumor growth delay was obtained in cohorts vaccinated with Xcl1-(OVA SLP)-Fc and OVA SLP + Xcl1-Fc fusion proteins, as compared to mice not receiving Xcl1 (OVA SLP and CpG only), while only a tendency to a higher delay was observed against the OVA SLP + free Xcl1 cohort (**Figure 3B**). To assess a non-specific adjuvant effect of the fusion proteins due to potential traces of endotoxin, two groups were vaccinated with the Xcl1-Fc and Xcl1-(OVA SLP)-Fc fusion proteins without CpG. However, both groups of mice showed fast tumor growth (**Figure 3B**), confirming the adjuvant effect of Xcl1 fusion proteins. As seen in the blood on day 7 post-vaccination in both EG7 and B16.OVA tumor challenge experiments, the vaccination with Xcl1-(OVA SLP)-Fc fusion proteins plus CpG led to similar expansions of OVA-specific CTLs, which was best with Xcl1-(OVA SLP)-Fc, when compared to any other cohort, likely resulting from the co-delivery of the antigen to cross-presenting DCs via its fusion to Xcl1 (**Figures 3C,D**). Combined immunization with the mixture of the fusion Xcl1-Fc protein and the free OVA SLP + CpG still resulted in a significantly better CTL expansion than in the group receiving free Xcl1 mixed with the OVA SLP + CpG, which only showed a trend for higher OVA-specific CTLs as compared to only OVA SLP + CpG.

Tumors of Mice Vaccinated With Xcl1 Fusion Proteins Show Higher Infiltration of OVA-Specific $CD8^+$ T Cells Characterized by an Increased Functionality

In order to dissect the mechanisms by which therapeutic vaccinations using Xcl1 fusion proteins showed better tumor control, B16.OVA tumors from mice immunized as described in **Figure 3B**, were harvested 10 days post vaccination in order to quantify TILs and characterize their functionality. Frequencies of OVA-specific $CD8^+$ T cells in the spleen (**Figure 4A**) and in the tumors (**Figure 4B**, left panel) were higher in the cohorts of mice vaccinated with Xcl1 fusion protein as compared to the other cohorts. When normalized by the tumor volume, mice vaccinated with the Xcl1 fusion proteins also showed higher numbers of OVA-specific $CD8^+$ T cells, as compared to cohorts vaccinated with free OVA SLP + CpG, with or without free Xcl1 (**Figure 4B** right panel). Upon *in vitro* restimulation of tumor-infiltrating lymphocytes (TILs) with SIINFEKL as illustrated in **Figure 4C**, we found that cohorts vaccinated with Xcl1 fusion proteins showed higher frequencies of $IFN\gamma^+$ TILs than the other cohorts (**Figure 4D**). Furthermore, increased frequencies of $CD8^+$ TILs expressing the lysosomal marker CD107a were also observed (**Figure 4E**), associated with higher CD107a mean fluorescence intensity (data not shown), indicative of increased degranulation capacity. Altogether, these results suggest not only a higher frequency but also a higher functionality of CTLs within tumors of mice vaccinated with Xcl1-OVA SLP-Fc or Xcl1-Fc + free OVA SLP.

Immunization With Xcl1 Fusion Proteins Generates an Endogenous OVA $CD8^+$ T Cell Response as Efficient as Upon OT-1 T Cell Transfer

To be closer to a clinical situation, we wanted to assess the tumor protection capacity of the Xcl1 recombinant proteins in therapeutic vaccinations without OT-1 adoptive cell transfer. To this aim, C57BL/6 mice were grafted s.c. with 2 x 10^5 B16.OVA melanoma cells as described in **Figure 3**. Mice were vaccinated 3 days later, when tumors were all visible in the flank of the mice. As in the previous experiment involving

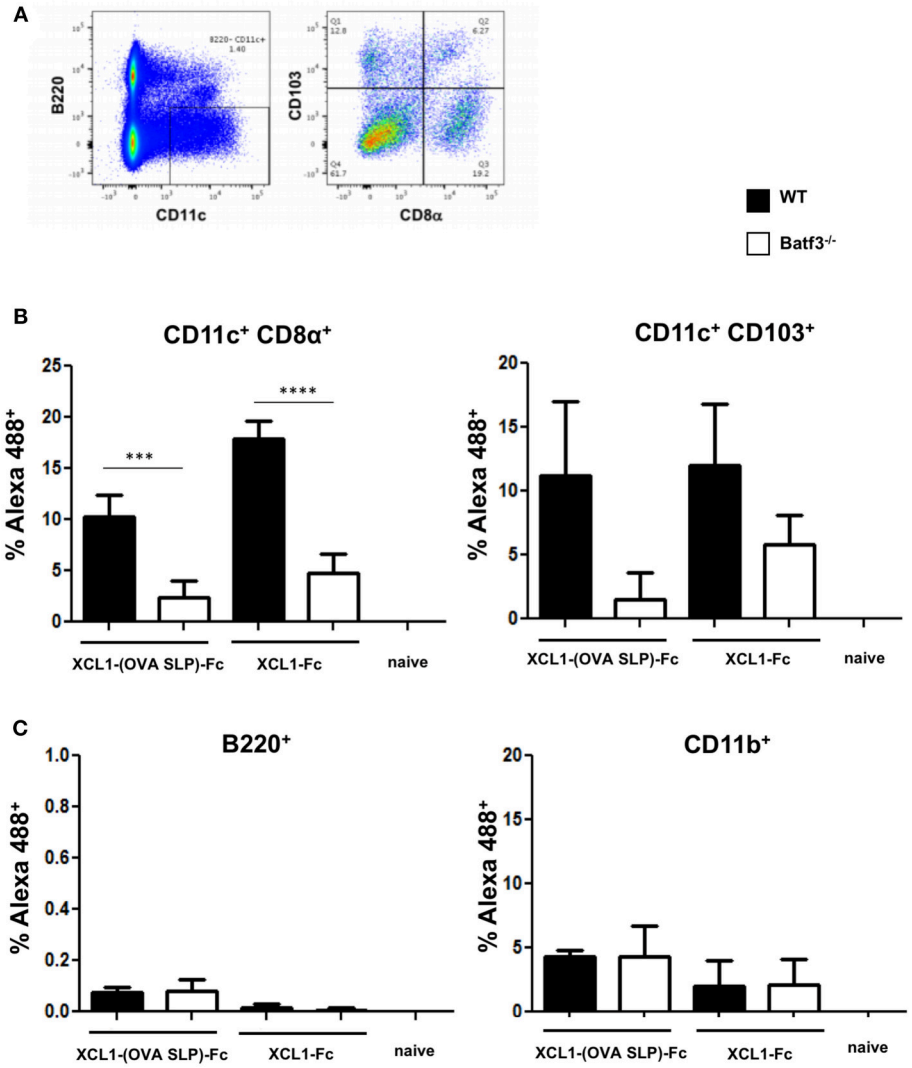

FIGURE 2 | *In vivo* uptake of Xcl1-(OVA SLP)-Fc in skin draining LN. WT and Batf3 KO mice were injected intradermally in the footpad with a mix of 50 μg of CpG and 6 μg of Alexa 488-labeled Xcl1-(OVA SLP)-Fc or Xcl1-Fc fusion proteins. Inguinal LNs were harvested 16 h post injection and the uptake of labeled fusion proteins was measured in different populations of APCs, **(A)** gating strategy for identifying CD103+ and CD8α subtypes in $CD11c^{+}B220^{neg}$DCs isolated from inguinal LNs. **(B)** Uptake of labeled Xcl1-fusion proteins by CD8α DCs (left), CD103+ DCs (right), and **(C)** B220+ B cells (left), and CD11b macrophages (right). Data are shown as mean +/- SEM ($n = 3$–4 mice/group). Results are representative of two independent experiments. $^{***}p < 0.001$, $^{****}p < 0.0001$.

OT-1 T cell transfer, mice vaccinated with Xcl1-(OVA SLP)-Fc fusion protein showed better control of B16.OVA tumor growth, compared to other cohorts (**Figure 5A**). Mice were bled 7 days after vaccination and the percentages of OVA-specific $CD8^{+}$ T cells followed the same pattern as seen upon OT-1 cell transfer, with the highest percentages in the Xcl1-(OVA SLP)-Fc and Xcl1-Fc + OVA SLP-immunized mice (**Supplementary Figure 2**). Strikingly, when comparing tumor growth kinetic with or without OT-1 T cell transfer (**Figures 3A**, **5A**), the tumor control was quite similar, despite a 10-fold lower frequency of endogenous OVA-specific T cells, as seen in the blood on day 7 post vaccination (**Supplementary Figure 2**). Moreover, when analyzing tumors 10 days post vaccination, we observed that the frequency of OVA-specific CTLs infiltrating the tumors of Xcl1-(OVA SLP)-Fc- and Xcl1-Fc + OVA SLP-immunized mice was only 2–3 fold lower in the absence of OT-1 cell transfer (**Figure 5B**), which confirmed their efficient homing to the tumor, as compared to mice vaccinated with free OVA SLP + free Xcl1. In addition, these settings also revealed that the ratio between antigen-specific $CD8^{+}$ T cells and Tregs inside the tumor mass was 4-fold higher in Xcl1 fusion proteins-vaccinated cohorts when compared to mice vaccinated with free OVA SLP with or without free Xcl1 (**Figure 5C**). Representative profiles of the gating strategy for identifying T regs and OVA-specific CTLs are shown in **Supplementary Figure 4**.

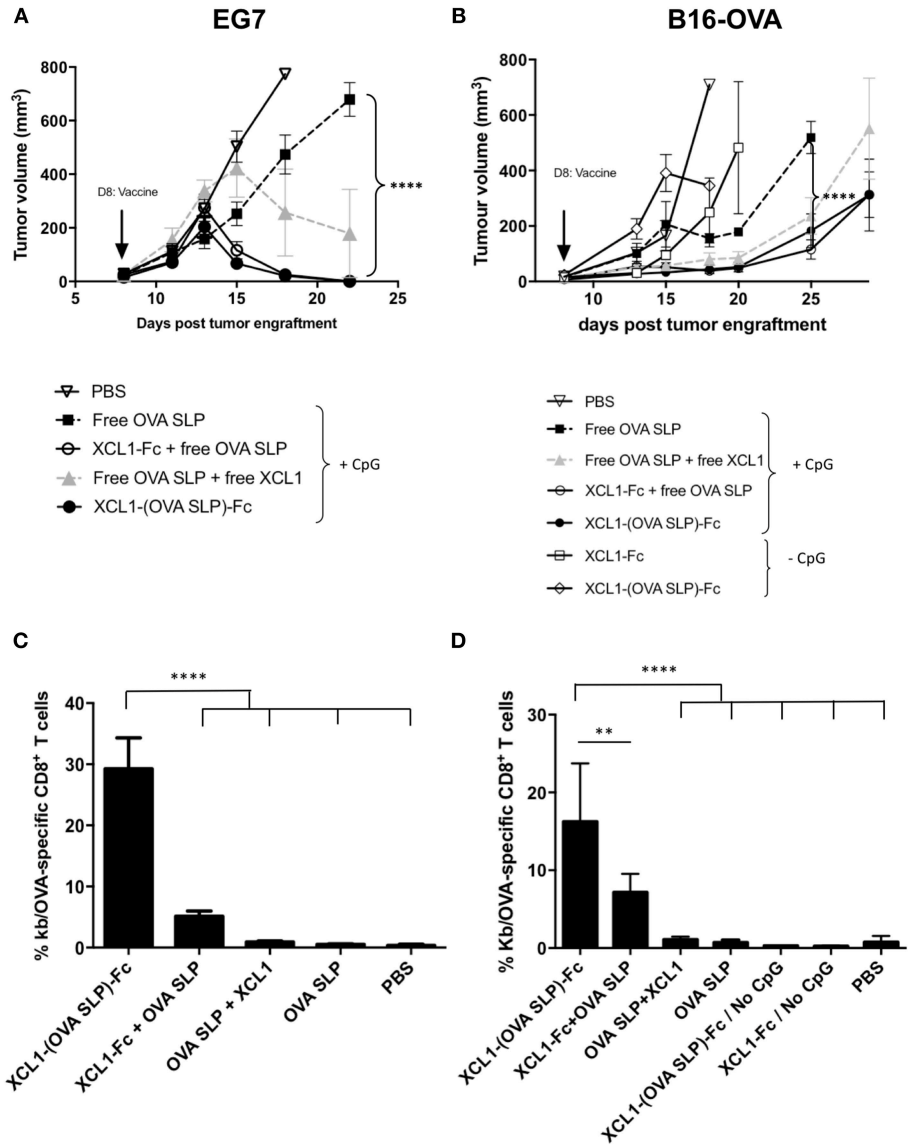

FIGURE 3 | Anti-tumor immunity upon Xcl1-(OVA SLP)-Fc therapeutic vaccinations in tumor bearing mice. **(A)** Tumor growth of the T lymphoma EL4-OVA cell line (EG7) grafted s.c. on the flank of mice (1 x 10^6 cells), followed on day 7 by the i.v. transfer of 10^5 OT-I cells and on day 8 by i.d. vaccination on the left foot (arrow). Cohorts of mice received equimolar amounts of Xcl1 and OVA SLP antigen as described in Materials and Methods. All cohorts received 50 μg of CpG-ODN, except the PBS control **(B)** Tumor growth of B16.OVA tumors engrafted s.c. on the flank of mice (2 x 10^5 cells), followed on day 7 by the i.v. transfer of 10^5 OT-I cells and on day 8 by i.d. vaccination on the left foot (arrow), as described in **(A)**. Graphs represent tumor kinetic as the mean of tumor volume of 6 mice per group ± SEM. $n = 6$ (except in groups with only fusion protein where $n = 3$). Results are representative of three independent experiments. **(C)** Frequencies of H-2Kb/OVA tetramer positive $CD8^+$ T cells in the blood 7 days after vaccination of EG7 and **(D)** of B16.OVA tumor bearing mice as described, respectively, in **(A,B)**. Data are shown as mean ± SEM ($n = 6$ mice/group). Results are representative of two independent experiments. $^{**}p < 0.01$, $^{****}p < 0.0001$.

DISCUSSION

The goal of therapeutic cancer vaccines is to elicit a tumor-specific T cell-mediated immune response, and their success will rely on the use of adjuvants able to break immune tolerance, given that in most cases tumor antigens are derived from self-antigens. In that context, cross-presenting DCs are the APCs of choice, as they are the only subtype of DCs capable of diverting part of endocytosed antigens, such as peptides, from the endocytic pathway to the cytosolic compartment where antigen is degraded by the immunoproteasome before being loaded on to MHC class I molecules for $CD8^+$ T cell presentation (1). The aim of the present study was to develop a strategy to harness these essential cross-presenting DCs.

To do so, we took advantage of the uniquely selective expression of the Xcr-1 chemokine receptor by cross-presenting DCs, essential for their chemotaxis toward primed T cells at the site of infection. We showed that fusion proteins of Xcl1, fused

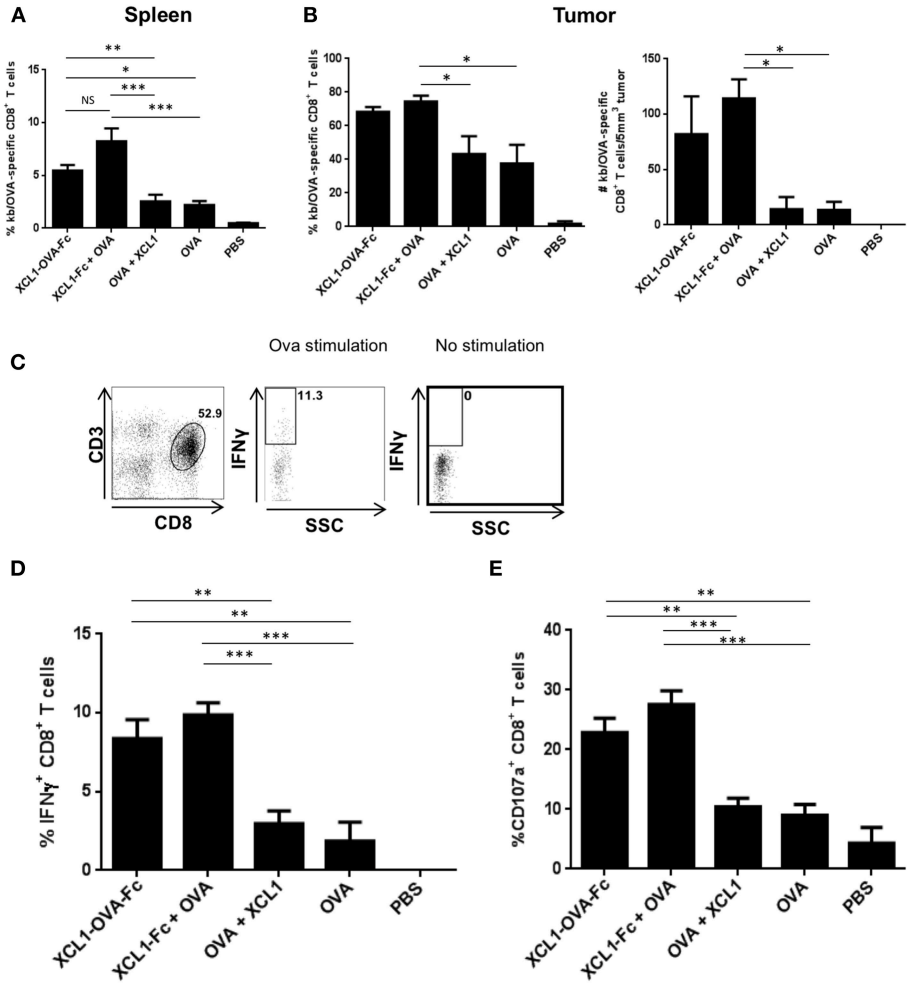

FIGURE 4 | *Ex vivo* function of TILs following Xcl1-fusion proteins vaccination. **(A)** Frequencies of OVA-specific $CD8^+$ T cells in the spleens harvested 10 days post vaccination of mice challenged with B16.OVA on day 0, adoptively transferred with OT-I cells on day 7 and vaccinated on day 8. Data is shown as mean ± SEM ($n = 5$ mice/group). Results are representative of two independent experiments. **(B)** Frequencies of H-2Kb/OVA tetramer positive $CD8^+$ T cells present in the B16.OVA tumors of vaccinated mice (left panel), and absolute number of SIINFEKL-specific $CD8^+$ T cells per 5 mm^3 of tumor mass (right panel). Data is shown as mean ± SEM ($n = 5$ mice/group). Results are representative of two independent experiments. **(C)** Representative plots depicting the IFNγ content of $CD3^+CD8^+$ T cells isolated from B16.OVA tumors 10 days post-vaccination and restimulated with or without SIINFEKL peptide. **(D)** Frequencies of $IFN\gamma^+$ $CD8^+$ T cells and **(E)** of $CD107a^+$ $CD8^+$ T cells isolated from B16-OVA tumors. Data is shown as mean ± SEM ($n = 5$ mice/group). Results are representative of two independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

or not to a peptide antigen and dimerized on a Fc domain were significantly internalized by lymph node-resident $CD8\alpha^+$ DCs, and a trend for preferential uptake by migratory $CD103^+$ DCs was also observed [of which ∼80% express the Xcr1 chemokine receptor (7)]. With regard to T cell antigen priming, we have recently shown that the magnitude of tumor control depends on the avidity of TAA recognition by tumor-infiltrating T cells (26). In the present study, we have used the OVA antigen as a surrogate neoantigen, since it is not subjected to central tolerance and hence allows the priming and recruitment of high affinity T cells to the tumor site. Indeed, therapeutic vaccination with the Xcl1-(OVA SLP)-Fc fusion proteins was able to induce complete tumor regression in the EG7.OVA model and a delayed tumor growth in the more stringent B16.OVA melanoma model.

Previous studies have exploited Xcr1-antigen targeting either in the context of Flu (27) or cancer vaccines. For instance, Xcl1 or an anti-Xcr1 mAb have been fused to the full OVA protein and tested in antitumor vaccinations, albeit in a tumor prophylaxis setting (28). During the same year, another study has targeted $Xcr1^+CD103^+$ DCs via laser-assisted intradermal ear vaccination with Xcl1-OVA fusion protein on day 3 post tumor graft (29). We now further demonstrate the vaccine potency of Xcl1-antigen fusion proteins when injected on day 7 post-tumor graft, when EG7 tumors or the more aggressive B16.OVA tumors are fully established. Our study shows the monitoring of tumor growth over a long period of time and, instead of LPS, our vaccine formulation included the TLR9 ligand CpG-ODN, which is a clinically accepted adjuvant (30). Moreover, our study shows

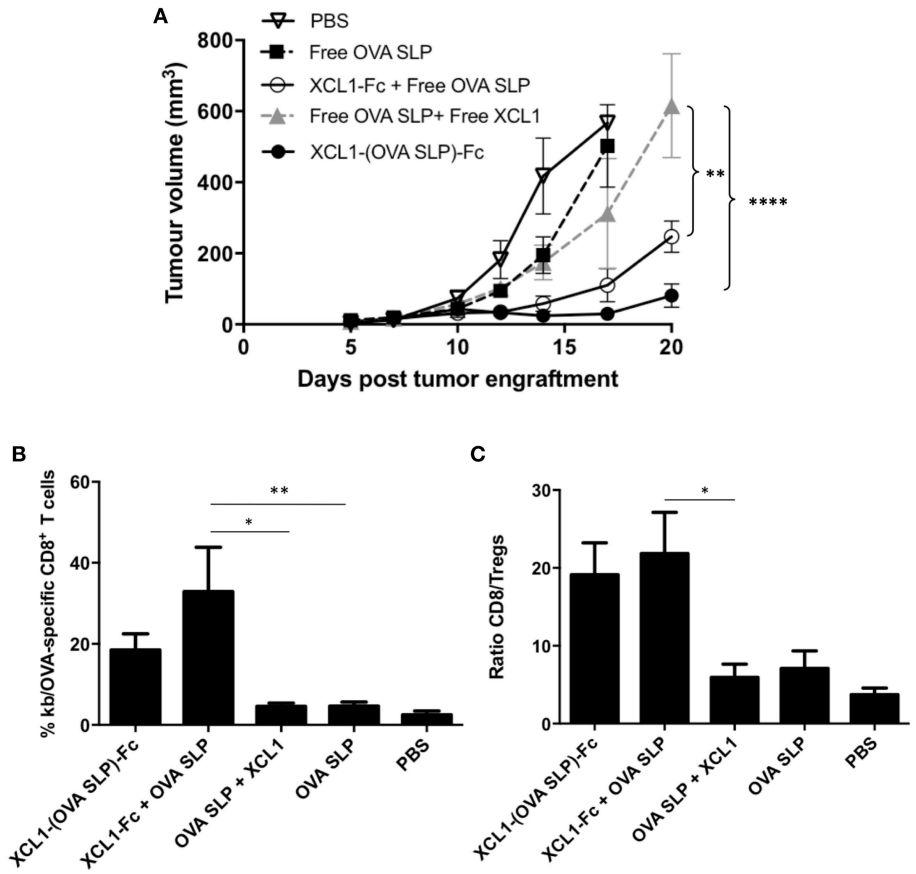

FIGURE 5 | Effective therapeutic vaccinations with Xcl1-(OVA SLP) fusion proteins even in the absence of OT-1 T cell transfer. **(A)** B16.OVA tumor growth of C57BL/6 mice engrafted subcutaneously on the left flank with of 2 x 10^5 B16.OVA cells followed by intradermal vaccination on day 3. Data is shown as mean ± SEM (n = 5–6 mice/group). Results are representative of three independent experiments. **(B)** Frequencies of SIINFEKL-specific $CD8^+$ T cells present in the B16.OVA tumors harvested 10 days post vaccination. **(C)** Ratio of SIINFEKL-specific $CD8^+$ T cells vs. Tregs within B16.OVA tumors. Data is shown as mean ± SEM (n = 5–6 mice/group). Results are representative of two independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

the extent to which vaccination impacts the immune response within B16.OVA tumors, which showed a potent recruitment of OVA-specific T cells to the tumor even in the absence of OT-1 T cell transfer. In addition to their tumor targeting, these tumor-specific CTLs also showed better effector functions, such as IFNγ production and degranulation capacity.

Various strategies have used other surface markers to deliver antigens to cross-presenting DCs, such as DEC205 (19) and CLEC9A (20). Moreover, chemokine receptors common to several subpopulations of DCs were also used to deliver antigens fused to a chemokine such as the gp100 melanoma antigen fused to CCL20 (31). The authors showed that such fusion proteins are endocytosed via binding to the chemokine receptor and are delivered to the cytosol for proteasomal processing, resulting in their loading on MHC class I molecules in a TAP-1-dependent manner, leading to potent tumor control. Alternative strategies to target antigens to other subsets of DCs have also been shown, for example by using glycoliposomes targeting DC-$SIGN^+$ DCs (32), or adenylate cyclase-based vector (CyaA) that target $CD11b^+$ DCs (33). Unfortunately, the large variability between all these vaccination protocols does not allow evaluating which DC marker is the most efficient for T cell priming.

In both of our tumor models, the frequencies and functionality of tumor infiltrating T cells as well as associated tumor control were similar, whether the OVA SLP was fused with the Xcl1-Fc or was co-delivered, which suggests that the signaling machinery induced by the internalization of the cargo via the Xcr1 receptor was instrumental for efficient antigen internalization and processing for MHC class I-mediated presentation. We can also speculate that the intradermal delivery of the combined Xcl1-Fc + OVA SLP vaccine formulation has reached the inguinal lymph nodes in the form of aggregates, which were engulfed by the same DCs. Additional experiments are required to clarify that aspect. Of note, in our *in vitro* testing, both Xcl1 fusion proteins showed similar binding to $Xcr1^+$ DCs as well as similar *in vivo* uptake by $CD8\alpha^+$ DCs. Importantly, vaccination with Xcl1 fusion proteins did not only elicit a quantitatively higher CTL response, but also a qualitatively increased recruitment and functionality at the tumor site. In this context, it will be important to evaluate if tumor control could be further enhanced

by combining Xcl1-SLP-Fc vaccination with immune checkpoint blockade, as demonstrated by us and others in pre-clinical and clinical settings (26, 34–36). Lastly, it will be also important to study the $CD4^+$ T cell response to Xcl1 fusion proteins vaccinations, which we failed to do in this work. Of note, Terhorst et al. (29), who used laser-assisted delivery of Xcl1-OVA fusion protein have reported $CD4^+$ T cell responses, which may well-participate in the efficient $CD8^+$ T cell priming.

DCs are key players in initiating anti-tumor responses and are considered as an essential target in the context of cancer vaccinations (37). Some cancer vaccines directly target DCs, such as Sipuleucel-T, which is the first FDA-approved DC vaccine for the treatment of refractory prostate cancer (38). Moreover, several clinical trials are currently testing the allogenic GM-CSF-secreting whole tumor cell vaccine GVAX in pancreatic cancer patients (39). However, there is so far no DC vaccine that specifically targets cross-presenting DCs in cancer patients. A harmonization of all the strategies tested so far would help in choosing the best DC-specific receptor(s) for delivering tumor antigens to cross-presenting DCs. Such DC targeting strategies may prove very attractive for personalized cancer vaccines using tumor-derived neo-antigens as identified by mass-spectrometry based antigen discovery (40–42).

Our data demonstrate the applicability of Xcl1/Xcr1-mediated DC vaccine for clinical development, given that $Xcr1^+$ cross-presenting DCs have also been well-described in humans. Moreover, developing Xcl1-SLP-Fc fusion proteins as an off-the-shelf DC vaccine might be a more economical and easier alternative to *ex vivo* DC vaccines. Interestingly, the efficacy of the Xcl1-Fc to promote effective targeting of the synthetic long peptide immunogen as a mixture might greatly facilitate the formulation of cancer type-specific, and neo-antigen therapeutic vaccines.

AUTHOR CONTRIBUTIONS

NB performed the experiments and participated to the manuscript preparation. BT performed the experiments in the late stage of the study. JH and MS made substantial contributions to conception, experimental design and analysis of results. AD supervised the study and the mansucript preparation. PR designed and supervised the study and manuscript preparation. All co-authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00294/full#supplementary-material

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REVIEW
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The Journey of *in vivo* Virus Engineered Dendritic Cells From Bench to Bedside: A Bumpy Road

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Dendritic cells (DCs) are recognized as highly potent antigen-presenting cells that are able to stimulate cytotoxic T lymphocyte (CTL) responses with antitumor activity. Consequently, DCs have been explored as cellular vaccines in cancer immunotherapy. To that end, DCs are modified with tumor antigens to enable presentation of antigen-derived peptides to CTLs. In this review we discuss the use of viral vectors for *in situ* modification of DCs, focusing on their clinical applications as anticancer vaccines. Among the viral vectors discussed are those derived from viruses belonging to the families of the *Poxviridae, Adenoviridae, Retroviridae, Togaviridae, Paramyxoviridae,* and *Rhabdoviridae.* We will further shed light on how the combination of viral vector-based vaccination with T-cell supporting strategies will bring this strategy to the next level.

Keywords: viral vaccine, dendritic cell, T cell, cancer, immunotherapy, preclinical and clinical

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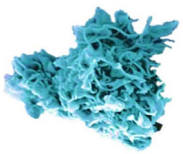
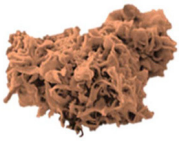
DENDRITIC CELLS: NATURE'S ADJUVANT

Since their discovery in 1973, it was clear that dendritic cells (DCs) stood out above the immune cell pack (1, 2). They are morphologically distinct from all other immune cell types and are gifted with an unparalleled capacity to take up, process and present self and foreign antigens to both $CD4^+$ and $CD8^+$ T cells. DCs are critical intermediaries between the innate and adaptive immune systems, as they stimulate, regulate, and shape both immunity and tolerance in all its disguises. Ralph Steinmann, who discovered these cells, was awarded the Nobel Prize for Medicine in 2011, because the discovery of DCs changed medicine (3).

Dendritic cells in both humans and mice represent a population of at least four different subtypes with distinct phenotypical and functional characteristics (4–7). These subsets are: plasmacytoid DCs (pDCs), two subsets of conventional DCs (cDC1 and cDC2), and inflammatory DCs. The latter represent a monocyte-derived subset that appears during inflammatory responses (**Table 1**). Recently, additional types of human blood DCs, monocytes, and progenitors were revealed using single cell RNA-sequencing. The group of Prof. Nir Hacohen identified pDCs next to cDC progenitor-derived cDC1 ($Clec9A^+$) and two types of $CD1c^+$ cDC2, of which one can also be derived from $CD14^+$ DCs. Furthermore they found a $CD141^-$ $CD1c^-$ $CD11c^+$ DC subset derived from $CD16^+$ monocytes and an AXL^+ $Siglec6^+$ subset (8). Future research will have to unravel a possible murine representative for the human cDC2 and AXL^+ $Siglec6^+$ DC subset. Also, Langerhans cells have been considered an important DC subset for vaccination as they are localized in the epidermis ($HLA\text{-}DR^+$ $CD11c^+$ $CD1a^+$ $CD207^+$). However, recent evidence suggests that they are related to macrophages, another antigen-presenting cell (APC) type with potential antitumor activity (9).

TABLE 1 | Overview of currently described murine dendritic cell subsets with their human counterparts.

	cDC1	**cDC2**	**pDC**	**Infl DCs**
				
Mouse				
Common name	**CD8α$^+$ cDC (LT) CD103$^+$ cDC (NLT)**	**CD4$^+$ CD11b$^+$ cDC (LT) CD11b$^+$ cDC (NLT)**	**SiglecH$^+$ BST2$^+$ pDC**	**Ly6C$^+$ monocyte derived infl DCs**
Other markers	TLR3$^+$ CADM1$^+$ XCR1$^+$ BATF3$^+$ CLEC9A$^+$ FLT3$^+$ CD205$^+$	CD24+,SIRPα$^+$ CD11c$^+$ FLT3$^+$	B220$^+$ Ly6C+ TLR7^{hi} TLR9^{hi}	FcεRI$^+$ CD11b$^+$ CD206$^+$ CD115$^+$ CD64$^+$ DC-SIGN$^+$ MAC-3$^+$
Human				
Common name	**CD141$^+$(BDCA-3) cDC**	**CD1c$^+$(BDCA-1) cDC**	**CD123$^+$ pDC**	**CD14$^+$ monocyte derived infl DCs**
Other markers	TLR3$^+$ CADM1$^+$XCR1$^+$ FLT3$^+$ CLEC9A$^+$ CD162^{hi} CD205^{hi}	SIRPα$^+$ CD11b$^{lo/+}$ FLT3$^+$ CD11c$^+$	CD45RA+,BDCA-2$^+$, BDCA-4$^+$ TLR7^{hi} TLR9^{hi}	FcεRI$^+$ CD11b$^+$ CD206$^+$ CD115$^+$ CD64$^+$ BDCA-1$^+$ CD1a$^+$ CD172a$^+$ DC-SIGN$^+$ CD1c$^+$
Conserved				
Phenotype	**TLR3$^+$ CADM1$^+$ XCR1$^+$ CLEC9A$^+$**	**CD1c$^+$ SIRPα$^+$ CD11b$^+$**	**TLR7^{hi} TLR9^{hi}**	**FcεRI$^+$ CD11b$^+$ CD206$^+$ CD115$^+$ CD64$^+$ DC-SIGN$^+$ ZBTB46$^+$**
Functions	Cross-presentation IL-12 secretion T_H1/2 polarization TLR3-induced IFN-λ production	Presentation to CD4$^+$ T cells IL-1β, IL-6, and IL-23 production T_H2 and T_H17 polarization	Viral sentinels TLR7/9-induced IFN-α/β and IFN-λ production	Highly adaptable with amongst others IL-12 or IL-23 secretion + **TipDCs** = TNFα and iNOS producing subset of infl DCs

General hallmarks not included in this table are MHCII^{hi} and CD11c$^+$, LT, lymphoid tissue; NLT, non-lymphoid tissue.

Different DC subsets are endowed with distinct functions. pDCs are specialized in sensing viral infections. To that end, pDCs use toll-like receptor 7 (TLR7), TLR9 and stimulator of interferon genes (STING) for sensing of nucleic acids (ssRNA, dsDNA, and cytosolic DNA, respectively). Triggering these receptors results in the production of high levels of type I interferon (IFN) (10). A key function of cDC1 that requires the production of IL-12 and/or type I IFN, is activation of cytotoxic CD8$^+$ T lymphocytes (CTLs) via cross-presentation of antigens and linked herewith stimulation of CD4$^+$ T helper 1 (T_H1) responses (11–14). cDC1 selectively express TLR3 enabling them to sense dsRNA, and similar to pDCs, cDC1 express TLR9 for sensing of dsDNA (15). The expression of TLR3 and TLR9 explains the cDC1s' ability to produce type I IFN. cDC2 and inflammatory DCs are also able to produce IL-12, stimulate CD4$^+$ T_H cells and CD8$^+$ T cells by cross-presentation. Depending on their activation, they will instigate a specific immune response. Both cDC2 and inflammatory DCs are equipped with a wide range of TLRs allowing them to become activated upon contact with various stimuli like polyI:C (TLR3), LPS (TLR4), and R848 (TLR8) (15, 16). The DC subsets co-operate in a wide range of immune responses, through mechanisms that are relatively conserved across mammalian species. The knowledge that human DC subsets have counterparts in mice enables the use of murine models to study the potential of DCs for cancer vaccination.

In general, antitumor vaccines comprise one or more tumor-associated antigens (TAAs) and an adjuvant to avoid induction of TAA-specific tolerance. Due to the exquisite capacity of DCs to cross-present and stimulate antitumor immunity, they have been applied as nature's adjuvant in cancer vaccination studies. Therefore, autologous DCs are generally loaded *ex vivo* with one or more TAAs, possibly with additional DC activating stimuli. Subsequently, they are transferred back to the patient to induce a TAA-specific CTL response. To exemplify, Sipuleucel-T, trade name Provenge (Dendreon), was the first autologous DC-vaccine that was approved by the FDA in 2010. More specifically it was approved for the treatment of metastatic, hormone-refractory prostate cancer. This vaccine consisted of autologous DCs that were loaded with a fusion protein consisting of prostatic acid phosphatase (PAP) and granulocyte macrophage-colony stimulating factor (GM-CSF) (17).

In most clinical trials with DC-based vaccines, autologous monocyte-derived DCs (moDCs) are used (18). However, these moDCs do not recapitulate the natural diversity of DCs, but rather mimic inflammatory DCs. The awareness that moDCs might not be ideally suited for vaccination purposes together with their overall limited efficacy in clinical trials, has stimulated research in the use of cDCs or pDCs in the clinic (19, 20). Comparing clinical trials is a challenging task, as there are significant differences in (i) type of antigens used, (ii) type of system used to deliver the antigens, (iii) protocol used to activate

the DCs, (iv) route of DC administration, and (v) heterogeneity of inclusion criteria with patient selection bias. Nonetheless, we dare to state that clinical data do not hint at a better outcome upon cDC- or pDC-based cancer vaccination compared to the clinical data obtained with moDC-based vaccines (21–23). This could suggest a need for cooperation between multiple APC subsets to induce effective antitumor immunity (24, 25). When optimal priming of antiviral $CD8^+$ T cells was investigated, a response fundamentally similar to an antitumor immune response, accumulation of pDCs at sites of $CD8^+$ T cell activation led to local recruitment of cDC1 via XCL1 chemokine secretion by the $CD8^+$ T cells. The $CD8^+$ T cell-mediated reorganization of the local DC network allowed the cooperation of cDC1 and pDCs, and enhanced the maturation and subsequent cross-presentation of antigens by cDC1 (26). These findings suggest that stimulation of only one DC subset is most likely not optimal for CTL stimulation. Together with the fact that vaccination with patient-specific, *ex vivo* engineered DCs is a very costly and cumbersome method (27–30), research moved to the *in situ* engineering of DCs. This allows targeting of natural DC subsets. Moreover, it implies an assent for cooperation with other subsets and as such optimal CTL activation *in situ* (24).

We can roughly distinguish four types of *in situ* DC-directed vaccines: naked proteins, naked nucleic acids, viral vectors and nanoparticles (25, 31–34). In general, naked protein- and nucleic acid-based vaccines are relatively easy to generate. However, they need to be co-delivered with an adjuvant to achieve robust antitumor immunity. In contrast, nanoparticles and viral vectors represent more immunogenic vaccines. For viral vectors, this is explained by the fact that TAAs are truly produced by the viral vectors upon infection next to the delivery of intrinsically immunogenic viral proteins that trigger a type I IFN response (35–37). When *in vivo* vaccination of mice with a viral vector was compared to peptide, DNA, or DC-vaccination, the strongest tumor-specific immune responses were elicited with viral vectors (38–40).

Despite this knowledge, viral vectors have not taken the lead in clinical antitumor vaccination trials. Therefore, we review the use, advantages as well as shortcomings of viral vector vaccines, highlighting their potential. In particular, we focus on their clinical application. Furthermore, we touch upon pre-clinical data for the viral vector types that have not been clinically tested yet.

VIRAL ANTICANCER VACCINES THAT HAVE ENTERED THE CLINICAL ARENA: FROM BENCH TO BEDSIDE

Antitumor vaccination strategies using viral vectors can be subdivided into two main classes. The first class comprises viral vectors that encode TAAs to engineer tumor-specific DCs *in situ*. The second class consists of non-replicating apoptosis-inducing vectors or oncolytic viruses that are used to induce tumor cell death, and as such stimulate local and systemic immunity toward released TAAs (41). Oncolytic viruses are designed in such a way that they selectively replicate in tumor cells leading to their lysis without affecting normal cells. Therefore, they cannot be considered as TAA-encoding, DC-targeted therapeutic vaccines, and are not within the scope of this review. A comprehensive review on oncolytic viruses is provided elsewhere (42).

In search of clinically relevant viral approaches to deliver TAAs to DCs *in situ*, we turned to "ClinicalTrials.gov." As depicted in **Figure 1**, viral vectors derived from viruses of the *Poxviridae* family are most often used in clinical trials in the framework of antitumor immunotherapy with over 85 registered clinical trials. In comparison, less than 15 registered clinical trials involve therapeutic antitumor vaccination with viral vectors derived from viruses of the *Retroviridae, Togaviridae, Paramyxoviridae,* or *Rhabdoviridae* families. In this section we provide an overview of the journey these viral vectors made from the bench to the bedside.

Viral Vectors Derived From Viruses of the *Poxviridae* Family

Poxviruses are enveloped dsDNA viruses with a linear genome that can infect mammalian cells. A major advantage of poxvirus-derived vectors is their ability to accept large inserts of foreign DNA and as such deliver large transgenes to target cells, including DCs (**Table 2**). Since viral replication and transcription occurs solely in the cytoplasm of host cells, the risk of insertional mutagenesis is precluded. By attenuating the viral system via deletion of certain pathogenic genes, the safety of poxvirus-derived vectors is enhanced, as this disables them to generate infective viral particles and complete their life cycle. This is exemplified by the recombinant vaccinia virus, which is based on the attenuated Wyeth strain. Another interesting asset is the fact that poxvirus-derived vectors are relatively easy to produce at high-titers and stability (43).

There are currently about 69 species divided over 28 genera described for this family. Humans, vertebrates and arthropods can serve as natural hosts. Vaccinia virus is the prototypical poxvirus that has been administered to roughly one billion people through the profoundly successful smallpox eradication program. The latter paved the way for its clinical evaluation as an anticancer vaccine. Accordingly, extensive evaluation of therapeutic vaccination with live recombinant vaccinia virus encoding TAAs such as carcino-embryonic antigen (CEA) or prostate specific antigen (PSA) started more than 20 years ago. For example, recombinant vaccinia virus expressing CEA or PSA (rV-CEA or rV-PSA) was administered to advanced carcinoma or metastatic androgen independent prostate cancer patients, respectively. This induced elevated levels of anti-TAA antibodies next to TAA-specific CTLs, capable of lysing TAA-expressing tumor cells *in vitro* (44, 45). Despite these immunologic occurrences, a lack of clinical response with tumor regression in most patients was observed. This may be explained by inadequate clonal expansion and/or cytotoxicity *in vivo* next to low antibody titers with low affinity (44, 46). Importantly though, as long as 10^7 plaque forming units (PFU) were injected, no significant treatment-related toxicities were observed, apart from

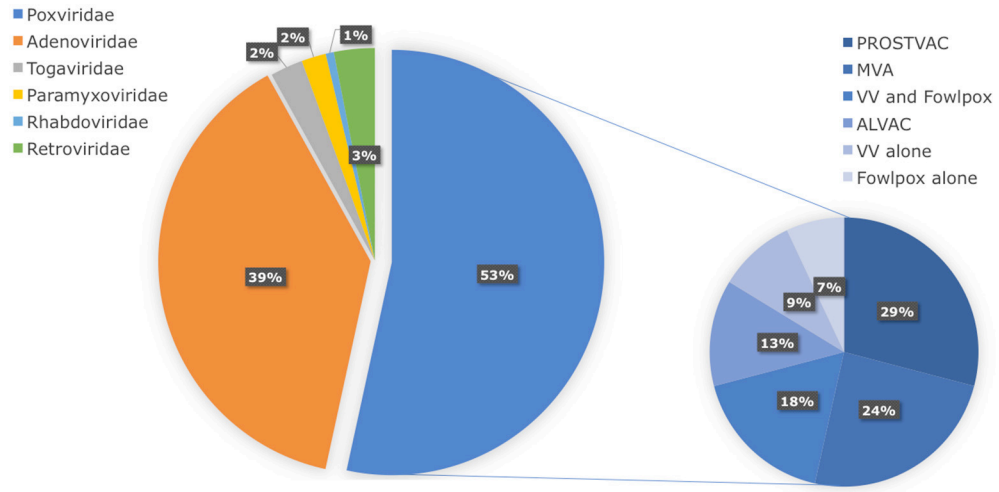


FIGURE 1 | Distribution of viral vector families involved in ongoing or completed clinical trials. Within the search engine ClinicalTrials.gov from the National Institute of Health (NIH), the search terms "virus," "cancer," and "vaccine" yielded 325 search results, of which only 75 trials were selected based on the following criteria: *in situ* therapeutic viral vaccinations encoding TAAs with or without extra adjuvant. Oncolytic virus-based vaccines, preventive virus-based vaccines, virally modified DCs, tumor, or T cell-based vaccines were excluded.

injection site reactions such as erythema and pustule formation in all patients, who were previously vaccinated against smallpox.

These results were in marked contrast with the preclinical evaluations of TAA-expressing recombinant viral vaccines showing significant anticancer activity in animal models. Suggested reasons for the marginal clinical effects are the intrinsic tolerance of the TAA in humans and the immunosuppressive effects of the tumor and its microenvironment. Furthermore "epitope dominance" of viral antigens over TAAs could derivate the immunological focus from the cancer cells toward the viral vectors themselves. A phenomenon that was reinforced by the observation that rV-CEA or -PSA could only be administered once, at most twice, to result in a measurable immune response as after the third injection, viral vector-neutralizing antibodies completely diminished the cellular and/or humoral anti-TAA effect. The search for alternatives that had less compunction with pre-existing immunity led to evaluation of two Avipoxviral strains namely canarypox (ALVAC) and fowlpox. Furthermore, an attenuated strain of vaccinia, named modified vaccinia Ankara (MVA) was generated via repeated passaging (>350 times) in chicken embryo fibroblasts. Interestingly, ALVAC, fowlpox and MVA can infect but not replicate in mammalian cells. This increases the overall patient safety, while ensuring TAA-expression for up to 3 weeks after infection before cell death is induced within the virally infected cells.

Since clinical responses with replication-deficient poxviral vectors was also marginal and repeated vaccination still suffered from viral epitope dominance, it was suggested to use prime-boost regimens to increase the therapeutic outcome. These regimens generally consist of at least two different consecutively administered poxviral strains expressing the same TAA. In an attempt to determine which prime-boost regimen to use, a small randomized trial compared rV-CEA as the initial priming vaccination with three ALVAC-CEA injections (VAAA), vs. three vaccinations with ALVAC-CEA, followed by one rV-CEA (AAAV) (47). The IFN production by T cells in response to CEA peptide was much higher in the VAAA arm than the AAAV arm, which was furthermore correlated with a striking difference in overall survival of five vs. zero patients out of nine respectively. This and other studies suggested that optimal usage of poxviral vaccinations is done by priming with recombinant vaccinia, followed by booster vaccinations with recombinant non-replicating vaccines and/or vectors. One of the most applied poxviral vaccines (>25 clinical trials) is represented by the PSA-encoding PROSTVAC, which is most often delivered via a prime-boost regimen consisting of recombinant vaccinia followed by fowlpox virus injection.

As outlined in the first chapter of this review, DCs are the main drivers of immunity and as such represent the leading targets in vaccination. Since several DC subtypes with different maturation and polarization states co-exist *in situ*, the induction of a T_H1 polarized antitumor CTL response requires their proper stimulation. However, direct injection of a TAA-encoding viral vaccine can result in the infection of both APCs and non-APCs. In the latter case, TAAs will be expressed *via* MHC-I by the infected cells and only via MHC-II by an APC, if the infected non-APC released TAAs upon cell death or via secretion. Only when the viral vaccine directly infects DCs, processed TAAs will be abundantly presented via MHC-I and MHC-II together with the appropriate co-stimulatory molecules to initiate a cytotoxic T_H1-supported CTL response. Especially if a MHC-II targeting signal, such as invariant chain (Ii) or LAMP-1/2, and/or cross-presenting stimulators, such as calreticulin or the non-hemolytic part of the Listeria monocytogenes virulence factor, listeriolysin O, are co-delivered (48). Injection of mice, bearing Human Papilloma Virus (HPV)-16 immortalized tumor,

TABLE 2 | Overview of clinical and preclinically tested viral vaccines for cancer.

	Poxviridae	Adenoviridae	Retroviridae	Togaviridae	Rhabdoviridae	Paramyxoviridae	AAV	Coronaviridae	Papillomaviridae	Baculoviridae
Genomic material	dsDNA	dsDNA	ssRNA	ssRNA	ssRNA	ssRNA	ssDNA	ssRNA	circular DNA	circular DNA
Insert capacity	>30 kb	<7.5 kb (though HC-AdV 35 kb)	12 kb	8 kb	6 kb	6 kb	<4 kb	6 kb	8 kb	>38 kb
Production (titers)	High	High	Moderate	High	High	Low	High	Low	Low to high	High
Efficacy of tg delivery to DCs	Broad tropism	Serotype dependent tropsm, infects dividing and non-dividing cells, transient expression	Psedotype dpendent tropism, infects dividing or/and non-dividing cells, stable integration with long term expression	Broad tropism with strong neuronal preference + high expression level	Broad tropism, highly transient expression	No	Serotype dependent tropsm, infects dividing and non-dividing cells, slow expression onset	DC-specific tropism	Epithelial tropism	Broad tropism
DC stimulatory potential	High	High	Wild type low, but attenuated vector high	Low but cytotoxic	Wild type low, but attenuated vector high	Induction of DC maturation	Moderate	High	High	High
Pre-existing immunity	High	High	Low	Low	Low	High	Moderate	Most likely high	Most likely high	Low (but serum complement inactivation)
Biosafety level	BSL-2	BSL-2	BSL2-3	BSL-1-2	BSL-2	BSL-1	BSL-1	BSL-2	BSL-2	BSL1-2
Clinical phase as vaccine	Phase I-III	Phase I-II	Phase I-II	Phase I-II	FDA-approved	Phase I	Not applicable	Not applicable	Not applicable	Not applicable

with vaccinia encoding E7 fused to listeriolysin O or LAMP-1, resulted in enhanced uptake and presentation *via* MHC-I, or MHC-I and MHC-II, respectively. What's more, tumors appeared to regress because of increased amounts of IFN-γ and TNF-α secreting CTLs within the spleen. Of note, only the vaccines with MHC-I directing listeriolysin O resulted in high intratumoral CTL infiltration as well (45).

Due to the abiding relatively weak clinical response rates, viral vaccines were pimped with co-stimulatory signals to skew a T_H1 climate. Multigene constructs were generated that included both a TAA as well as one or more co-stimulatory genes such as CD80 (B7.1) or CD154 (CD40L) that could aid in the stimulation of DCs *in situ* and as such in the proper stimulation of TAA-specific CTLs. Building on promising preclinical data, ALVAC–CEA–B7.1 was injected intramuscularly into patients with advanced, unresectable CEA-expressing malignancies. The virus could induce CEA-specific peripheral blood T cells in a proportion of patients, and 3 out of 16 patients demonstrated transient disease stabilization, but no disease regression (49). Interestingly, preclinical efficacy of MVA was mainly attributed to $CD4^+$ T cells and polyclonal h5T4-specific antibodies, as only weak $CD8^+$ T cell responses were induced (50). Therefore, the addition of stimulatory immune checkpoints like inclusion of CD70 or mGITRL-fusion proteins has been tested preclinically to enhance CTL responses (51). More robust tumor regression with improved overall survival was reported when using viral vectors encoding mGITRL-fusion proteins. This was linked to stimulation of strong antitumor CTL-responses and depletion of $FoxP3^+$ regulatory T cells (Tregs) (52).

Current observations point out in favor of adding several co-stimulatory molecules in one vaccine. The MVA-based cancer vaccine TG4010 targeting the MUC1 antigen has been tested in a phase II trial for renal cell carcinoma (37 patients, metastatic) combined with IFNα2a and IL-2. Though no objective clinical responses were observed in the form of complete or partial tumor regression, improved overall survival was demonstrated. Antivaccine and antiIL-2 antibodies, $CD4^+$ T cells, and MUC1-specific CTL responses were reported. Importantly, patients that had MUC1-specific CTLs showed a longer survival compared to the overall population (53). Also, several clinical-grade poxviral vaccination approaches such as PROSTVAC and ALVAC are regularly tested with the inclusion of a triad of immune enhancing co-stimulatory molecules, namely CD80 (B7.1), CD54 (intercellular adhesion molecule-1 or ICAM-1), and CD58 (leukocyte function-associated antigen- 3 or LFA3), collectively designated as TRICOM. When this formula was used to vaccinate mice, superior TAA-specific responses were described compared to constructs that only contained one or two of these molecules (54). A vaccinia prime–fowlpox boost regime encoding two TAAs (CEA and MUC1) for the treatment of pancreatic cancer, termed PANVAC, has also been evaluated alongside TRICOM. Phase II results have been promising with increased median survival in those patients with a pre-trial life expectancy of 3 months. However, a phase III trial did not demonstrate any survival benefit. More encouragingly, two different studies enrolling patients with metastatic ovarian or breast cancer, showed

TAA-specific immunity after administration of a CEA-MUC-1-TRICOM poxviral-based vaccine (55, 56). This immunity did result in stable breast cancer disease (5/13), tumor shrinkage (1/13) and even one complete response with a significant drop in serum IL-6 and IL-8.

Interestingly, poxviruses have also been injected intratumorally to bring TAAs and co-stimulatory signals in close proximity. When melanoma lesions were injected with a recombinant vaccinia virus expressing TRICOM, clinical responses were shown in more than 30% of patients (57). Furthermore, when a vaccinia-based vaccine encoding both PSA and TRICOM was injected intratumorally in 21 patients with locally recurrent prostate cancer, higher numbers of tumor-infiltrating $CD4^+$ and $CD8^+$ T cells could be demonstrated. Furthermore, local Treg function was reduced and up to 76% of patients had stable or improved serum PSA levels (58). Finally, ALVAC has also been tested as an intratumorally delivered adjuvant by combining ALVAC encoding human CD80 with ALVAC encoding human IL-12 in patients with surgically incurable melanoma. Fourteen patients received intratumoral injections on days 1, 4, 8, and 11. Unexpectedly, tumors injected with ALVAC-B7.1 and ALVAC-IL-12 showed higher intratumoral levels of immunosuppressive cytokines like IL-10 and VEGF, and decreased intratumoral levels of pro-inflammatory cytokines IL-12 and IFN-γ, when compared to tumors injected with saline. While no tumor regression was observed, all patients did develop neutralizing antibodies against ALVAC, suggesting that pro-inflammatory intratumoral strategies can also lead to the induction of negative feedback mechanisms that aggravate the immunosuppressive tumor climate (59).

In addition to co-stimulatory molecules, adjuvant or growth factors such as GM-CSF have been added to increase the targetable DC load. This approach was shown to induce local and systemic tumor immunity with effective clinical responses. To exemplify, in a randomized study with PROSTVAC and GM-CSF, or empty viral vector and saline injections, primary objectives of improved progression-free survival were not reached. However, an increased median overall survival compared with control subjects was reported (25.1 vs. 16.6 months; $P = 0.015$) (60, 61). Also when ALVAC-CEA with CD80 was compared to its combination with the adjuvant GM-CSF, disease stabilization was seen in 26% compared to 37% of patients, who received the combination (62).

Next to co-stimulatory cytokines and growth factors, a few trials with poxviral vaccines evaluated its combinatorial potential with other anticancer treatments, such as targeted therapy, chemo- or radiotherapy. A large randomized phase III trial involving 733 patients with metastatic renal cancer was conducted using MVA-5T4 in combination with first-line treatment of receptor tyrosine kinase inhibitor sunitinib, IL-2 or IFN-α. No overall survival benefit was seen in the vaccine arm. However, analysis in this larger trial did reveal a significant correlation between the magnitude of 5T4-specific antibody responses and improved patient survival (63). In contrast, a phase II trial of TG4010 combined with first-line chemotherapy (cisplatin plus gemcitabine) in advanced non-small cell lung cancer (NSCLC) demonstrated a significant 6 month increase in median survival (64). It was recently shown in a randomized phase II study with 220 NSCLC patients that the combination of TG4010 with several chemotherapy regimens led to responses against MUC1, which correlated with improved survival under TG4010 treatment. Furthermore, these responses were associated with CTL responses against non-vaccine TAAs, thus evidencing epitope spreading (65). Finally, recombinant vaccinia virus encoding the HPV16 and 18 E6 and E7 fusion protein, was evaluated with heat shock protein 70 (HSP70) encoding DNA and TLR7-stimulating imiquimod. This led to a potent antigen-directed antibody and cytotoxic response in a phase I/II clinical trial for patients with (pre-)malignant cervical lesions (66–68). Since the arrival of antagonistic checkpoint inhibitor therapies, also their combinatorial potential with poxviral vaccination has been tested in metastatic castration-resistant prostate cancer. No dose limiting effects were observed while 58% of the chemotherapy naïve patients had a PSA decline from baseline (69).

Despite the growing use of poxviral vectors as antitumor vaccine candidates for cancers encoding a diverse range of TAAs such as CEA, PSA, MUC1, NY-ESO, Epstein Barr Virus nuclear antigen-1 (EBNA1), latent membrane protein-2 antigens (LMP-2), 5T4, melanoma antigen recognized by T cells-1 (MART-1), gp100, tyrosinase, HPV16 and 18 E6 and E7; their innate stimulatory properties remain poorly characterized. Interestingly, when the innate immune profiles elicited by ALVAC, MVA, and New York vaccinia virus (NYVAC) were compared *in vivo* in rhesus monkeys and *in vitro* in human peripheral blood mononuclear cells (PBMC), they appeared to be all distinct. ALVAC elicited a higher induction of proinflammatory and IFN-related antiviral cytokines with chemokines on day 1 following immunization. In addition, ALVAC's stimulatory phenotype was influenced by several PBMC subsets such as T cells, monocytes, macrophages, and pDC. Furthermore, the stimulatory phenotypes observed following priming with ALVAC, MVA, or NYVAC were all reduced when these poxviral vectors were used as a boost (70). Interestingly, Hanwell et al., compared TAA-expression and immunogenicity of 5T4 or gp100 delivered by ALVAC or MVA (71). While 5T4 expression in chicken embryo fibroblasts was equal for both vector systems, ALVAC-derived gp100 was much faster degraded compared to MVA-derived gp100. Furthermore, the HLA-A2 transgenic mouse model was used to measure CTL-responses upon vaccination. It was shown that vectors encoding 5T4 elicited low to immeasurable responses irrespective of the virus strain used. In contrast, MVA-vectors encoding gp100 elicited a significantly higher gp100-specific response than ALVAC-vectors encoding gp100, reflecting the *in vitro* TAA expression and stability (72). The above studies confirm the complexity of the possible immunological outcomes that depend on immunogenicity of the vector as well as the transgene it encodes, *in vivo* stability of transgene expression and order of vaccination in prime-boost regimens. Additional studies are required to evaluate the correlation between these different innate signatures, subsequent adaptive immune responses, and protective efficacy.

Viral Vectors Derived From Viruses of the *Adenoviridae* Family

Adenoviruses are non-enveloped dsDNA viruses efficient at delivering DNA to both dividing and quiescent cells, like DCs. Furthermore, they can be readily produced with high titers up to 10^9 IFU/ml that can be concentrated to 10^{13} IFU/ml (43). Early cancer vaccination studies used replication-incompetent variants (deletions in E1 and E3 region) of serotypes Ad2 and Ad5 encoding a range of TAAs. However, most humans show pre-existing immunity against these viruses, as a result of lifelong exposure to the wild type virus, especially against the most common serotype (Ad5). This hampers therapeutic efficacy through induction of neutralizing antiviral antibodies and/or CTL-mediated immunity, and moreover entails the risk of toxicity upon systemic adenoviral vector administration. In search for safer adenoviral vectors, a third generation high capacity HC-AdV, stripped of all viral coding sequences was engineered (73). Consequently, this HC-AdV is less immunogenic. Furthermore, this HC-Adc has a larger packaging capacity of up to 35 kb. From the adenoviral vector trials related to DC activation *in situ*, about 50% of the trials use TAA-encoding vaccines, while the other 50% only encode pro-inflammatory factors such as IL-12, type I or type II IFN, TNF-α, Flt3L, *et cetera* or co-stimulatory molecules such as CD40L.

Preclinical testing of various adenovirus-based antitumor vaccines demonstrates the induction of both protective humoral and cellular immunity as well as eradication of established tumors in mice (74–83). When different routes of administration were compared, intravenous and intradermal delivery appeared the most efficacious for antitumor immunity (79). Though preclinical animal models often respond well to vaccination, more variable vaccine responses are elicited in cancer patients with little therapeutic benefit (41, 84, 85). A phase I study for metastatic melanoma, showed that Ad2 encoding MART-1 ($n = 36$) or gp100 ($n = 18$), were safe, but failed to induce immunological or clinical efficacy (86). Remarkably, in one patient receiving the Ad2-MART-1 vaccine, a complete response was observed that could be attributed to the vaccination (86). One way to decrease vector neutralizing antibodies was by delivering a heterologous prime-boost. While only 50% of patients receiving naked DNA encoding CD86 and prostate-specific membrane antigen (PSMA) showed signs of successful immunization, this was 100% when they were inoculated with 5×10^8 PFUs of PSMA-encoding viral vectors followed by PSMA plasmid boosts (87). On the other hand, when 13 NSCLC patients received sequential DNA and adenoviral vaccines coding for the lung tumor antigen L523S intramuscularly, this only resulted in L523S-specific sero-reactivity in one patient (88).

Pre-existing immunity to the adenoviral serotypes might be explanatory for their variable efficacy. This is supported by studies designed to circumvent antibody-mediated neutralization such as the *ex vivo* approach, i.e., infecting DCs and using these as a cellular vaccine. In one such study, advanced melanoma patients received DCs transduced with adenoviral vaccines encoding MART-1 and gp100. While one out of 17 patients experienced a complete response, three developed post-vaccination vitiligo. The latter signifies the generation of antigen-specific immunity that was even able to break tolerance to self-antigens (89, 90). In another phase I/II study, metastatic melanoma patients received three intradermal injections of adenoviral transduced DCs. Vaccination-induced $CD8^+$ and $CD4^+$ T cell responses to MART-1 were found in 6/11 and 2/4 evaluable patients, respectively. Evidence of epitope spreading was obtained in two patients, implying that the elicited T cells showed strong tumor reactivity. Out of the 14 patients receiving all three vaccines, one was considered tumor free, four had durable stable disease, and one remained disease-free after becoming eligible for a surgical resection (91). This positive outcome is not limited to highly immunogenic melanoma. A phase I trial was also performed in NSCLC patients, showing success in individual cases. Patients received multiple vaccines of DCs transduced with p53 encoding adenoviral vectors, 28% of patients demonstrated partial tumor regression or stable disease (92). Recently, a multi-genetically modified DC vaccine was generated based on an adenovirus that delivered two different TAAs (survivin and MUC1), the TLR5 agonist flagellin for DC maturation and a RNA interference moiety to silence the intracellular immune checkpoint molecule SOCS1. This vaccine was found to be safe and induce a complete remission rate of 83% in a phase I trial with 12 acute myeloid leukemia patients (93).

In conclusion adenoviral vaccines are mainly evaluated for *ex vivo* modification of DCs since pre-existing immunity hampers repeated injections *in vivo*. Whether *in situ* targeting of DCs with next-generation adenoviral vectors can lead to tumor regression, remains to be evaluated.

Viral Vectors Derived From Viruses of the *Retroviridae* Family

All members of the *Retroviridae* are characterized by a ssRNA genome that is reverse transcribed into pro-viral DNA in the cytoplasm of the infected host cell. Subsequently this pro-viral DNA is inserted in the host cell genome, leading to permanent gene transfer. This asset makes retroviruses ideal blue prints for development of gene therapy vectors as they permanently modify the target cell of choice (94). Two genera within the *Retroviridae* family are most commonly applied namely the γ-retroviruses and the lentiviruses. While most members of the *Retroviridae* only replicate in dividing cells, lentiviruses uniquely replicate in non-dividing cells. However, lentiviral vectors (LVs) are not very efficient at transducing DCs as the reverse transcription process requires cellular deoxynucleoside triphosphates, which are extremely low in DCs. Interestingly, the addition of the lentiviral accessory protein Vpx to the LV is able to enhance their DC-specific infectivity by countering the low dNTP levels (95, 96). Furthermore LV transduction of DCs does not affect their immunophenotype, viability, or maturation capability while lack of pre-existing immunity allows repeated injections (25, 97).

However, the very first clinical trials performed with a γ-retrovirus-derived vector to successfully treat X-linked severe combined immunodeficiency, resulted in the development of leukemia in four out of nine children due to oncoretrovirus-mediated activation of the *LMO2* oncogene (98, 99). This

unfortunate event created a major setback for the translation of vectors derived from the *Retroviridae* family to clinical applications. Though LVs are derived from a different genus and have a lower propensity for integrating in potentially dangerous regions within the human genome (100), these studies instigated the optimization of safer LV systems with engineered envelopes, pro-viral and/or packaging proteins (101–103). An additional safety feature comprises the mutation of the LV integrase, which impairs pro-viral integration into the host genome. Although this feature reduces the risk of insertional mutagenesis, non-integrative LV expression is less stable because it remains episomal and loses the transgenes after target cell replication, as with adenoviral vectors.

Despite the ample preclinical evidence that LVs represent safe and potent anticancer vaccines (25, 97, 104–107), their clinical use for this purpose remains low. Only in the field of adoptive transfer with *ex vivo* transduced chimeric antigen receptor T cells (CAR-T cells), LVs have taken a prominent place in cancer therapy with about 60 clinical trials registered today. The few active vaccination-related clinical trials involve subcutaneously delivered integrase-deficient LVs encoding NY-ESO-1. In addition, these are directly targeted to DCs *in vivo* through pseudotyping with a modified Sindbis virus envelope protein (DC-SIGN) and are termed LV305 (108). Preclinical murine models showed that the LV305 could be injected more than three times to recall peak-levels of CTLs. Furthermore, biodistribution appeared to be limited to the site of injection and draining lymph node with therapeutic efficacy in tumor bearing mice. Currently LV305 is being evaluated in phase I and II clinical trials for advanced, relapsing or metastatic solid tumors that express NY-ESO-1 such as melanoma, sarcoma, ovarian cancer, and small cell lung cancer. The vaccine is either being used as a single agent or in combination with other cancer drugs. These other drugs include anti-programmed death 1 (PD-1) therapy (pembrolizumab). So far, the first female patient with metastatic and recurrent synovial sarcoma, induced a robust NY-ESO-1-specific T cell response after three injections of LV305 with subsequent disease regression of 85% over 2.5 years (109). Furthermore, intradermal LV305 together with intramuscular delivery of G305 is studied as a combination product termed the CMB305 vaccine regimen for the treatment of sarcoma. G305 comprises a NY-ESO-1 recombinant protein and a TLR4 triggering glucopyranosyl lipid adjuvant stable emulsion (GLA-SE), with potential synergistic immunostimulatory and antineoplastic activities. So far, the vaccine regimen was well tolerated and generated a strong anti-NY-ESO-1 specific immune response in more than 50% of sarcoma patients with significant growth arrest and an overall survival rate (110). In general, CMB305 results in stronger and broader integrated responses than LV305 alone, underpinning the potential of heterologous prime-boost regimens. Finally, a fully enrolled, open-label, randomized phase II study is currently evaluating the safety and efficacy of CMB305 in combination with anti-PD-L1 therapy (atezolizumab) in 88 patients with advanced sarcoma. So far, patients receiving the combination experienced greater clinical benefit, more robust immunity and improved overall survival compared to atezolizumab alone.

Viral Vectors Derived From Viruses of the *Togaviridae* Family

Togaviridae comprises alphaviruses which are small enveloped viruses that transfer a self-replicating ssRNA genome (111). Advantages of alphaviruses for therapeutic vaccination are their high-level expression of encoded proteins due to genomic replication next to lack of pre-existing immunity. Additionally, high-titer virus production is achieved in less than 2 days, be it at a high cost. Their strong preference for expression in neuronal cells has made alphaviruses particularly useful in neurobiological studies (112). In general alphavirus-based vectors are replication-deficient and require a helper vector for packaging of recombinant particles (113). Semliki Forest virus (SFV), Venezuelan Equine Encephalitis (VEE) and Sindbis virus have all been engineered as efficient replication-deficient or -competent vectors. Moreover, variants of the Sindbis virus have been preclinically explored for their differential abilities to target and activate DCs *in vitro* and *in vivo* (114). Importantly, human and mouse DCs were differentially infected by selected variants, suggesting differences in receptor expression between human and murine DCs. Despite these results, only the SFV and VEE have been tested clinically for their potential to engineer DCs *in situ*.

The SFV is an insect alphavirus that is able to infect dividing and non-dividing cells. A replication-incompetent SFV-based vector encoding the HPV derived antigens E6 and E7 has been evaluated preclinically (115, 116). This vector is currently tested in a phase I clinical trial for the treatment of (pre)-malignant cervical lesions (Vvax001). Furthermore, this replication-defective SFV-vector has been evaluated as an IL-12 encoding adjuvant that is encapsulated in cationic liposomes (LSFV-IL-12). This encapsulation approach tends to passively target the LSFV-IL-12 to tumors and enables repeated administration without the generation of antiviral immunity. The safety of administering these SFV-based vectors intravenously was shown in a phase I clinical study in melanoma and renal cell carcinoma patients. In addition, this LSFV-IL-12 has been described in a phase I/II protocol for the treatment of glioblastoma multiforme in which the vaccine will be infused intratumorally (117).

Secondly, virus-like replicons have been generated from an attenuated strain of VEE with potential antineoplastic activity (118–120). This self-amplifying replicon was evaluated in a phase I clinical trial for its safety and efficacy to deliver HER2 and is termed AVX901 (121). More specifically 22 patients with HER2-overexpressing (breast) cancer were evaluated, alone or in combination with other HER2-targeted therapies such as trastuzumab. Importantly, early clinical data did not report any dose-limiting toxicities, supporting the safety of this vaccine. In addition, two trials with the same virus-like replicon, but then encoding CEA termed AVX701, are registered for the treatment of colon and/or colorectal, breast, lung, and pancreatic cancers (122, 123). When the immune responses generated with AVX701 in colorectal cancer patients were compared between stage III and IV patients, the latter showed a trend for longer survival. In contrast, the antibody and T cell response tended to be higher in stage III patients, possibly reflecting a less immunosuppressive milieu in the latter.

The strong cytotoxic effect of alphavirus-based vectors on host cells, holds drawbacks for their use as anticancer vaccine moieties. In contrast, this feature is highly appreciated for oncolytic vectors as reflected in the amount of ongoing studies with oncolytic alphavirus-based vectors (124).

Viral Vectors Derived From Viruses of the *Rhabdoviridae* Family

Rhabdoviridae are enveloped, bullet-shaped (rhabdos refers to rod) virions encapsulating ssRNA. In cancer therapy, this family is mainly known because of its oncolytic virus members derived among others from Vesicular Stomatitis Virus or Maraba virus (125, 126). In the framework of antitumor vaccination, this family is clinically represented by only one vaccine termed YS-ON-001. This is an inactivated rabies vaccine combined with TLR3-stimulating polyI:C for advanced solid malignancies. In 2016 and 2018, this was granted an orphan drug designation by the FDA for the treatment of hepatocellular carcinoma and pancreatic cancer, respectively (127, 128). The vaccine was shown to re-activate the suppressed tumor microenvironment with stimulation of T_H1 cells, DCs, macrophages, B cells, CTLs and NK cells while downregulating Tregs. Currently also a phase I trial for the treatment of liver and breast cancer upon its intramuscular administration is ongoing.

Viral Vectors Derived From Viruses of the *Paramyxoviridae* Family

Paramyxoviridae are represented by measles virus-derived vectors, which are enveloped ssRNA viruses that are mainly tested as oncolytic therapeutics (129). Confusingly, two clinical trials evaluated the therapeutic vaccination potential of oncolytic CEA-encoding vectors derived from the Edmonston measles strain (MV-CEA). Importantly, here CEA was not used as a TAA but to facilitate the *in vivo* monitoring of viral gene expression and replication (130). A first study (NCT00408590) started in 2004 with 37 participants for the treatment of ovarian epithelial cancer or primary peritoneal cancer. Intraperitoneal delivery of MV-CEA was well tolerated and resulted in stable disease for about 66% of patients. In 2006, the NCT00390299 trial was initiated to assess the safety and toxicity of intratumoral administration of MV-CEA for the treatment of recurrent glioblastoma multiforme (131). As this trial was suspended, no results have been disclosed so far.

The general consensus from published (pre-)clinical studies is that virus-based vaccines have the potential to be both safe and efficacious. Nevertheless, to raise the overall survival rates, further fine-tuning and clinical testing are imminent.

PRECLINICAL EVALUATION OF NOVEL VIRAL VACCINES

Viral Vectors Derived From Adeno-Associated Viruses (AAVs)

AAVs are small replication-defective non-enveloped ssDNA parvoviruses. They can only replicate inside the cell in the presence of a helper virus, such as adenovirus. However, AAV genomes can establish latency and persist as episomes in the absence of a helper virus or, in some rare cases, can even integrate into the host genome, particularly in a specific region of chromosome 19 (AAVS1). AAVs are able to infect dividing and non-dividing cells, making them attractive for delivery of transgenes to DCs. Moreover, they sustain long-term gene expression with low immunogenicity. These characteristics and their good safety profile make them appealing candidates for immunotherapy.

When an AAV vector containing the HPV16 E7 gene was used to infect mouse DCs, efficient gene transfer and DC activation was observed with upregulation of CD80 and CD83 next to T cell stimulation (132). Similarly, AAVs have been used to infect human DCs with HPV16 E7 (133), cytomegalovirus antigens (134), PSA (135), Her2/neu (136), or lactadherin, a membrane-associated self-glycoprotein that is expressed in breast cancer cells (137). Analogous to the observations with mouse DCs, efficient activation and priming of antigen-specific CTLs upon infection was observed. Furthermore, when an AAV-derived vector encoding HPV16 L1 protein, was used to immunize BALB/c mice intramuscularly, strong antibody titers were observed next to accumulation of APCs such as macrophages and DCs. In addition, the added benefit of co-vaccination with an adenovirus encoding murine GM-CSF was shown (138). Also the addition of a minimal CD11c promotor in the AAV expression cassette improved the infected DCs' ability to stimulate CTLs (139).

Even though AAVs are less immunogenic than adenoviral vectors, antibody neutralization due to previous exposure of the patient to multiple AAV serotypes, remains a common limitation for successful gene therapy and repeated vaccination (43, 140). Numerous AAV serotypes have been identified so far, with variable tropism depending on their route of administration (141). Therefore, an obvious approach to overcome neutralizing antibodies a specific AAV serotype is the use of a different serotype or naturally occurring AAV variant (142). To further enhance the outcome of AAV immunization, a rational design of its capsid can be performed by site-directed mutagenesis of surface-exposed serine and threonine residues. As such, a capsid-optimized AAV (serotype 6) showed a 5-fold increase in its transduction efficiency of bone-marrow derived DCs. In addition its intramuscular injection in prostate tumor bearing mice, resulted in PAP-specific CTL induction and tumor growth suppression (143). While these studies set the stage for clinical applications with capsid-optimized AAVs, the only clinical studies employing AAVs so far aim to use *ex vivo* AAV-modified DCs to expand CEA-specific CTLs present in blood of patients with grade IV gastric cancer and use these T cells for adoptive transfer (NCT01637805).

Viral Vectors Derived From Coronavirus

The enveloped coronaviral vectors carry a 31 kb autonomously replicating ssRNA genome and offer the advantage of being safe, since they do not create a DNA intermediate upon infection. Furthermore, they are able to exploit a diverse range of surface molecules to infect target cells. Some of them recognize the DC-specific C-type lectin DC-SIGN, which

endows them with the ability to target DCs *in vitro* and *in vivo* (144). The group of Volker Thiel evidenced this with a biosafe coronavirus-based vector encoding human Melan-A with or without GM-CSF. In addition they reported that a single intravenous immunization with only 10^5 PFU, resulted in a prophylactic and therapeutic immune response against metastatic melanoma (145). Furthermore, they also showed that human DCs, transduced with Melan-A-recombinant human coronavirus 229E, efficiently activated tumor-specific CTLs. That same group also demonstrated that vectors encoding Flt3L, exhibited a higher capacity to induce DC maturation compared to vectors delivering IL-2 or IL-15. The former more efficiently induced tumor-specific CTLs with expanded epitope repertoire, resulting in therapeutic tumor immunity (146).

The natural DC tropism combined with relative low doses needed, hold high potential for future clinical evaluation. However, as the *Coronoviridae* are believed to cause a significant amount of common colds in human adults, the risk of vaccination-limiting pre-existing immunity issues will need to be investigated.

Viral Vectors Derived From Papillomavirus

Papillomaviruses are small non-enveloped, circular dsDNA viruses. As widely accepted, chronic infection with certain HPV genotypes forms a major etiological factor for cervical cancer. For prophylactic vaccination, the HPV-derived capsid proteins L1 and L2 embedded in virus-like particles are profoundly exploited (147). For therapeutic vaccination, the oncogenic E6 and E7 antigens represent ideal targets because they are essential to the induction and maintenance of cellular transformation. Today several therapeutic vaccines for the treatment of HPV^+ cervical malignancies are being investigated (148). However, when a prime/boost with an adenovirus type 5 vector was performed to a cervicovaginal model antigen, the high systemic $CD8^+$ T cell response failed to induce intraepithelial $CD103^+$ CTLs, necessary for protection against local challenge (149). These observations suggest that the epithelial tropism of HPV itself endows them with an interesting feature for their use as therapeutic vaccines. A major advantage of HPV as a viral vector system (HPV pseudovectors), is its capacity to package plasmids up to 8 kb in length, completely devoid of viral sequences (150). Upon an HPV intravaginal prime/boost with different HPV serotypes, a durable cervicovaginal antigen-specific CTL response was induced by promoting local proliferation and retention of primed CTLs (149).

Viral Vectors Derived From *Baculoviridae*

The enveloped family of *Baculoviridae* has been preclinically evaluated to develop anticancer vaccines. This family forms an exception in the sense that they normally infect insects at larval stage. Hence since the 1940s, they have proven to be useful biopesticides in the field of agriculture (151). Furthermore, baculovirus-mediated expression of recombinant heterologous proteins in cultured insect and mammalian cells also represents a widely used and robust protein production method (152). Vaccination with the tumor-specific immunoglobin Id is considered a valuable approach for the treatment of lymphoma patients. Methods to improve its immunogenicity have been explored, leading to Id production via baculovirus-infected cells. Due to the addition of terminal mannose residues, typical for recombinant proteins expressed by insect cells, the Id proteins had enhanced immunostimulatory properties. Moreover, these Ids showed higher binding and activation capacity for human DCs next to higher elicitation of tumor-specific CTLs and eradication of pre-established murine lymphoma (153).

More recently, baculoviruses have been considered useful in gene therapy as well, as they (1) infect though not replicate in mammalian cells, (2) show low cytotoxicity, and (3) are able to carry large foreign genes into their 80–140 kb spanning genome (154). Baculovirus was shown to efficiently transduce and activate DCs *ex vivo* with upregulation of co-stimulatory molecules, MHC, type I IFN and other pro-inflammatory cytokines (155). Moreover, these DCs generated robust antitumor immunity in tumor bearing mice (154). Intradermal injection of wild type baculovirus (adjuvants) together with tumor cell lysates has also shown antitumor efficacy in several murine cancer models (156). Finally, a CEA-specific $CD4^+$ T cell response was observed upon intramuscular injection of a CEA encoding baculovirus-derived vector (157).

Although there is no reported pre-existing anti-baculovirus immunity, these vectors could be highly immunogenic and as such rapidly inactivated by human serum complement upon systemic delivery (152, 158). Further preclinical studies are warranted though, their DC-transducing capacity, large gene insert capacity and biosafety profile represent promising features for future development of potent anticancer vaccines.

CONCLUSIONS AND FUTURE DIRECTIONS

While TAA-specific CTL responses are frequently induced upon vaccination with TAA-encoding viral vectors, most responses poorly translate into prolonged survival benefit for cancer patients (159, 160). The lack of overall clinical efficacy can be assigned to: (1) the fact that most patients received immunosuppressive (chemo)therapeutic regimens prior to vaccination, (2) pre-existing or induced vector-neutralizing antibodies, (3) lack of eligible TAAs, and (4) established tolerance to the TAA and linked herewith presence of a CTL suppressing tumor microenvironment.

The immunosuppressed status of heavily pretreated patients, as well as the immunosuppressive status of the tumor microenvironment, argues for the exploration of viral vaccines in earlier disease stages with less tumor burden. As the first virus-based vaccines have been approved by the FDA, their evaluation as early line treatments instead of last line becoming more likely. The immunogenicity of *in situ* administered viral vectors acts as a double-edged sword. The activation of DCs by viral vectors through recognition of pathogen-associated molecular patterns by pattern recognition receptors, such as TLRs, obviates the need for adjuvant (161, 162). Moreover, type I IFN-driven antiviral immunity is characterized by a T_H1 response. Therefore, strong CTL responses are generated against TAAs that are delivered

by viral vectors, as these are sensed as viral antigens. However, this immunogenicity entails that immunity is also build against viral components. This antiviral immunity precludes repeated injection of the viral vaccine, hampers prolonged transgene expression, neutralizes the vaccine and hinders the strength of TAA-specific cellular immunity (163, 164). Importantly, most of the clinically evaluated vectors like pox- and adenoviral vectors, show pre-existing immune responses in the host (165). A careful review of the literature on the topic of pre-existing immunity to viral vectors, suggests that this is indeed a hindrance. How pre-existing immunity impacts on the viral vaccine efficacy depends on the natural immunity to the vector. In essence all viral infections can elicit robust B and T cell memory responses (166), which can reduce antigen delivery by the viral vector due to neutralizing antibodies (167). Moreover, the pre-existing antiviral response will lead to rapid vector clearance and as such reduce exposure of the heterologous antigen (TAA) to the immune system. Finally, the immune response could focus on the strong viral antigens and "ignore" the co-expressed TAAs via the process of "epitope dominance." Importantly, several approaches have been applied to avoid the downsides of pre-existing vector immunity, such as the use of vectors derived from non-human sources or from rare serotypes (83, 168). An alternative approach is provided by the "prime–boost" regimen in which two different recombinant viral vaccines expressing the same TAA are used consecutively (169). What's more, one can also alter the viral surface epitopes (envelope or capsid proteins) that might elicit neutralizing antibodies (170, 171). The inhibitory effect of pre-existing immunity can also be avoided by masking the viral vector inside DCs as discussed in the section on adenoviral-based vaccines (172). Besides, mucosal or high dose vaccination have also been shown to overcome pre-existing immunity problems (164, 173–175). A recent study showed that COX2 inhibitors, such as Celecoxib, can prevent the generation of neutralizing antibodies to vaccinia, allowing repeated administration without losing infectivity (176). Pre-existing immunity is however not an issue for all virus-based vaccines. For instance, the majority of the population has never been in contact with lentiviruses, making their vector derivatives attractive candidates for further vaccine development. Therefore, it may not be a surprise that the only lentiviral vaccine (LV305) that has been clinically evaluated in a handful of trials, all showed improved and durable responses in sarcoma patients (109, 110).

It should be noted that the route of administration profoundly affects the biodistribution of viral vectors, which can in turn influence their therapy efficacy and toxicity profile (43). While for example intravenous injection of AAVs via the tail vein triggers a $CD4^+$ T cell-dependent humoral response, its delivery via the portal circulation leads to a T cell-independent B cell response (177). Importantly, while tissue-specific delivery can be an issue for naked protein or nucleic acid-based vaccines, viral vectors often hold a natural tropism for specific cells or tissues. As such, virus-based vaccines are excellent vehicles for tissue-specific delivery of transgenes together with its intrinsic immunogenicity. For example, adenoviral vectors are scavenged by the reticuloendothelial system after systemic injection, especially by Kupffer cells in the liver. However, upon intranasal administration of an IL-12 encoding adenoviral vector, pulmonary metastasis in a murine model of osteosarcoma could be treated without putative risks (178). As discussed, the epithelial tropism of the HPV-derived vectors themselves could endow them with the most optimal features for prophylactic and therapeutic HPV-related cancer vaccination. Additionally, some viral vectors have been extensively re-engineered in order the alter their tropism or transgene expression, as extensively discussed elsewhere (24). Targeting viral vectors to DCs has been explored as a means to tighten the control on where the viral vector is delivered to enhance the safety and efficacy. An approach that has been adapted to both lentiviral and adenoviral vectors is the use of single domain antibodies or so-called nanobodies that specifically bind APCs, albeit DCs or both DCs and macrophages (102, 179). Although it was expected that such

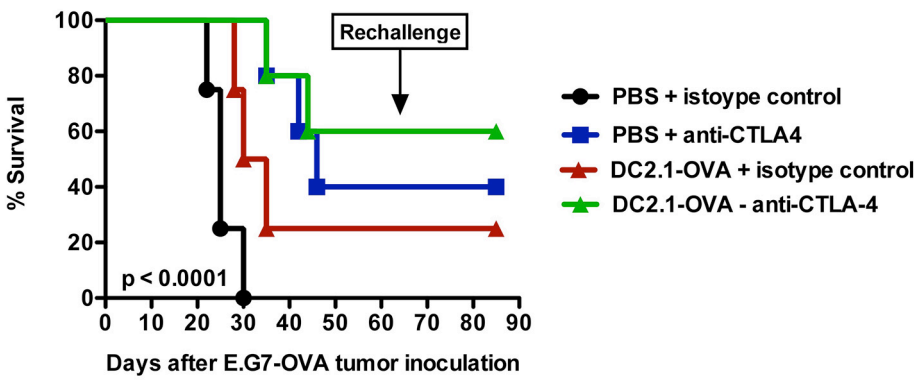


FIGURE 2 | Intranodal vaccination of DC-targeted LVs in combination with anti-CTLA4 results in prolonged survival. To evaluate the therapeutic potential of DC-targeted LVs in combination with anti-CTLA4, C57BL/6 mice were challenged on day 0 with 3×10^5 cells of an ovalbumin positive EL4 lymphoma line termed E.G7-OVA. Ten days later, mice were intranodally immunized with PBS or 10^6 transducing units of single chain antibody or nanobody (Nb) DC2.1 pseudotyped LVs encoding OVA. Seven days later, the treatment was repeated. Furthermore, mice were treated on days 13 and 20 intraperitoneally with 50 μg isotype control or anti-CTLA4 antibody. Tumor growth and survival were examined every 2 days. The results shown are representative for one experiment with five mice per group.

an approach would enhance the vaccine efficacy, by avoiding presentation by non-professional APCs, this strategy did not deliver on its promise (180). This is in part explained by an enhanced anti-viral type I IFN response next to the lack of stromal cell transduction with reduced MHC-I mediated antigen presentation (181).

The ever-growing field of cancer antigen target identification should lead to a knowledge platform that can develop complete tumor eradicating vaccines. So far however, large clinical trials did not meet the expectations. This is most likely explained by the very inconsistent expression pattern of TAAs within the heterogenous tumor mass as well as their (vaccine-induced) tumor evasion over time (182, 183). The concept of neo-antigens harboring high-affinity T cell recognizable and tumor-unique epitopes, will become indispensable for the next generation antitumor viral vaccines. So far, mainly oncolytic viral systems have been linked to modulate the spectrum of neo-antigen specific CTLs with subsequent abrogation of systemic resistance to checkpoint inhibitor immunotherapy (184). Furthermore, both adenoviral and MVA vectors have been tested as neo-antigen encoding vaccines in the framework of human immunodeficiency virus related disease. More specifically, a genetic algorithm-based mosaic method was developed to generate artificial protein sequences that could increase the cross-reactivity of vaccine responses for diverse HIV-1 isolates. When these "mosaic" HIV sequences were delivered via adenovirus or MVA, this resulted in a strong protective effect against subsequent infection in non-human primates (185). These findings are encouraging for the development of cancer neo-antigen encoding viral vectors for the treatment of cancer.

Tumor-derived DCs are most often dysfunctional. As such they are less mature with low sensitivity to TLR activation, which is associated to STAT3 hyperactivity. Ideally, a vaccine should therefore consist of TAAs together with adjuvants to overcome the DCs' anergic state. While in the field of nanovaccines, several combinations have been explored (186), the delivery of more than one antigen/adjuvant/genetic silencer (e.g., small interfering RNA against STAT3) (187) is exactly what viral vectors could do. Especially viral vectors with a large genetic insert capacity such as poxvirus or baculovirus could be used for this purpose. Furthermore, viral vectors could also be used to target the delivery of proteins to cells of interest a.k.a. protein transfer vector or PTVs (188). Therefore, research into strategies to exploit the advantageous traits of viruses (e.g., high infectivity, adjuvant potential), while avoiding their traits developed to avoid immune responses (e.g., decreasing the translational machinery) should be continued.

Finally, it also makes sense to combine DC-targeted vaccines, purposed to elicit antitumor T cell responses, with strategies designed to support the function of T cells in the tumor microenvironment (148). In this regard immune checkpoint inhibitors might be ideal candidates. These drugs are able to release the brakes on T cells imposed by inhibitory receptors, such as CTLA-4 and PD-1. This is nicely exemplified by the combination of an adenoviral vector, encoding the murine breast TAA TWIST1, with intraperitoneal injection of a bifunctional anti-PD-L1/TGFβ fusion protein. This combination was shown to induce a more active CTL and NK cell phenotype within the tumor microenvironment (189). Previously, we performed a therapy experiment with the ovalbumin (OVA) expressing EL-4 thymoma model (E.G7-OVA) by combining a DC-targeted LV encoding OVA with anti-CTLA-4 treatment. This led to prolonged overall survival compared to the injection of LVs or anti-CTLA-4 antibodies alone (**Figure 2**). Moreover, this resulted in protection against a subsequent challenge with a lethal dose of E.G7-OVA cells, suggesting that DC-targeted LVs can be promising immunotherapeutics if combined with a T cell suppression counteracting strategy.

Nature has fine-tuned viruses to highly efficient gene transmitters in a cell-specific fashion with intrinsic adjuvant-like features. Hence an abundant range of viral vectors has been explored and tweaked substantially to develop anticancer vaccines with specific features. As a result we believe it will not be a matter of finding the "one-fits-all" vector but the "most appropriate combination" for the cancer type and stage at issue.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Radiation and Local Anti-CD40 Generate an Effective *in situ* Vaccine in Preclinical Models of Pancreatic Cancer

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Radiation therapy induces immunogenic cell death, which can theoretically stimulate T cell priming and induction of tumor-specific memory T cell responses, serving as an *in situ* vaccine. In practice, this abscopal effect is rarely observed. We use two mouse models of pancreatic cancer to show that a single dose of stereotactic body radiation therapy (SBRT) synergizes with intratumoral injection of agonistic anti-CD40, resulting in regression of non-treated contralateral tumors and formation of long-term immunologic memory. Long-term survival was not observed when mice received multiple fractions of SBRT, or when TGFβ blockade was combined with SBRT. SBRT and anti-CD40 was so effective at augmenting T cell priming, that memory CD8 T cell responses to both tumor and self-antigens were induced, resulting in vitiligo in long-term survivors.

Keywords: radiotherapy, abscopal effect, immunotherapy, pancreatic cancer, CD40, vitiligo

INTRODUCTION

Successful generation of an anti-tumor CD8 T cell response involves multiple steps. First, local dendritic cells, laden with antigens from dying tumor cells, become activated and migrate to the draining lymph node (1). There, activated dendritic cells interact with naïve T cells which become primed, proliferate, and acquire effector capabilities. These activated effector T cells then traffic to the tumor, and ideally are able to kill tumor cells via direct cytolysis or production of interferon (IFN)γ. Immunosuppressive myeloid cells in the tumor microenvironment, as well as nutrient starvation and expression of inhibitory ligands such as PD-L1, may prevent CD8 T cell-mediated killing even when CD8 T cell priming has occurred. The fact that immune checkpoint blockade has single-agent efficacy in some cancer patients indicates that CD8 T cell priming successfully occurs in a significant fraction of humans with cancer (2, 3).

However, anti-tumor CD8 T cells are not found in all patients, and therapeutic cancer vaccines have been developed to induce T cell priming *de novo* (4–6). Systemic vaccines require knowledge of the antigens of interest, or at a minimum, cumbersome preparation of tumor cell lysates. Perhaps the simplest and most effective vaccination strategies involve direct delivery of immune stimulatory agents to the tumor microenvironment (7). These so-called "*in situ*" vaccines operate under the idea

that induction of tumor cell death releases tumor antigens, which are phagocytosed and presented by local dendritic cells that become activated and prime naïve T cells in the draining lymph node (1). Successful *in situ* vaccines require both a means of tumor cell death and a source of adjuvant to activate local dendritic cells. Oncolytic viruses serve both functions, and local injection of TVEC is approved for metastatic melanoma patients (8, 9). Local delivery of adjuvants such as STING agonists or TLR ligands have been proposed, although these agents do not induce cell death on their own, and may be more efficacious when combined with radiation or with certain chemotherapies or targeted therapies (7, 10–13).

Radiation has long been used to treat cancer patients, usually for local control or palliation (14). In rare cases, regression of lesions outside the field of radiation have been observed (14, 15). This so-called abscopal effect is due to induction of adaptive immunity and recognition of tumor antigens at distant sites by effector CD8 T cells. Although many agents that induce cell death may be predicted to synergize with immunotherapy, radiation may be particularly good at inducing T cell priming. Radiation has pleiotropic effects on the tumor microenvironment, including induction of MHC expression on tumor cells and upregulation of costimulatory ligands on dendritic cells (16, 17). Indeed, several studies have shown that radiation broadens the oligoclonality of the T cell response, presumably by inducing T cell responses against a wider array of tumor antigens (18, 19). At the same time, radiation induces production of myeloid cell attracting chemokines such as CCL2 that can establish an immunosuppressive microenvironment (20). Combination of radiation and immune stimulating adjuvants is therefore critical.

CD40 is a TNF family member expressed on dendritic cells, macrophages and B cells. When engaged by CD40L or by an agonistic antibody, CD40 signaling leads to NF-κB upregulation and expression of costimulatory ligands, production of IL-12 and other cytokines, enhanced antigen presentation, and in the case of dendritic cells, upregulation of CCR7, and trafficking to the draining lymph node. Agonistic antibodies to CD40 have been successful in generating limited responses in both mice and humans with pancreatic tumors, in some cases via enhanced T cell priming, and in other cases through activation of myeloid cells (21–24). In mouse models of pancreatic ductal adenocarcinoma, SBRT was shown to transiently deplete CD8 T cells, increase MHC class I expression on tumor cells and be synergistic with checkpoint blockade (18, 25). SBRT combined with systemically delivered anti-CD40, anti-PD1, and anti-CTLA4 led to durable remissions of the majority of subcutaneous tumors, in a manner that was dependent on endogenously primed T cells and IFNγ (25), although the dual combination of SBRT and anti-CD40 was not evaluated. In pancreatic neuroendocrine tumors, radiation, and agonistic anti-CD40 together were insufficient to induce T cell priming, although these two agents served as preconditioning regimens for successful adoptive T cell therapy (26).

Pancreatic tumors are notoriously refractory to therapy, including immunotherapy (27). Adjuvants that stimulate dendritic cell activation and T cell priming in other cancer types may have tumor promoting effects in pancreatic cancer. Pancreatic tumor cells constitutively express TLR7, secrete myeloid cell recruitment and maturation factors such as GM-CSF, and have chronic STING pathway activation due to chromothryptic events and the formation of micronuclei (28–32). TGFβ blockade is effective at inducing CD8 T cell influx (33, 34), and synergizes with radiation in other tumor types (35, 36); however whether blockade of TGFβ signaling in pancreatic tumors would synergize with radiation is unclear given that pancreatic cancer cells rely on TGFβ signaling to maintain radiosensitivity (37).

Here we defined the effects of radiotherapy on anti-tumor immunity in two mouse models of pancreatic cancer. A single moderate dose of stereotactic body radiotherapy (SBRT), along with intratumoral injection of agonistic anti-CD40 induced complete regressions in both treated and non-treated lesions. Tumor regression was associated with decreased myeloid populations and increased percentages of CD8 T cells. Cured mice were refractory to rechallenge, indicating successful generation of immunologic memory. CD8 T cell priming was robustly induced, with mice generating not only anti-tumor T cells, but also auto-reactive T cells capable of inducing vitiligo.

RESULTS

Single but Not Multiple Dose SBRT Combined With Intratumoral Anti-CD40 Leads to Regression of Contralateral Panc02 Pancreatic Tumors

We used image guidance to deliver precise doses of SBRT to defined areas in mice using a small animal radiation research platform (SARRP) (**Figures 1A–C**). Mice bearing subcutaneous Panc02 tumors on each flank were treated unilaterally with 5×2 Gy, 6×5Gy, or 3×10 Gy. Pancreatic tumors are relatively resistant to radiotherapy, and both irradiated and non-irradiated lesions grew progressively (**Figure 1D** and **Supplemental Figure 1**). Addition of intratumoral anti-CD40 administered concurrently with the first and last fractions of SBRT improved local control of treated tumors at the 10Gy dose, but did not induce regression of contralateral tumors (**Figure 1E**).

Radiation damages not only tumor cells, but also immune cells that may be present. In the case of radioresistant pancreatic tumors, additional fractions of radiation have little impact on the overall tumor burden. Previous reports of fractionated radiation combined with immunotherapy used checkpoint blockade immunotherapies, which act on T cells that infiltrate tumors a week or more after treatment and are thus temporally protected from the damaging effects of radiation. We hypothesized that multiple fractions of SBRT delivered over several days may be detrimental to local dendritic cells which are required for crosspresentation of tumor antigens to naïve CD8 T cells and are likely the cellular targets of anti-CD40 (38). To address this issue, single dose SBRT of 5Gy with or without intratumoral anti-CD40 was administered to mice bearing Panc02 tumors. Therapy was initiated 2 weeks post-implantation, at a time

when all tumors were palpable (~25 mm^3). SBRT and anti-CD40 administration alone each provided some local control of the treated tumor, but complete regressions of the contralateral tumors were only observed in mice receiving combination SBRT and anti-CD40 (**Figures 2A–C**). Mice were followed long-term, and overall survival was 80% in the combination group vs. zero in control or single agent treated mice (**Figure 2D**). We therefore used single dose SBRT in all subsequent experiments.

Combination Therapy Induces CD8 T Cell Infiltration in Panc02 Tumors

Although CD8 T cells can mediate tumor rejection, they are largely excluded from pancreatic tumors at baseline due, at least in part, to immunosuppressive macrophages (39). Two weeks following therapy, we examined CD8 T cell infiltrates in treated and contralateral Panc02 tumors by histology (**Figures 3A,B**) and by flow cytometry (**Figures 3C,D**). Consistent with previous reports, CD8 T cells were infrequent in the interior of control tumors (39). Radiation led to an increase in intratumoral CD8 T cells in both RT and combination treated mice at 3 weeks post therapy. Flow cytometry revealed a decrease in granulocytic ($Gr1^{high}$, $CD11b^+$) and monocytic ($Ly6C^+CD11b^+$) myeloid suppressor cells in response to anti-CD40, resulting in an increased CD8 to CD11b ratio that was most striking in the combination treated group. Increased CD8 T cell infiltration was observed in both treated and contralateral tumors, suggesting that CD8 T cells primed against tumor antigens from one tumor were capable of accumulating in non-treated tumors expressing similar antigens.

Combination SBRT With Intratumoral Anti-CD40, but Not TGFβ Blockade, Leads to Regression of Contralateral KPC Pancreatic Tumors, and Formation of Immunologic Memory

The Panc02 cell line is notable for a high mutational burden and increased susceptibility to CD8 T cell responses. To better model pancreatic tumors with lower endogenous CD8 T cell responses, we used a cell line derived from the *LSL-Kras;p53+/floxed,Pdx-cre* mouse (KPC). These tumors grow similarly in both immunodeficient and immune competent mice, and are resistant to T cell augmenting therapies (40). We tested a similar regimen of single dose SBRT (10Gy) with or without intratumoral anti-CD40 in mice bearing palpable KPC pancreatic tumors on each flank, and again observed significant regression of non-treated tumors and increased overall survival in combination treated mice (**Figures 4A–C**).

TGFβ has been reported to synergize with radiation therapy in mouse models of breast cancer (36, 41). Furthermore, TGFβ has been shown to restrict CD8 T cells to the periphery of tumors (33), and TGFβ production in pancreatic cancer leads to increased fibroblast activation and stromal deposition, both of which are likely tumor promoting (27). We therefore administered systemic blocking antibodies to TGFβ in combination with SBRT with or without anti-CD40. Contrary to expectations, TGFβ blockade had no effect when combined with SBRT, and triple combination of SBRT, anti-CD40, and TGFβ blockade resulted in regression of the treated tumor, but complete loss of efficacy at the contralateral lesion (**Figures 4D–F**).

Intratumoral anti-CD40 was more effective in the KPC as compared to the Panc02 model, and long term survivors were observed in both anti-CD40 single agent and in the combination treated groups. To determine whether tumor regression was associated with induction of immunologic memory, surviving mice were rechallenged with a higher dose of KPC cells (4×10^5). All mice rejected rechallenge in the absence of further treatment, indicative of immunologic memory (**Figure 5A**). To determine whether T cells were required for the immunologic memory observed, cured mice that survived rechallenge were depleted of CD4 and CD8 T cells and again rechallenged with a two-fold dose of KPC cells. Although all of these mice had demonstrable immunologic memory, T cell depletion allowed for outgrowth of KPC tumors in all cases (**Figure 5B**). Memory T cells generated in combination treated mice are superior to mice treated with single agent alone. We collected CD4 and CD8 T cells from mice 12 days after therapy and transferred these into naïve recipient hosts. We then challenged the new hosts with KPC tumors and found that only mice receiving T cells from combination treated donors were protected from tumor growth (**Figure 5C**). Thus we confirm that memory T cells capable of preventing tumor recurrence are generated with combination of SBRT and intratumoral anti-CD40.

Mice that had been treated with combination SBRT anti-CD40 also developed vitiligo at the site of rechallenge (**Figure 5D**). Vitiligo was not observed in mice that received radiation only and were monitored for 8 weeks following SBRT, suggesting that radiation-induced tissue damage was not responsible for depigmentation. Immunohistochemistry of affected skin revealed CD8 T cells residing in the hair follicles (**Figure 5E** and **Supplemental Figure 2**). Vitiligo responses have been reported previously in both mice and humans with melanoma treated with checkpoint blockade (42, 43), usually explained by T cells primed against self antigens shared between melanoma and melanocytes (44). In this case, we postulate that SBRT may be inducing death of surrounding normal tissues, and antigens from dying melanocytes may be acquired by dendritic cells. Antigen presentation is enhanced by anti-CD40, suggesting a means for development of autoreactive CD8 T cells, and ensuing destruction of healthy melanocytes by memory CD8 T cells recalled to the site of tumor rechallenge. Encouragingly, these autoreactive responses were restricted to melanocytes, as the skin epithelial cells and other normal tissues of the mouse were unaffected.

DISCUSSION

Radiation therapy is a promising adjunct to immunotherapy as it is widely used clinically and generates a source of immunogenic cell death. However, radiation treatment alone rarely generates productive CD8 T cell responses capable of clearing distant lesions. Case reports of abscopal effects induced

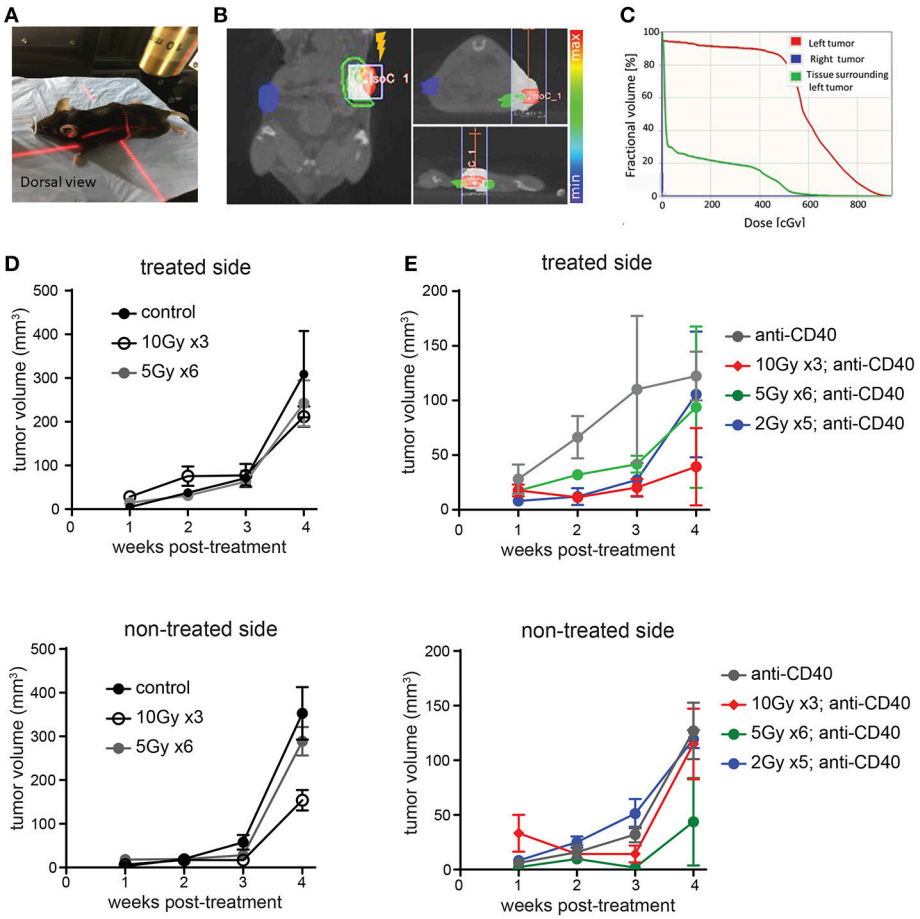

FIGURE 1 | Multiple fractions of image guided SBRT delivers radiation precisely to pancreatic tumors, but fails to achieve an abscopal effect. **(A)** A Small Animal Radiation Research Platform (SARRP) was used for RT. Image guided RT was given to the left tumor only. **(B,C)** Dosimetry showing the CT view of a mouse during image guided RT and dosimetry showing RT distribution in treated and contralateral tumors, as well as surrounding normal tissue. **(D)** C57BL/6 mice were inoculated with Panc02 tumors on each flank. Once tumors were palpable, mice were treated on one flank with no SBRT, 10Gy on three consecutive days, or 5Gy on 6 consecutive days. Tumor growth on each side was measured. $n = 5$ mice/group. **(E)** Mice were treated as in **(D)**, except that anti-CD40 was administered (10 μg, intratumoral) with the first and last dose of SBRT, or two injections 5 days apart in mice receiving no SBRT. $n = 5$ mice/group. Error bars are SD.

in a few patients receiving checkpoint blockade prompted much excitement among clinicians (15, 18), although attempts to use SBRT to rescue patients who had failed ipilimumab (anti-CTLA-4) were less successful than might be hoped (16, 45). The sequence of radiation and immunotherapy, the SBRT dose and fractionation schedule, and which particular immunotherapy agent(s) are used likely make an enormous difference in the clinical outcome (46, 47). Indeed, we showed that multiple fractions of SBRT distributed over a week long period were far less effective in our Panc02 model in combination with anti-CD40 than a single SBRT dose. Other groups similarly reported a heavy reliance on timing and dose fractionation in mice, and clinical trials designed specifically with one or a few high doses of SBRT in combination with immunotherapy are now underway (48).

Currently approved checkpoint blockade therapies sustain productive T cell responses and can prevent or reverse T cell exhaustion. While certainly an important component of combination immunotherapy, checkpoint blockade does little to enhance dendritic cell activation, and T cell priming. To this end, local administration of adjuvants is most effective, and efforts to study combination of adjuvants with radiation have met with some success across a range of tumor types. Notably, STING agonists, TGFβ blockade, anti-CD40, checkpoint blockade, and TLR 7/8 ligands have been reported to synergize with radiation therapy in mice (12, 19, 35, 36, 49–51). We caution that the tumor microenvironments are different across different tumor types, and that agents used in one setting may not be amenable in another. TGFβ blockade, for example, although strikingly effective in combination with radiation in breast cancer (36, 41), had negligible effect in our KPC pancreatic tumor model, and in fact, reversed the efficacy of anti-CD40. We did observe improved local control of the treated tumors with anti-CD40, SBRT, and anti-TGFβ, with all mice fully clearing their tumors. Blockade of TGFβ signaling in pancreatic stellate cells promotes radiosensitivity (52), potentially rendering tumor cells better

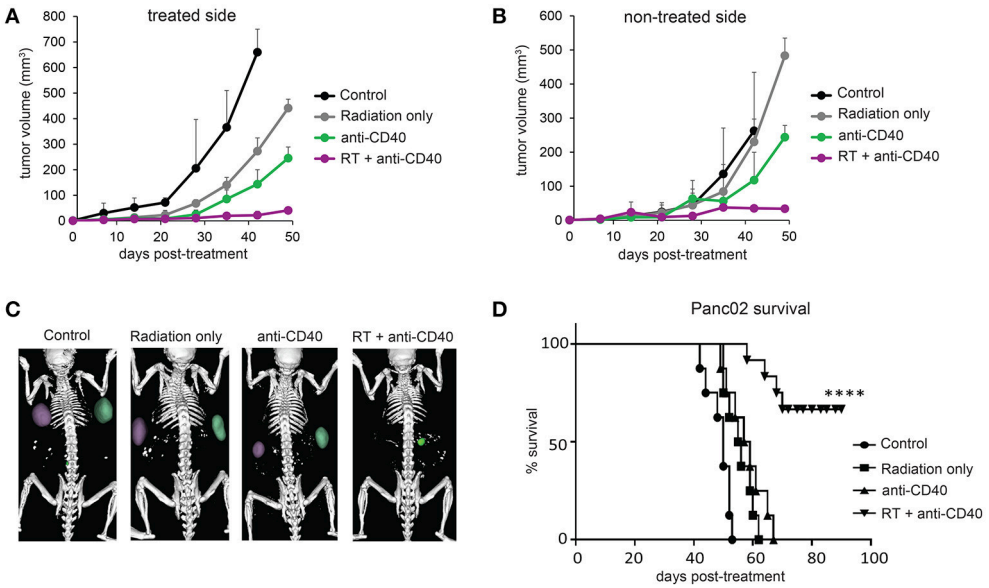

FIGURE 2 | Single dose 5Gy SBRT combined with anti-CD40 induces regression of contralateral Panc02 tumors. C57BL/6 mice were inoculated with Panc02 tumors on each flank. Once tumors reached palpable size, the right flank was treated with RT and/or a single dose of anti-CD40 (20 μg) as indicated. **(A)** Volumes of treated tumors over time, measured by CT. **(B)** Volumes of contralateral tumors over time, measured by CT. **(C)** Representative CT imaging of mice at 3 weeks post-treatment. **(D)** Overall survival. $n = 8$/group. $^{****}p<0.0001$.

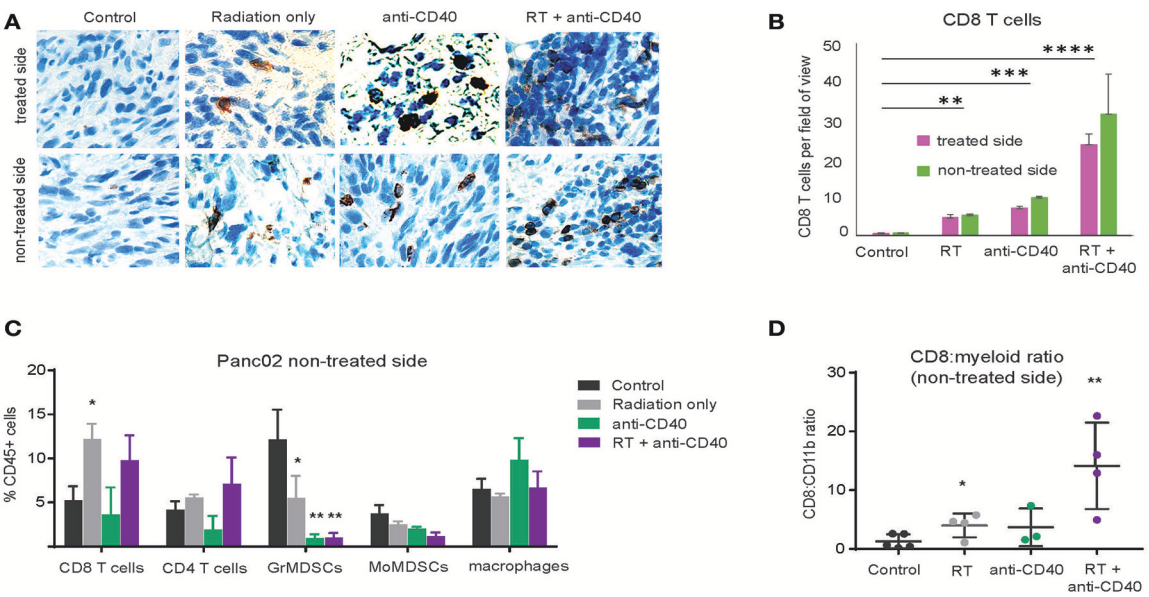

FIGURE 3 | Combination RT and anti-CD40 leads to increased intratumoral CD8 T cells. **(A)** Tumors from mice treated with 5Gy SBRT and/or a single dose of anti-CD40 (20 μg) were harvested at 2 weeks post-treatment and analyzed by immunohistochemistry for CD8. **(B)** Quantification of a. $n = 5$ mice/group. **(C)** Tumors from mice treated as in **Figure 2** were harvested at 2 weeks post-treatment, digested and analyzed by flow cytometry. GrMDSCs: $CD11b^{+}Gr1^{high}$; MoMDSCs: $CD11b^{+}Ly6C^{+}$; macrophages: $CD11b^{+}$,$Gr1^{-}$. **(D)** Ratio of CD8 T cells to total $CD11b^{+}$ myeloid cells. $n = 5$ mice/group. Error bars are SD. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

able to be cleared by CD40-activated local macrophages. TGFb signaling also promotes fibroblast deposition of extracellular matrix, and interrupting this pathway is likely to be more effective in combination with locally delivered therapies (53, 54). However, these striking local effects did not translate to improved systemic immunity, since adding anti-TGFβ to combination SBRT and anti-CD40 resulted in progressive outgrowth of non-treated tumors. We selected anti-CD40 as a rational choice

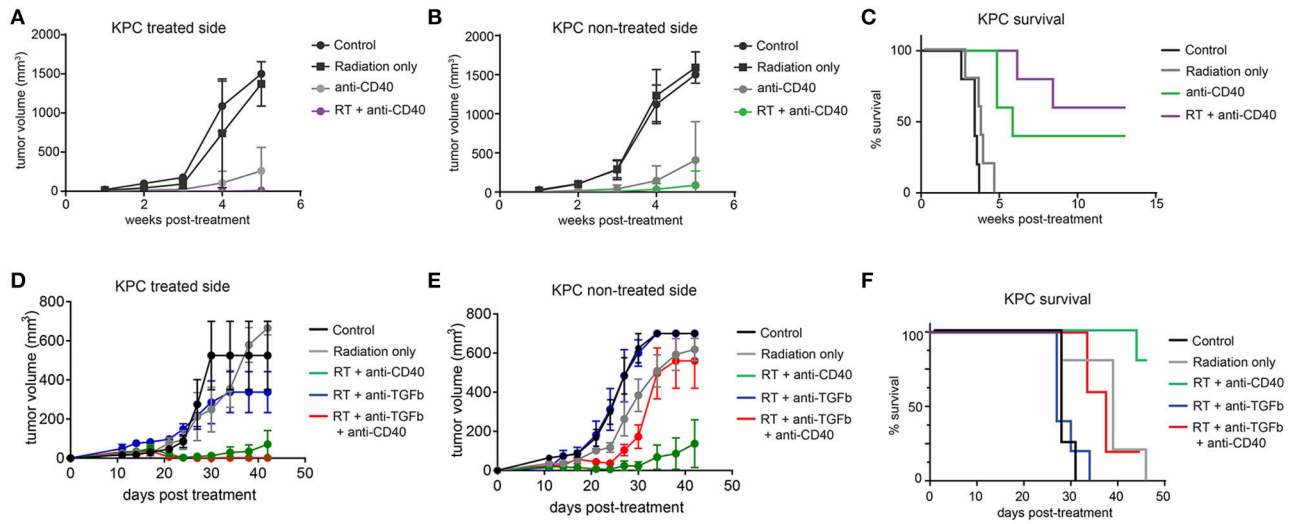

FIGURE 4 | Combination 10Gy RT and anti-CD40 induces regression of contralateral KPC tumors, but TGFβ blockade counteracts the abscopal effect of anti-CD40. C57BL/6 mice were inoculated on each flank with 150,000 KPC cells. Once tumors reached palpable size (11–14 days post-implantation), mice were treated with 10Gy SBRT, anti-CD40 (20 μg once, intratumoral), both RT and anti-CD40, or PBS control. **(A)** Volume of treated side tumors over time. **(B)** Volume of contralateral tumors over time. **(C)** Overall survival. **(D–F)** C57BL/6 mice were treated as in **(A–C)**, except anti-TGFβ (200μg intraperitoneal every 3 days starting at the time of SBRT) was included where indicated. $n = 5$/group. Error bars are SEM.

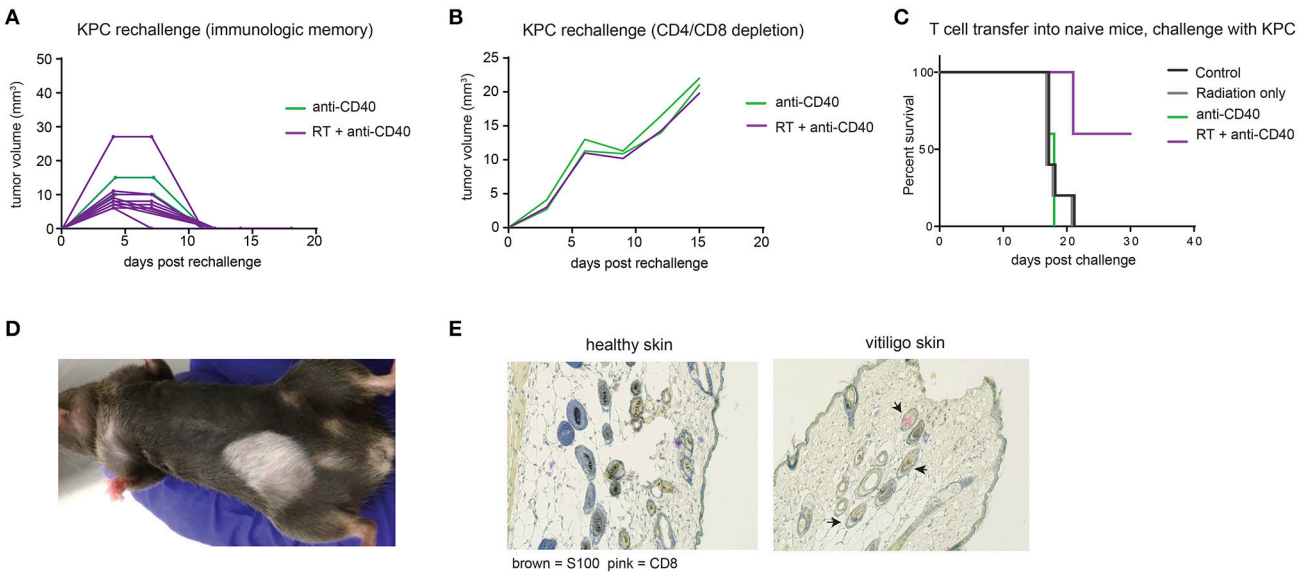

FIGURE 5 | Combination RT and anti-CD40 induces immunologic memory and autoimmune vitiligo. **(A)** Mice from **Figure 4** that were cured of their tumors by anti-CD40 or RT+anti-CD40 were rechallenged with 400,000 KPC cells in the absence of further treatment. **(B)** Mice from **Figure 4** that were cured of their tumors were treated with depleting antibodies to CD4 and CD8 (100 μg every 3–4 days) and inoculated with 500,000 KPC cells. **(C)** Mice were treated as in **Figure 4** with RT, anti-CD40 or RT+anti-CD40. Twelve days post treatment, spleens and lymph nodes were harvested, and total T cells isolated by magnetic bead selection. T cells from the indicated groups of donor mice were transferred into naïve recipient C57BL/6 mice that were then challenged with 200,000 KPC cells subcutaneously. Tumor growth was monitored until all mice were euthanized or tumor-free. **(D)** Representative picture of vitiligo development in combination treated mice that had been rechallenged with KPC tumors. **(E)** Histology of skin from an untreated mouse or a mouse with vitiligo shown in **(C)**. Immunohistochemistry stains for CD8 (pink) and the melanocyte marker S100 (brown). Arrowheads indicate CD8 staining in the hair follicles. Representative of 3 mice per group.

for combination with SBRT in the setting of pancreatic cancer due to previous activity of this agent in mouse models and human pancreatic cancer patients (21–23) and the potential for augmentation of T cell priming in combination with radiation (18, 25, 51). Although previous studies administered anti-CD40 systemically (18, 25, 51), we found that local injection into the irradiated tumor site required five-fold less antibody, and was still effective at generating T cell-mediated immunity.

While overall less toxic than conventional cancer therapies, immunotherapy is not without risk (55). The development of autoimmune vitiligo in mice treated with radiation and anti-CD40 underscores the fact that augmentation of T cell priming may induce priming of both autoreactive and tumor-reactive T cells. In some cases, these two groups may overlap; tumors often overexpress tissue-restricted self antigens that may be recognized by T cells. In general, central tolerance results in deletion of overtly self-reactive T cells during thymic development, but weakly self-reactive T cells, or T cells recognizing antigens not displayed in the thymus may escape into the periphery. Despite their relatively low affinity, these T cells may be useful components of the anti-tumor immune response (56), and priming self-reactive T cells may be the major mechanism by which radiation and anti-CD40 synergize. T cell priming may be too effective, as it is unlikely in this case that KPC pancreatic tumors and healthy melanocytes share common tumor rejection antigens. Indeed vitiligo has now been observed outside of melanoma, in patients treated with radiation or checkpoint blockade for other malignancies (55, 57, 58). Limiting the field of radiation and the damage to healthy tissues may be critical to restricting immune-related toxicities.

Local delivery of adjuvants is key for combination with radiation therapy. Adjuvants must be present at the site of cell death for activation of tumor-antigen loaded dendritic cells (1, 7). Although intratumoral injection has thus far been attempted in melanoma, lymphoma, head and neck cancer and other tumors with skin-accessible lesions, technologies for local delivery to other sites are progressing. Interventional radiologists currently can access nearly any site for biopsy or placement of fiducial markers. Local adjuvants that can be administered with, or incorporated into, fiducial markers may be a practical approach for clinical delivery in combination with radiation therapy to generate *in situ* cancer vaccines.

MATERIALS AND METHODS

Cell Culturing

Panc02 was obtained from the National Cancer Institute (59). KPC cells derived from a *LSL-Kras;p53+/floxed,Pdx-cre* mouse were a gift from Dr. Anirban Maitra (MD Anderson). Cells were cultured at 37°C in a humidified incubator with 5% CO_2. RPMI media was supplemented with 10% FBS, 2 mmol/L L-glutamine, 1% penicillin/streptomycin, 1% MEM non-essential amino acids, 1 mmol/L sodium pyruvate, and 0.1 mmol/L β-mercaptoethanol. Cells used for *in vivo* experiments had been passaged for less than 2 months, were negative for known mouse pathogens, and were implanted at >95% viability.

Mouse Pancreatic Subcutaneous Tumor Model

Female 6–8 week old C57BL/6J mice purchased from Jackson labs and used for KPC experiments. Panc02 experiments were replicated in both C57BL/6J (Jackson Labs) and C57BL/6NTac mice from Taconic. Syngeneic Panc02 or KPC cells were inoculated subcutaneously into both flanks of wild-type C57BL/6 mice at 2×10^5 or 1.5×10^5 cells, respectively. When tumors reached palpable size (week 2–3), mice were randomized and treatments were administered. Mice were observed at least twice per week and tumor measurements were performed using precision calipers at least once per week. In some experiments, CT scans were periodically performed to corroborate manual measurements. Mice were euthanized when either tumor exceeded 1 cm in diameter, or when tumors ulcerated. For mice that were cured of their initial tumors and rechallenged with KPC cells, 5×10^5 cells were inoculated. Animals were maintained and experiments were conducted at the DFCI Animal Resources Facility in accordance with IACUC guidelines. Animals were treated according to protocols approved by the Dana-Farber Cancer Institute IACUC.

Radiation Therapy (RT) and CT Image Analysis

A Small Animal Radiation Research Platform (SARRP) was used to administer RT at 220 kVp and 13 mA using either a 10 × 10 or 5 × 5 mm collimator and a 0.15 mm copper filter. Mice were anesthetized with isoflurane and image-guided RT was used to specifically irradiate tumors on the right flank. Panc02 tumors receiving a single dose of radiation were given 5 Gy whereas KPC tumors were given 10 Gy. For cohorts receiving fractionated radiation, a total of 30 Gy was administered over the course of three (10 Gy × 3) or six (5 Gy × 6) consecutive days. Whole-body CT images were manually segmented using Preclinical Imalytics Software (developed at ExMI, Aachen, Germany, along with Philips Research, Aachen, Germany) (60), allowing three-dimensional measurement of tumor volume.

Antibodies

Monoclonal anti-CD40 (clone FGK, BioXcell) was injected intratumorally into the treated tumors of relevant mice. Anti-CD40 or PBS was administered either as a single 20 μg dose or as two 10 μg doses spaced 3 days apart as indicated in the figure legends. Mice receiving both RT + anti-CD40 were treated with anti-CD40 within 3 h after radiation was administered.

Histopathology

Tumors from both flanks, as well as lung tissue in applicable cases, were extracted and fixed in 10% formalin. Sections were stained with hematoxylin and eosin (H&E), and images were obtained using an Eclipse E1000M microscope (Nikon). For CD8 immunohistochemistry, paraffin-embedded tumor tissue was sliced into 5 μm-thick sections with a microtome, air-dried, fixed with acetone, and stained by the DFCI Rodent Histopathology Core. Immunostaining was performed using anti-CD8 (Abcam) according to the manufacturer's protocol. Multi-color images were obtained using a Zeiss fluorescent microscope.

Flow Cytometry

Tumors were extracted from mice, digested in RPMI supplemented with type II collagenase (Sigma) and soybean trypsin inhibitor (Life Technologies), and dispersed into a single-cell suspension by filtering with a 40 micron cell strainer. Cell preparations were stained and analyzed using a Sony spectral cytofluorimeter (SP6800). Flow cytometry antibodies used in

this study were purchased from BioLegend (anti-CD45-BV711 [clone 30-F11], anti-CD11c-APC [N418], anti-CD11b-FITC [M1/70], anti-Gr-1-PE-Cy7 [RB6-8C5], anti-I-A/I-E-BV510 [M5/114.15.2], anti-CD4-BV421 [GK1.5], anti-CD103-PE [2E7], anti-B220-BV605 [RA3-6B2], anti-Ly6C-BV570 [HK1.4], anti-CD8-PacificBlue [53-6.7]).

Statistical Analysis

Groups were compared using a two-tailed Student's *t*-test. All reported tests were two-tailed and were considered significant at $p < 0.05$. Survival assays were plotted using Graphpad Prism and were analyzed using Log-rank (Mantel-Cox) and Gehan-Breslow Wilcoxon tests. Error bars are SD unless otherwise noted.

AUTHOR CONTRIBUTIONS

SY-K, PB, and SG designed and performed experiments and analyzed data. MM, SK, and GC performed experiments. RK contributed to experimental design. SD and WN supervised the project, analyzed data, and wrote the manuscript with input from all of the authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02030/full#supplementary-material

Supplemental Figure 1 | Pancreatic tumors are resistant to radiation. **(A)** Mice bearing palpable subcutaneous KPC tumor were treated with the indicated doses of SBRT. Tumors were harvested 14 days later. **(B)** Mice bearing palpable subcutaneous Panc02 tumors were treated with the indicated doses of SBRT. Tumor growth was monitored over time.

Supplemental Figure 2 | Histology of control and vitiligo skin. Skin from 8 week old untreated C57BL/6 mice, contralateral skin from a mouse with vitiligo, and white vitiligo skin were paraffin-embedded, sectioned and stained with antibodies to CD8 (pink) and the melanocyte marker S100 (brown). Two representative images are shown in **Figure 5.**

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REVIEW
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Immunomodulation of the Tumor Microenvironment: Turn Foe Into Friend

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Immunotherapy, where the patient's own immune system is exploited to eliminate tumor cells, has become one of the most prominent new cancer treatment options in the last decade. The main hurdle for classical cancer vaccines is the need to identify tumor- and patient specific antigens to include in the vaccine. Therefore, *in situ* vaccination represents an alternative and promising approach. This type of immunotherapy involves the direct intratumoral administration of different immunomodulatory agents and uses the tumor itself as the source of antigen. The ultimate aim is to convert an immunodormant tumor microenvironment into an immunostimulatory one, enabling the immune system to eradicate all tumor lesions in the body. In this review we will give an overview of different strategies, which can be exploited for the immunomodulation of the tumor microenvironment and their emerging role in the treatment of cancer patients.

Keywords: immunotherapy, oncolytic virotherapy, radiotherapy, cancer, *in situ* vaccination

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INTRODUCTION

Already in 1909, Paul Ehrlich postulated that the immune system has the ability to suppress the majority of carcinomas and thus plays an important role in the protection against tumor development (1). Instrumental to this idea is the capacity of the immune system to distinguish "self" from "non-self" and to eliminate the latter without damaging the former.

To pursue the specificity of immunotherapy, various efforts have been made to identify cancer-associated antigens to use in therapeutic vaccination strategies. The first tumor-associated antigens (TAAs) identification was made in the context of melanoma with melanoma antigen family A1 (MAGE-A1) identified in 1991 (2). MAGE-A1 is a member of a large gene family, comprising 25 cancer-germline genes. This identification was followed by the observation that T cells frequently target proteins associated with pigment production in melanomas (3). These tissue differentiation antigens, which are normal proteins with a specific function in the target tissue, constituted the majority of initially discovered TAAs. However, targeting these antigens can lead to severe, life threatening side effects due to expression of these antigens, even in low amounts, by normal tissue (4, 5). Tumors can also overexpress normal self-proteins, that are important for their malignant phenotype, such as p53 and human Telomerase Reverse Transcriptase (hTERT). Given the important role of these proteins for the survival and phenotype of cancer cells, tumors cannot downregulate these molecules and this makes them an attractive target for immunotherapy. However, since they have normal functions in some tissues and under certain conditions, off-tumor reactions can occur when targeting these proteins (6). In recent years, with the development of

deep sequencing technologies, studies have revealed the presence of antigens resulting from somatic mutations and giving rise to proteins with altered sequence. These mutation-derived antigens, also known as neo-antigens, are tumor- and patient-specific. Targeting neo-antigens would overcome self-tolerance and lead to stronger immune responses (7, 8). Due to the heterogeneity within tumors and since cancer vaccines only target a limited number of antigens, cancer cells that do not express these antigens can escape immune control and give rise to new tumor populations that can resist treatment with a vaccine encoding the same TAAs (9). Moreover, T cells evoked after vaccination often fail to infiltrate in the tumor or fail to exert their function due to immunosuppression in the tumor (10).

With *in situ* vaccination these problems can be circumvented. *In situ* vaccination refers to any approach where the tumor vaccine antigens are processed in the patients own body following intratumoral (IT) treatment with immunostimulatory drugs. These immunomodulators have the capacity to stimulate tumor cell death and therefore enhance the uptake and presentation of TAAs by APCs. With this strategy, the need to identify TAAs to include in the vaccine is circumvented thereby limiting labor-, time-, and cost-intensive *ex vivo* efforts. The generation of anti-tumor T cells at one tumor site should allow them to attack distant tumor lesions resulting in a systemic immune response. Moreover, since *in situ* vaccination depends on the local injection of immunostimulatory molecules, systemic toxicities are limited (11). Overall, lower amounts of reagents are required when administered locally, significantly reducing the cost of therapies (e.g. for checkpoint inhibitors). Since *in situ* vaccination is not personalized but available off-the-shelf, this therapy can be combined with other standard of care treatments, such as surgery and radiotherapy, in order to find the most optimal treatment schedule resulting in curing the patient.

IN SITU VACCINATION: ACTIVATION OF THE IMMUNE SYSTEM

An *in situ* vaccine should be able to convert an immunosuppressive or dormant tumor microenvironment (TME) into an immunostimulatory one, which allows effector T cells to enter the tumor bed and to kill the tumor cells. Such an anti-tumor immune response will only lead to effective killing of cancer cells when a series of events occurs in a specific order, resulting in the proper activation of the immune system.

The innate immune response starts with the recognition of pathogens (characterized by Pathogen-Associated Molecular Patterns, PAMPs) or indicators of danger (Damage-Associated Molecular Patterns, DAMPs) by pathogen-recognition receptors (PRRs). Immature dendritic cells scan the periphery and when they encounter such a PAMP or DAMP, they efficiently take up antigens and undergo maturation under the influence of a number of danger signals, various cytokines and tissue factors. These DCs present antigens in the context of Major Histocompatibility Complex (MHC) class I and II molecules to activate both $CD8^+$ and $CD4^+$ T cells. Different activation signals are needed for a T cell before they can exert their function. The initial interaction between the DC and the T cell, through the MHC complex and the T cell receptor, provides the first signal. A so-called second signal concerns a costimulatory interaction between CD28 on T cells and CD80 or CD86 on APCs, and is also required for T cell activation. $CD8^+$ T cells also require additional cytokine signals (signal 3), for the optimal generation of effector and memory populations and for their survival (12, 13). The absence of these signals and the presence of immunosuppressive cytokines could either activate T helper 2 cells or attract and activate regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs) or dysfunctional DCs leading to immunosuppression (14). Tumors can increase the production of immunosuppressive cytokines, reduce the expression levels of MHC I molecules, downregulate their expression of TAAs, thereby evading immune recognition and eventually escape immune control.

With *in situ* vaccination, changes in cytokine secretion patterns are induced, leading to changes in the type, number and activation status of tumor-infiltrating lymphocytes (TILs), resulting in an effective anti-tumor immune response (15, 16). A second important feature of an *in situ* vaccine is the ability to induce immunogenic cell death (ICD). ICD is defined as a specific form of regulated cell death that induces the release of TAAs and triggers an anti-tumor immune response (17). During ICD, there is a timely release of DAMPs that warns the organism of a situation of danger, resulting in the induction of an immune response associated with the formation of an immunological memory. Although ICD is a very complex process, six DAMPs are mechanistically linked to the induction of this type of cell death and the subsequent immune response. Firstly there is calreticulin (CRT), an ER-associated chaperone protein that promotes phagocytosis of dying cells by attracting DCs (18). The second DAMP is high mobility group box 1 (HMGB1), a histone-chromatin binding protein passively released from stressed or dying cells. HMGB1 exerts potent immunomodulatory effects by binding to Toll Like Receptor (TLR) 4 and TLR9, which both play crucial roles in driving inflammatory responses (19). Extracellular ATP is the third DAMP, that is sensed by the purinergic receptor P2X7, a key regulatory element of the inflammasome, leading to the secretion of pro-inflammatory cytokines resulting in the attraction of DCs toward the dying tumor cells (19–22). The fourth DAMP is type I IFN, which is produced by cancer cells undergoing ICD in response to endogenous double stranded (ds) RNA detected via TLR3 (23) or in response to dsDNA sensed by cGAS (24–26). Type I IFN mediates various immunostimulatory effects on immune cells (27). Cancer cell-derived nucleic acids are the fifth DAMP that play a role in ICD. Cancer cell-derived nucleic acids are taken up by DCs, neutrophils and macrophages, resulting in a potent type I IFN response (28–31). Lastly there is extracellular ANXA1, which supports the activation of adaptive immune response by engaging formyl peptide receptor 1 (FPR1) on DCs (32). All these DAMPs play a role in the outcome of ICD and will determine the strength and the durability of the anti-tumor responses.

In this review we will discuss preclinical and clinical data of different *in situ* vaccination strategies that stimulate anti-tumor immune responses through the induction of ICD, the

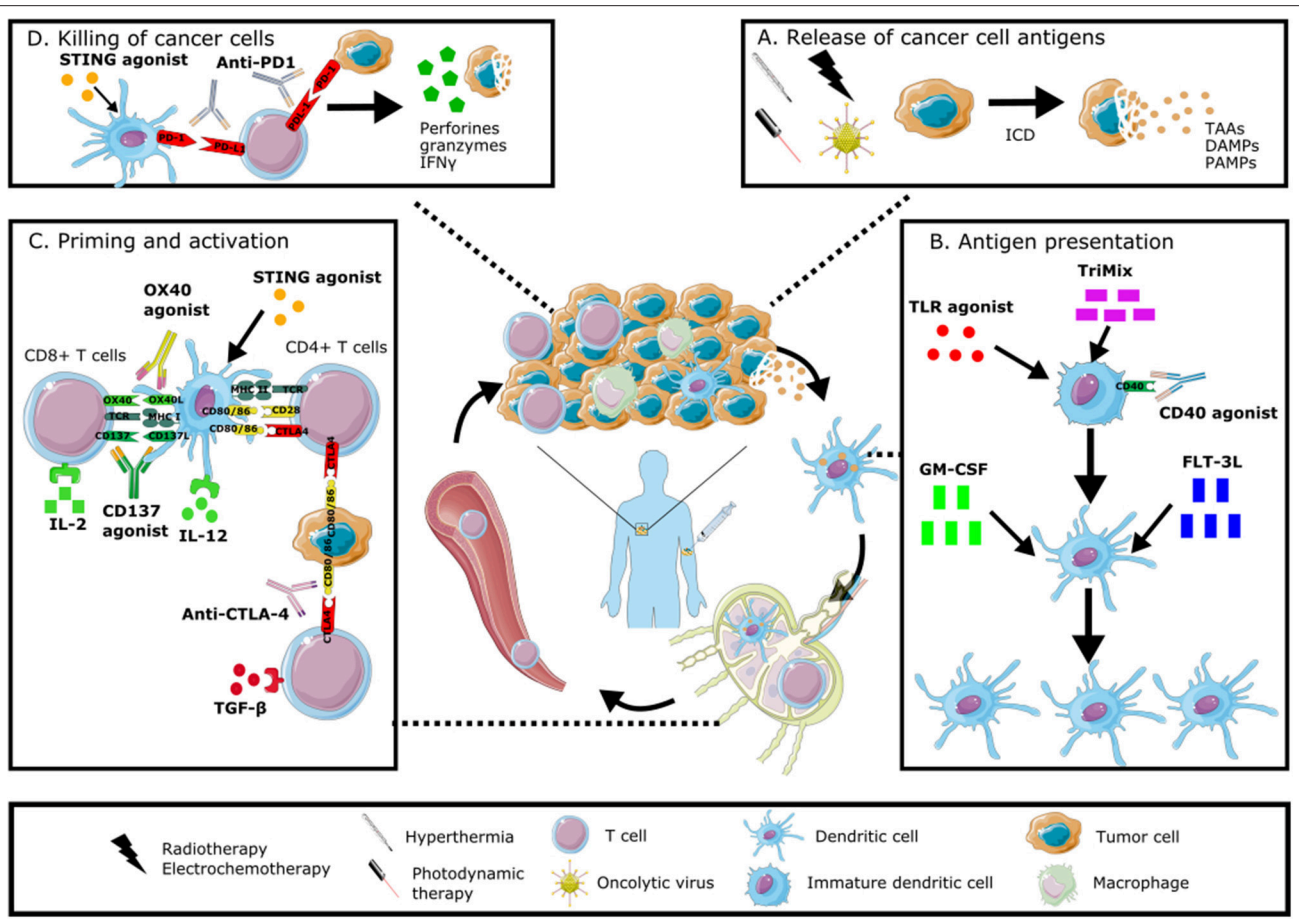

FIGURE 1 | Immunomodulation of the tumor microenvironment to induce anti-tumor immune responses. *In situ* vaccines result in intratumoral modulation to attract and activate dendritic cells able to present the full antigenic repertoire to tumor-specific T cells able to kill tumor cells. This immunomodulation can occur at different levels: stimulating the induction of immunogenic cell death with radiotherapy, electrochemotherapy, hyperthermia, photodynamic therapy or oncolytic viruses **(A)**, increasing the number and maturation of dendritic cells through the administration of growth factors, cytokines or TLR agonists **(B)**, stimulating the priming and activation of T cells through the intratumoral injection of checkpoint inhibitors, cytokines or other immunomodulating agents **(C)**, promoting the direct killing of cancer cells through the local administration of STING agonists or checkpoint inhibitors **(D)**. All of these modalities can be combined in order to induce a robust anti-tumor immune response. Graphical elements are adapted from Servier medical art repository (https://smart.servier.com).

attraction of different immune cell populations and by alleviating immune suppression. The discussed immunomodulators include oncolytic viruses, radiotherapy, physical therapies, growth factors and cytokines, as well as combinations of these modalities. An overview of these modalities and their mechanism of action is given in **Figure 1**.

IMMUNOMODULATORY APPROACHES: HOW TO MAKE A COLD TUMOR HOT?

Oncolytic Viruses (OVs)

The interest in oncolytic virotherapy is not a new concept, but has grown exponentially during the last years alongside the advancements in molecular biology, virology, immunology and genetic engineering (33).

Oncolytic viruses (OVs) are attenuated, mutated, or benign viruses that preferentially target cancer cells and do not infect normal, non-transformed cells. The list of OVs used for therapy is rapidly growing and includes reovirus, vesicular stomatitis virus, vaccinia virus, Newcastle disease virus, measles virus, poliovirus, herpes simplex virus, coxsackievirus, adenovirus, and Maraba virus.

The anti-tumor effect of OVs arises from a dual mechanism of action: the selective replication of the virus in tumor cells will result in cell killing while simultaneously stimulating the immune system through the induction of ICD. Via the recruitment and activation of cross-presenting DCs followed by the stimulation of specific lymphocytes this ICD will induce an effective anti-tumor immune response (34). The key desirable characteristics of OVs are therefore the specificity for the targeted cancer cells, their potency to induce ICD and safety to avoid adverse reactions and pathogenic reversion (35). Numerous naturally occurring OVs exist, but recently immense interest has revolved around genetically modifying viruses to improve their safety, specificity, immunogenicity, oncolytic potency, and drugability (35). All clinical related OVs have been genetically modified with one or

more immunomodulating agents (As described in the section Immunomodulatory factors).

Immune Modulation by OVs

Originally OVs were designed to be cytolytic agents, but it is now clear that they have pleiotropic effects on the TME through activation of different signaling pathways (36). Triggering of ICD in OV-infected cancer cells results in the release of PAMPs in the TME. Tumor cell derived PAMPs, for example viral capsids, DNA, RNA, and proteins, are important drivers of adjuvanticity and effective APC engagement, and are even more important than the mode of cell death (37, 38). The innate immune pathways and sensors that can be triggered by OVs induced PAMPS have been largely uncovered. This innate immune response is mainly mediated by a set of TLRs (expressed on the plasma membrane and in endosomal compartments), cytoplasmic receptors, and intracellular NOD-family of receptor complexes. The most important TLRs are TLR3/TLR7, which recognizes viral double stranded (ds) RNA and single stranded (ss) RNA and TLR9, which recognizes ss DNA. Upon infection of tumor cells with RNA/DNA-based OVs these TLRs may promote the intrinsic (in the tumor) and extrinsic (in the phagocyte) production of cytokines in the TME (39, 40). The cytoplasmic receptors Retinoic acid Inducible Gene 1 (RIG-I) and Melanoma Differentiation-Associated protein 5 (MDA-5) play a crucial role in the recognition of RNA from OVs. Both receptors can activate cytokine production through the mitochondrial antiviral signaling (MAVS) adaptor protein upon infection with OVs such as vesicular stomatitis virus (VSV) and measles viruses (40). In addition, it has become clear that innate immune STimulator of Interferon Genes (STING) signaling through the cGAS-STING complex plays a vital role in directing T cell responses toward infected tumor cells. After phagocytosis of the tumor cells, the partially degraded genomic DNA, which was compartmentalized in the nucleus, is efficiently processed by DNase II in the lysosomal compartment (41, 42). However, a small fraction of genomic DNA can leak out the lysosomal compartment resulting in activation of the STING pathway. Cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS), a cellular synthase, binds to these cytosolic nucleic acids, which generates self-DAMPS referred to as cyclic dinucleotides. At this point the cGAS-STING signaling complex is formed which triggers type I interferon (IFN) production required for cross-priming of TAAs and the generation of tumor specific T cells (43).

The intercellular transfer of a TAA released in the TME induced by different OVs upon infection has recently been reported, allowing recognition of TAA-loaded cancer cells by specific effector $CD4^+$ T cells. The generation of tumor-reactive cytotoxic T lymphocytes (CTLs) is mostly driven by the antigenicity of the dying tumor cells (44). The capacity of OVs to induce T cells specific for the entire TAA repertoire is an important feature of this therapy. OV-induced tumor cell death and the following epitope spreading in the TME can be seen as a personalized immunotherapeutic approach, without the need for prior identification of the TAA.

Although OV therapy has beneficial effects on the immune system the strength of the induced immune response depends on the particular virus strain that is used, the tumor burden and the immunogenicity. This will determine the outcome of the therapy (45). At this moment the first generation of OVs has been validated in recent clinical trials for their anti-cancer potential (46).

Radiotherapy

Photon and Particle Radiotherapy

In the past century, radiotherapy (RT) has been a strong pillar in the treatment of cancer. Currently, RT is the frontline therapy for approximately 50% of all patients with newly diagnosed cancer, alone or in combination with surgery or chemotherapy (47). Recent advances in RT technologies and approaches have focused on limiting toxicity and on achieving greater therapeutic effectiveness (48). The clinical efficacy of ionizing radiation comes principally from the induction of DNA damage, which can result in tumor cell death. The conventional fractionated regimes used in the clinic are built on four biological processes, called the "4Rs of fractionated radiobiology": Reoxygenation of hypoxic regions in the tumor, Repopulation of tumor cells, Repair of sublethal damage in normal cells and Redistribution of cells to a cell cycle phase which is more radiosensitive (49). However, Golden and Formenti proposed a fifth R: immune-mediated Rejection of the tumor. The "5th R" is based on preclinical studies that demonstrated an important contribution of RT on the TME and on the induction of anti-tumor immune responses (50). The abscopal effect of RT, originally described by Mole in 1953, is a phenomenon where localized radiation of a tumor results in a response at distant metastatic sites outside of the path of radiation (51). Over the last decade the rare abscopal effect has been reported for several cancers, including melanoma, renal cell carcinoma, breast cancer, hepatocellular carcinoma, and other metastatic solid tumors (52–57).

The immunogenic potential of particle radiation therapy (e.g., proton, carbon-ion, ...) has also been investigated by different groups. The main difference between particle radiation and x-rays are the physical properties of the beam. X-rays are absorbed in the tissue, leading to an exponential decay of the radiation dose by increasing depth. In contrast, charged particles lose little energy when they enter the body, when their velocity is high, and most energy deep in the tissue (= Bragg peak). Therefore, charged particle therapy produces a more conformal dose distribution thereby minimizing the area of normal tissue exposed to radiation (58). Moreover, heavy particles have a higher relative biological effectiveness (defined as the ratio of dose of a reference radiation (x-rays or γ-rays) and the dose of a rest radiation that produce the same biological effect) with high linear energy transfer (energy deposited per unit track in the tissue by charged particles) (59, 60).

Immune Modulation by RT

Preclinical evidence has demonstrated that tumor targeted RT can stimulate the immune system at least via three distinct mechanisms. First, RT can induce ICD, which leads to the release of neo-antigens. Thereby, RT can improve the recognition and killing of tumor cells by $CD8^+$ T cells. Moreover, RT can overcome T cell exclusion from the tumor by promoting the release of chemokines that attract effector T cells to the

TME. By surmounting the vascular barrier, T cell infiltration is also facilitated. Moreover, RT can upregulate MHC class I and other components of the antigen processing machinery (61, 62). Anti-tumor immune responses are also improved through the expression of pro-inflammatory cytokines and chemokines, as well as natural killer cell (NK) activating ligands that are produced in response to RT (29, 63–65). In addition, activation of cGAS-dependent and STING-dependent pathways trigger type I IFN signaling in DCs, further strengthening adaptive immune responses in response to RT (29). This shows that RT has the potential to trigger antigen-specific adaptive immunity, but in preclinical models radiation often fails to induce T cell responses to most TAAs (66).

Interestingly, radiation was shown to increase the intracellular peptide pool and induce T cell responses to these peptides. This observation suggests that radiotherapy can selectively boost anti-tumor T cell responses to unique radiation-induced antigenic peptides or tumor-related self-antigens (61). This could be extremely valuable in new strategies to combine radiotherapy and immunotherapy for locally advanced cancers. However, for metastatic diseases, it is unknown whether the different antigenic peptides are shared by the irradiated and non-irradiated metastases. Moreover, radiation has an effect on multiple surface molecules that facilitates recognition of irradiated tumor cells by T cells. Therefore, epitopes present in lower abundance or of low affinity for the TCR may not interact with T cells in the non-irradiated metastasis (67, 68). The presence of multiple antigenic targets, leading to polyvalent T cell responses, on irradiated and non-irradiated tumors may solve the concern about the differential specificity of T cells (69, 70).

Although there are multiple mechanisms by which RT can induce immune activation, for a long time, high-dose radiation was thought to be immune suppressive. The immune suppressive effects of RT can be explained by the fact that different immune cells are very sensitive to radiation and can be eradicated at much lower radiation doses than needed to kill cancer cells. Moreover, the TME also contains different subsets of inhibitory immune cells, including Treg, myeloid-derived suppressor cells and tumor-associated macrophages, that can be activated after RT (71–78) Furthermore, it was shown that RT can increase the expression of PD-L1 on melanoma and glioblastoma cells thereby hampering effecting killing of the tumor cells by cytotoxic T lymphocytes (79). This balance between immune activation and immune suppression caused by RT is nicely reviewed by Wennerberg et al. (80) and Lee et al. (81).

In *in vitro* tumor cell models it has been shown that proton radiation, compared to photon radiation, resulted in a higher translocation of calreticulin thereby increasing the cross-priming of TAA and the sensitivity of the tumor cells to CTL-mediated killing (82). *P*reliminary *in vivo* data suggest that carbon-ion radiation, combined with DC injection, correlated with a better activation of the immune system (83). Clinically, two patients experiencing abscopal responses following carbon ion RT without immunotherapy for recurrent colorectal cancer have been reported. However, the question remains whether these abscopal responses were due to ablative dose delivery afforded by particle therapy, an immunogenic effect secondary to high-LET radiation, or a combination of both (84, 85).

The use of localized RT with the goal to act as an *in situ* vaccine is a promising concept, especially when combined with other immunomodulating modalities (as described in sections Physical therapies and immunomodulatory factors). However, successful induction of antitumor immunity by RT is dependent on the balance of immune suppressive and immune activating signals that are generated by RT, depending on the dose and quality of the radiation.

Physical Therapies

Different destructive treatments that induce a local acute trauma at the tumor site, thereby inducing the release of TAAs, aim to initiate an innate immune response targeting both the treated lesion as well as distinct lesions. These physical therapies can be combined with classical treatment schedules or other immunomodulating factors, with the aim to enhance anti-tumor immune responses. An overview of these physical treatment modalities is given in **Table 1**.

Photodynamic Therapy (PDT)

Photodynamic therapy (PDT) or photochemotherapy is based on a reaction between light and a photosensitizer in the presence of oxygen. The combination of these components leads to a photochemical reaction that generates reactive oxygen species (ROS), which causes cell death. The localized acute trauma and oxidative stress induced by PDT, provokes a strong acute inflammatory reaction. Moreover, it has been established that PDT can induce an adaptive immune response, both humoral immunity as well as cell-mediated anti-tumor immunity. Different parameters, such as the treatment regimen, treated area and the type of photosensitizer, can influence the type and the strength of the immune response that is induced.

The major advantages of this technique include: the possibility to target any organ in the body, the limited invasiveness, the selective cytotoxicity toward the tumor and the complementarity with classical treatment modalities, including surgery, chemo- and radiotherapy. However, different parameters need to be defined for every patient and its specific tumor type since these can affect the outcome of the treatment. These parameters include the choice of and dose of the used photosensitizer, the time between administering the photosensitizer and exposure to light, the dosage of total light and its fluence rate and the oxygen concentration present in the tumor.

The first clinical use of PDT for cancer therapy dates back to the late 1970s, when five patients with bladder cancer were treated. From then on, many efforts are made to evaluate the effect of PDT in patients -currently over 400 clinical trials can be found on clinicaltrial.gov. The indications include premalignant conditions (e.g., mucous dysplasia, actinic keratosis (e.g., NCT03643744), carcinomas *in situ* (NCT03638622, NCT03133650, NCT03211078), and superficial tumors (such as superficially growing basal cell carcinomas (NCT02367547, NCT03467789). However, in most

TABLE 1 | Overview of different physical therapies.

Physical therapy	Advantages	Limitations	Indications
1. Photodynamic therapy	*Limited invasiveness *Selective cytotoxicity *Complementarity with standard of care treatments *All organs can be targeted	*Protocols need to be optimized for every patient and tumor type	Bladder cancer, Carcinomas *in situ*, Superficial tumors
2. Electrochemotherapy	*Increased drug levels at the tumor site *Induction of systemic immune response *Complementarity with other immunomodulating therapies *Favorable safety profile *Repeated treatments possible	*Protocol need to be adjusted for every tumor type *Choice of electrodes *Tumor size and location can limit the success delayed drug perfusion	Cutaneous tumors, Breast cancer, Pancreatic cancer, Colorectal cancer
3. Hyperthermia	*Suitable adjuvant for standard of care treatments	*Appropriate energy source *Non-selective tissue heating	Breast tumors, Gastrointestinal tumors, Melanoma, Brain tumors, Sarcomas
4. Tumor-treating fields	*Non-invasive anti-tumor effect *Complementarity with standard of care treatments	*Adverse events including skin irritations, rash, ulcerations and infections *Mechanism of action not clear *Cost-effectiveness	Glioblastoma

of the cases PDT is used in combination with other standard of care therapies (86).

Electrochemotherapy (ECT)

Electrochemotherapy (ECT) is based on the local application of electric pulses to deliver chemotherapeutic drugs at the tumor site. This reversible electroporation enhances the drug uptake by increasing the permeability of the cell membrane. Thereby potentiating the cytotoxicity of non-permeant chemotherapeutic drugs, such as bleomycin and cisplatin (87, 88). The cytotoxicity of ECT acts on the whole TME and therefore targets directly the tumor cells as well as the interwoven stromal and endothelial cells lining the tumor microvasculature. The cell death induced in these endothelial cells leads to the abrogation of tumor blood flow thereby impairing the viability of tumor cells surrounding the vessels. This results in a massive release of TAAs inducing a systemic immune reaction. This immune response can be enhanced when ECT is combined with other immunomodulatory factors, improving the antigen presentation and survival of effector T cells, such as IL-2, IL-12, GM-CSF, and TNF-α (88).

ECT is mainly used for the local treatment of accessible cutaneous and subcutaneous metastases (since different types of electrodes can be applied, from plate to needle electrodes). However, there are also some limitations to take into account. Different tissues need to be treated according to different protocols, the choice of the electrodes needs to be adapted in accordance with the size and type of the lesions, tumor size and location can determine the success of ECT and, due to delayed drug perfusion, there can be a decreased drug concentration at the tumor site.

Nevertheless, the use of ECT to treat cutaneous tumors has been proven to be a highly efficient and safe approach and is already widely accepted in clinical routine (89). Due to its simple application, favorable safety profile and the possibility of repetitive treatment, this treatment modality can be used for different tumor types with different histologies (88, 89). It has been shown that frequent administration of ECT led to an increase in the rate of complete remissions in breast cancer patients (90). During the years, efforts are made to extrapolate the ECT treatment of easily accessible lesions to non-superficial tumors. Safety, feasibility and efficacy of ECT in locally advanced pancreatic cancer patients in a phase I/II study (91) and in patients with bone metastasis (92) has already been reported. In the latter phase I/II clinical trial, 56% of the patients showed pain relief and in a few patients necrosis of the metastatic lesion was observed (92). A pilot study in patients with unresectable colorectal liver metastases revealed that 55% of the patient population were complete responders and 45% had a stable disease. Additionally, 80–100% of the treated patients had an overall and progression-free survival at 6 months (89, 93). At the moment ECT is usually applied in a palliative setting for patients with unresectable tumors, but it can also be an effective treatment option in minimally invasive oncologic treatments.

Hyperthermia

Hyperthermia can be defined as a treatment in which the target tissue, the tumor, is exposed to high temperature. Hyperthermia can be divided into thermal ablation, where the tumor tissue is destroyed directly, or thermal sensitization where the tumor is rendered more susceptible to other treatments (94). Thermal sensitization (40 – 45°C) is most used in the clinic and serves as adjuvant for standard of care treatments like chemotherapy and

radiotherapy (95, 96). An elevation in temperature causes tissue changes in the vascular permeability, increase in blood flow and eventually leads to tumor oxygenation.

Combinational strategies with radiotherapy or chemotherapy and hyperthermia have shown clinical benefit for the treatment of a wide range of cancers including breast cancer, gastrointestinal tumors, gynecological tumors, brain tumors, lung tumors, melanomas, and sarcomas (97). Although hyperthermia continues to show clinical benefits in randomized trials, widespread application remains omitted.

One of the challenging issues for hyperthermia is the appropriate means for heat delivery. At this moment four different energy sources can be used: microwave, radiofrequency, laser and ultrasound. In conventional local hyperthermia, the heating happens from the outside-in, which can lead to serious side effects through non-selectivity in tissue heating. Alternatively, the application of nanoparticles as hyperthermia agents was developed to increase the effectiveness of hyperthermia. Nanoparticle-mediated hyperthermia could help reduce the side effects by employing inside-out hyperthermia (94). There exist four different kinds of nanoparticle-mediated hyperthermia: nano-photo-thermal therapy, nano-magnetic hyperthermia, nano-radio-frequency ablation, and nano-ultrasound hyperthermia. Nano-magnetic hyperthermia is the only and first application of Nanoparticle-mediated hyperthermia that has been introduced in the clinic. The main advantage over conventional hyperthermia is the ability of the magnetic nanoparticles to distribute into the tumor hereby creating a difference in temperature between tumor and healthy tissue (98).

Tumor-Treating Fields (TTF)

Tumor-treating fields (TTF) represents a treatment modality designed to deliver alternating electrical fields to a malignant lesion. It concerns a cancer treatment specifically used for brain tumors, especially tested for glioblastoma. Different clinical trials have been performed to assess the benefits of this adjuvant therapy in combination with the standard of care in glioblastoma cancer patients. The EF-14 trial (NCT00916409), the largest multinational trial of TTF therapy, showed that both progression free survival and overall survival were prolonged in glioblastoma patients treated with TTF. Common adverse events are skin irritation, including rash, ulceration and infections (99).

TTF may also be synergistic with immunotherapeutic approaches. TTF have been shown to lead to an aberrant mitotic exit (which can induce ICD), expose CRT on cell surface and decrease tumor volume when combined with an anti-programmed cell death 1 (anti-PD-1) drug (100–104).

However, there still is significant skepticism about the TTF device. Questions about the clear mechanism of action, interpretation of the data from the clinical trials and cost-effectiveness of TFF therapy need to be elucidated (105). As such, more promising clinical data and research will be necessary to convince the physicians to apply TTF as standard treatment (106).

Immunomodulatory Factors

Through the local administration of growth factors, cytokines, and immunomodulatory molecules, we can enhance all the steps needed to induce an effective anti-tumor immune response and counteract the mechanisms that tumors use to escape immune control, while limiting toxicities associated with the systemic administration of these molecules.

These strategies, which can be used as a stand-alone therapy or in combination with OVs and/or RT, will be discussed in detail in the following section. An overview of these strategies is given in **Table 2**.

Growth Factors

Immune responses against malignant cells can be improved by increasing the number of APCs in the tumor that can cross-present TAAs to $CD8^+$ T cells (149).

Granulocyte macrophage—colony stimulating factor (GM-CSF)

GM-CSF plays an important role in DC recruitment and maturation but also facilitates the homing of CTLs in the TME. Multiple vaccine platforms include GM-CSF in their formulations and the goal of administering it intratumorally is to increase the number of DCs in the TME (149, 150). In different preclinical studies it was shown that the IT expression of GM-CSF resulted in an effective anti-tumor immune response (151, 152). In patients with melanoma, IT or peritumoral injection of recombinant GM-CSF results in an increase in the number of DCs in treated tumor lesions but this did not always result in better anti-tumor responses and effects on progression free survival (149, 153–155). A current phase I study investigates the IT administration of GM-CSF in pancreatic cancer patients (NCT00600002).

Although GM-CSF has therapeutic potential as a monotherapy, combinations with other immune modulating agents, such as OVs or radiotherapy, might potentiate the effects (149). Using OVs engineered to express cytokines to increase the number of APCs at the tumor site is also a solid strategy to enhance the anti-tumor effect of OVs. T-VEC, an attenuated herpes simplex virus incorporating a GM-CSF transgene, was granted marketing approval by FDA and EMA in 2015 for IT therapy in patients with unresectable stage 3 and 4 melanoma (107). Similar a vaccinia virus engineered to express GM-CSF, JX-594, has been shown to selectively target and replicate in tumor cells and has anti-tumor efficacy in both a preclinical and clinical setting (108). IT delivery of JX-594 is well tolerated in patients with liver cancer and melanoma, resulting in encouraging effects on the survival and overall response in both treated and untreated lesions (109–112). The combination of recombinant GM-CSF and RT is currently being evaluated in 5 phase II clinical trials in metastatic lung cancer and hepatocellular carcinoma.

Fms-related tyrosine kinase 3 ligand (Flt3L)

Flt3L is a key growth factor in the generation of DCs from hematopoietic progenitors present in the bone marrow (149, 156). Subcutaneous and systemic injection of Flt3L has proven

TABLE 2 | Overview of the different molecules and strategies used for the *in situ* modulation of the tumor microenvironment.

Immunomodulating factor	Mode of Action	Indication	References
GROWTH FACTORS			
1.GM-CSF	**Increase in the number of DCs in the TME**		
*T-VEC (OV)		Melanoma	(107)
*JX594 (OV)		Melanoma, Liver carcinoma	(108–112)
*Combined with RT		Lung carcinoma, Hepatocellular carcinoma	NCT02946138, NCT03113851
2. FLT3L	**Increase the mobilization of DCs**		
*Combined with chemotherapy		Preclinical	(113)
*Combined with RT		Low-grade B cell lymphoma	NCT01976585
CYTOKINES			
1. IL-12	**Polarization of type 1 helper T Increased IFNγ production by CTLs**		
*Systemic delivery		Melanoma, Renal cell carcinoma, Colon carcinoma	(114, 115)
*Encapsulated into nanoparticles		Preclinical Ovarian cancer	(116) (117)
*Gene electrotransfer		Triple Negative Breast Cancer, Lymphoma, Merkel cell carcinoma, Melanoma	NCT02531425, NCT01579318, NCT0144081
*Viruses expressing IL-12		Preclinical	(118–120)
2. IL-2	**Expansion and differentiation of effector lymphocytes**		
*Systemic delivery		Renal cell carcinoma, Melanoma	(121, 122)
*Encapsulated into nanoparticles		Renal cell carcinoma, Melanoma	(123–125)
*Combined with α-CTLA-4		Melanoma	NCT01480323, NCT01672450
*Combined with RT		Renal cell carcinoma, Melanoma, Non-small cell lung cancer	NCT01884961, NCT02306954, NCT030226236, NCT03224871
3. TGF-β (blocking)	**Associated with immunosuppression in the TME**		
*Combined with RT		Non-small cell lung cancer, Rectal cancer, Hepatocellular carcinoma, Solid tumors	NCT02581787, NCT02688712, NCT02906397, NCT02937272
IMMUNOMODULATORY FACTORS			
1. Checkpoint inhibitors	**Releasing the brakes on the immune system and promote function and survival of T cells**		
*Systemic delivery		Melanoma, Renal cell carcinoma	Different agents already FDA approved
*Combined with OVs		Preclinical Melanoma	(126–131) NCT02263508
*Combined with RT		Preclinical, >100 trials in different Solid tumors	(132–139)
2. CD40 agonist	**Initiation and propagation of adaptive immune responses**		
*Monoclonal antibodies		Preclinical Solid tumors	(140, 141) NCT02379741
*mRNA		Preclinical	(142)
*Combined with OVs		Preclinical	(143, 144)

(Continued)

TABLE 2 | Continued

Immunomodulating factor	Mode of Action	Indication	References
3. OX-40 agonist	**Delivering co-stimulatory signals to T cells needed for their full activation**		
*mRNA		Solid tumors, Lymphoma	NCT03323398
*Combined with checkpoint inhibitors		Preclinical	(145)
*Combined with OVs		Preclinical	(146–148)
*Combined with RT		Prostate cancer, Breast cancer, B cell Non-Hodgkin lymphoma	NCT01642290 NCT03410901
4. TLR agonist	**Activation of APCs**		
*Monotherapy		Advanced solid tumors, Prostate cancer, Basal cell carcinoma	NCT01984892, NCT03262103, NCT0066872,
*Combined with OVs			
*Combined with RT		B cell lymphoma, Merkel cell carcinoma, Solid tumors, T cell lymphoma	NCT01976585, NCT02501473, NCT02556463, NCT0088058, NCT02927964
5. STING agonists	**Activation of the innate immune system through upregulation of IFNs**		
*Monotherapy		Solid tumors, Lymphomas	NCT03172936
*Combined with checkpoint inhibitors		Solid tumors, Lymphomas	NCT02675439

to stimulate mobilization of different subsets of DCs to the peripheral blood of both healthy donors and patients with melanoma or colon cancer (157, 158).

Vaccination with Flt3L prior to tumor challenge has shown to be able to prevent tumor growth in mouse models of colon cancer and leukemia, however the therapeutic administration of Flt3L could not cure already established tumors. In contrast, IT administration of an adenovirus expressing Flt3L together with systemic chemotherapy induced complete remission of established murine hepatoma and colon cancer (113).

Systemic Flt3L combined with RT led to a significant growth delay of both the irradiated tumor and the non-irradiated tumor compared to the non-treated control groups. This abscopal effect was dependent on the induction and activation of T cells (159). Currently, one clinical trial is testing the combination of IT Flt3L and poly-ICLC with low dose RT in low-grade B-cell lymphoma patients (NCT01976585). This study reported partial and complete remissions of both treated and untreated lesions associated with increased DC numbers (160).

Cytokines

Cytokines are potent immune modulating proteins with an important role in the maintenance of immune homeostasis, initiation, and regulation of inflammatory responses, controlling pathogens and enforcing tolerogenic mechanisms. The *in situ* delivery of cytokines represents an attractive approach to remodel the immune system and their adjuvant properties can increase vaccine efficacy (123).

Interleukin-12 (IL-12)

IL-12 is a cytokine that plays a major role in the regulation of adaptive T cell responses. Various immune cell types—but particularly myeloid APCs—secrete IL-12 in response to infection or inflammation. IL-12 secretion leads to the polarization of type 1 helper T (Th1) cells and an increase in the activity and IFNγ production of CTLs, stimulating them to kill infected cells or tumor cells (123, 149).

The systemic delivery of IL-12 has been tested in melanoma, renal cell carcinoma and colon carcinoma patients, but unfortunately several patients experienced considerable hepatic and hematologic toxicity and only a modest anti-tumor efficacy could be observed (114, 115). In contrast, the IT administration of IL-12 is correlated with less toxicity and different methods are being evaluated in order to deliver IL-12 locally (149).

One approach is the use of particle-encapsulated cytokines in order to deliver the cargo in a specific (to certain cell types and tissues) and protected manner. IT administration of IL-12 encapsulated into polymer microspheres induces the regression of primary and metastatic murine lesions (116). These cytokine depots have shown their potential for anti-cancer therapies, but the challenge remains to translate their preclinical promise into a clinical application (123). The intra- or peritumoral use of a lipopolymer formulated human IL-12 plasmid has been tested in an early study including 13 ovarian cancer patients. An increase in IL-12 and IFNγ levels could be detected in peritoneal fluid (but not serum) and a minority of patients showed treatment-related decreases in serum levels of the tumormarker Cancer Antigen-125 (CA-125) (117).

Kamensek et al. tested the IT gene electrotransfer of TNF-α combined with IL-12 in murine melanoma tumors. This approach was proven feasible and effective in eliciting a potent and durable anti-tumor response, resulting in a delayed tumor growth and prolonged survival (161). This delivery method also found its way toward the clinic for the treatment of different cancer types including Triple Negative Breast Cancer (NCT02531425), lymphoma (NCT01579318), and Merkel cell carcinoma (NCT01440816), and the therapy induces objective systemic tumor responses in a significant number of melanoma patients (162).

Different preclinical studies using modified viruses expressing IL-12 resulted in strong anti-tumor immune responses associated with delayed tumor growth and increased survival in various murine cancer models (118–120).

Interleukin-2 (IL-2)

IL-2 is one of the most intensively studied cytokines in cancer immunotherapies, because of its important role in the development of an adaptive immune response. It has a wide spectrum of effects on the immune system including the expansion and differentiation of effector lymphocytes—crucial for the development of a specific anti-tumor response.

IL-2 is already approved by the FDA as a first-line treatment for patients with renal cell carcinoma and melanoma, although the systemic administration is associated with significant toxicity. To limit these toxicities, *in situ* delivery of soluble IL-2 has already been tested in a preclinical setting and resulted in the increased infiltration of $CD8^+$ T cells and reduced tumor growth in tumor bearing mice (121, 122).

Moreover, the IT injection of IL-2 encapsulated in polymeric microparticles for the treatment of brain or liver tumors, had better results than the use of modified tumor cells expressing IL-2 (123–125). Combining the IT injection of microparticles encapsulating IL-2 with microwave coagulation—to induce tumor cell death—resulted in a systemic tumor-specific immune response in mice bearing lung or hepatocellular carcinomas. These encouraging preclinical observations were extrapolated and tested in the clinic. Patients with renal cell carcinoma or melanoma who received IT treatment with either recombinant IL-2 or IL-2 encoding plasmids suffered from less toxicity (compared to systemic administration) and promising anti-tumor efficacy was observed. Although, treatment of renal cell carcinoma patients with an IL-2 encoding plasmid led to a low number of responses (163, 164), injection of recombinant IL-2 into melanoma metastases induced high response rates resulting in tumor regression. However, IT administration of one lesion failed to cause complete regression of untreated melanoma lesions and was not able to prevent the occurrence of metastases, indicating that the induced immune responses are not strong enough to result in an abscopal effect or to induce long-lasting memory responses (149, 165–167).

Different strategies combining IL-2 with other treatment modalities are heavily being investigated. The IT delivery of IL-2 together with the checkpoint inhibitor anti-CTLA-4, delivered either systemically or locally, represents a promising approach in melanoma patients (NCT01480323, NCT01672450). Preclinical data indicates that the use of TILT-123, a modified adenovirus expressing TNF-α and IL-2, in combination with checkpoint inhibitor or TIL therapy could be an effective treatment. The first phase I trial is planned in patients with advanced melanoma (168, 169). Moreover, different phase I and II studies investigating the combination of IL-2 and RT in renal cell carcinoma, melanoma and non-small cell lung cancer are ongoing (NCT01884961, NCT02306954, NCT030226236, NCT03224871).

Transforming growth factor-beta (TGF-β)

Inhibition of immunosuppression mediated by different soluble factors secreted by both the tumor cells and different immunosuppressive cell types infiltrating the TME can convert a "cold" tumor into a "hot" tumor. A known immunosuppressive cytokine that is often released after RT is TGF-β (66, 170, 171).

Preclinical studies have already investigated the effect of inhibiting TGF-β during and after RT and showed that this allows T cells to recognize multiple TAAs leading to a broad immune-mediated regression of both the irradiated tumor and the non-irradiated lesions (66). Currently, different clinical trials are ongoing where TGF-β inhibitors are combined with radiotherapy. Fresolimumab is being tested in the SABR-ATAC phase I/II trial in patients with stage Ia/Ib non-small cell lung cancer (NCT02581787). Two phase I studies are testing Galunisertib in rectal cancer and advanced hepatocellular carcinoma in combination with chemotherapy and RT (50.4–54 Gy in 1.8 Gy daily fractions; NCT02688712, NCT02906397). A phase I trial is testing LY3200882 and LY3300054 in combination with chemoradiotherapy in solid tumors (NCT02937272).

Immunomodulatory Molecules

In addition to the initial interaction between the TCR and MHC-molecules on APCs, costimulation of the T cells is crucial in order to develop an effective anti-tumor immune response. Different strategies can be envisaged to strengthen the costimulatory signals and prevent downregulation of these interactions in the TME.

Checkpoint inhibitors

To prevent auto-immunity and to control immune responses against self-antigens, inhibitory immune checkpoints are expressed on T cells. Currently approved checkpoint inhibitors target the molecules cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), PD-1, and PD-L1. These molecules play a key role in the regulation of immune responses and their expression is often dysregulated in the TME (both on tumor cells and immune cells) thereby preventing effective killing of the tumor cells by effector T cells. CTLA-4 blockade causes a broad enhancement of immune responses and the systemic delivery of anti-CTLA-4 blocking antibodies is currently FDA approved for the treatment of melanoma and renal cell carcinoma. However, the clinical success is hampered by dose-limiting toxicities and immune-related adverse events. Therefore, the IT administration of these checkpoint inhibitors is attractive. Most research is performed on the IT delivery of anti-CTLA-4 (since this was the first checkpoint inhibitor to be approved and is associated with higher toxicities then anti-PD-1/PD-L1).

The use of the slow-release agent Montanide ISA-51 to inject an anti-CTLA-4 antibody peritumorally resulted in local anti-tumor $CD8^+$ T cell activation and tumor eradication associated with thousand-fold lower serum levels of antibody compared to the systemic delivery—reducing the adverse events and the risk of auto-immunity (172).

OVs are ideal candidates to combine with monoclonal antibodies against inhibitory immune checkpoints. The IT injection of Newcastle disease virus combined with systemic injection of an anti-CTLA-4 antibody resulted in slower tumor growth, prolonged survival and protected the mice from a subsequent tumor rechallenge in a melanoma setting (126). The combination of T-VEC with ipilimumab was evaluated in a phase Ib study and showed a tolerable safety profile, with a greater efficacy of the combination compared to monotherapy with the single agents (127). More recently, preliminary data from an ongoing phase Ib trial (NCT02263508) showed a response rate in 62% of the treated melanoma patients with combination therapy of T-VEC and pembrolizumab (an anti-PD-1 antibody) (128). Moreover, oncolytic adenoviruses can be engineered to express blocking antibodies against CTLA-4. IT treatment with these viruses results in much higher concentrations of the antibody detected in the TME compared to the serum of mice, with the average plasma concentration staying below the limit that is well-tolerated in humans (129). Also other studies showed that treatment with attenuated viruses expressing blocking antibodies of CTLA-4 resulted in a delayed tumor growth and prolonged survival in murine models of both melanoma and lung cancer. Moreover, treatment with a combination of viruses expressing either an anti-CTLA-4 blocking antibody or GM-CSF resulted in complete tumor regression (130, 131).

Synergy between checkpoint inhibitors and radiation has been demonstrated in different preclinical tumor models, but at this moment the optimal timing of the treatment modalities, the dose, and fractionation regimen of the radiation, resulting in the highest responses are not yet clear warranting further research (69, 132–137). More than 100 clinical trials are currently testing the combinations of different checkpoint inhibitors with different radiotherapy regimens and preliminary data shows that there may be clinical benefit of the combination therapy in cancer patients (137–139).

CD40

CD40 is expressed by B cells, professional APCs, as well as non-immune cells and tumor cells. Under inflammatory conditions, CD40 ligand (CD40L) is transiently expressed on T cells and other non-immune cells, and binding to CD40 initiates a variety of molecular and cellular processes including the initiation and progression of cellular and humoral adaptive immunity (173).

Peritumoral injection of a slow-release formulation containing an agonistic anti-CD40 antibody was tested in preclinical tumor models and this treatment resulted in systemic tumor-specific CTL expansion and eradication of distant tumors (140). Another research group molecularly engineered an agonistic antibody with high affinity for CD40 (ADC-1013) and tested its effect in two different bladder cancer models. The IT administration of this immunostimulatory antibody resulted in a long-lasting anti-tumor response and immunological memory (141). A phase I clinical trial evaluating the safety and feasibility of the IT administration in patients with advanced solid tumors is already completed (NCT02379741).

mRNA vaccines can also be used to deliver activation stimuli in addition to TAAs to DCs. TriMix is a mix of three mRNA's encoding for a constitutive active form of TLR4 (caTLR4), CD40L, and CD70. The IT delivery of this mRNA mix (in various mouse cancer models) resulted in systemic therapeutic anti-tumor immunity. In addition, TriMix stimulated anti-tumor T cell responses to spontaneously recognized and internalized TAAs, including a neo-epitope (142).

Oncolytic adenoviruses expressing CD40L have been shown to induce significant anti-tumor effects in mice and patients (143, 144).

OX40 and CD137

OX40 and CD137 (4-1BB) are both members of the tumor-necrosis factor receptor superfamily, and are expressed on T cells, including TILs, as well as other immune cell subsets. Ligation of these receptors with their ligands delivers a costimulatory signal to T cells, necessary for their full activation. Targeting of both receptors has been assessed in early clinical trials and shows promising anti-tumor effects (145).

Two anti-CD137 monoclonal antibodies are currently in the clinic: Urelumab (Bristol-Myers Squibb) and PF-05082566 (Pfizer) (174). Unfortunately, Urelumab induced liver toxicity requiring dose reduction for subsequent trials and therefore the drug is now tested in different combination strategies but no longer as a monotherapy (145, 174). In contrast, PF-05082566 was not associated with any dose-limiting toxicities and is also under further investigation in combination with other immunomodulatory therapies (174).

A phase I clinical trial is ongoing where mRNA encoding for OX40 Ligand (OX40L) is intratumorally delivered in patients with refractory solid malignancies or lymphomas (NCT03323398). The anti-tumor effects of a mixture of mRNA molecules encoding for OX40L, IL-23, and IL-36γ in different mouse models after IT injection, either alone or in combination with checkpoint inhibitors is also being tested. Hebb et al. tested whether targeting both CD137 and OX40, in combination with the immune checkpoint inhibitor anti-CTLA-4, could result in a synergistic effect on tumor growth control and survival compared to the targeting of only one receptor. The triple combination administered intratumorally at low doses to one tumor had dramatic local and systemic anti-tumor efficacy in preclinical tumor models. Moreover, the IT administration resulted in superior local and distant tumor growth control, compared to the systemic delivery of the combination (145).

Targeting OX40 and 4-1BB with modified OVs has already proven their promise in preclinical mouse models and will soon be tested in a clinical setting (146, 147). In preclinical studies the use of OX40 led to an enhancement of T cell memory and proliferation, in combination with a suppression of Treg function showing the potential for combining OX40 agonists with RT, surgery or systemic agents (148). A phase I and a phase I/II clinical trial testing an agonistic antibody against OX40 with

cyclophosphamide and single fraction RT in metastatic prostate cancer (NCT01642290) and a OX40 agonist (MEDI6469) with different doses SBRT in metastatic breast cancer are currently active. A phase I clinical trial combining an anti-OX40 antibody (BMS-986178) with a TLR9 agonist (SD-101) and RT is tested in patients with low-grade B-cell Non-Hodgkin lymphomas (NCT03410901). This approach envisions the inhibition of tumor cell growth using the TLR9 agonist, activation of T cells by the anti-OX40 antibody and supplementary killing of cancer cells by radiation making them more visible for the immune system.

TLR Agonists

TLR2. Already 100 years ago William Coley injected Coley's toxins locally in the tumor resulting in regression of sarcoma. These data are translated in the use of Bacillus Calmette-Guérin (BCG) for the treatment of superficial urothelial carcinoma (175). BCG activates TLR2 and TLR4 in macrophages and DCs. This vaccine was primarily developed for the prevention of tuberculosis and is nowadays the standard treatment for patients with *in situ* or non-muscle invasive bladder cancer (176). The IT injection of a genetically engineered, lethal-toxin deficient strain of *Clostridium novyi*, that activates DCs via TLR2, can induce $CD8^+$ T cell mediated anti-tumor effects in preclinical renal cell carcinoma, colon carcinoma, and anaplastic squamous cell carcinoma models (177).

TLR3. A danger signal that is detected by endosomal TLR3 and the intracellular sensors RIG-I and MDA-5 is dsRNA (149). The IT delivery of poly-ICLC or Hiltonol, a synthetic analog of dsRNA, has already shown its potential in the clinic and a sequential treatment scheme of IT and intramuscular (IM) delivery of poly-ICLC was given to a young male patient with advanced facial embryonal rhabdomyosarcoma with extension to the brain. After treatment, the patient showed tumor inflammation, followed by gradual, marked tumor regression, with extended survival (178). Such results have prompted a phase II clinical trial (NCT01984892) in patients with advanced solid tumors receiving IT poly-ICLC to prime the immune system followed by IM poly-ICLC injections to boost the response. The idea is that these IT/IM booster injections will mimic a viral infection that will result in the release of TAAs upon IT injection and a strong activation of the immune response against these TAAs upon IM injection. Hiltonol is currently intratumorally tested in a phase I neoadjuvant setting in prostate cancer patients (NCT03262103). A phase I/II clinical trial combining IT Flt3L (CDX-301), Hiltonol and low-dose radiotherapy in B-cell lymphoma patients is ongoing (NCT01976585).

TLR4. In different transplantable murine tumor models it has been shown that IT treatment with TLR4 agonists, such as lipopolysaccharide (LPS) and monophosphoryl lipid A (MPL A), induces an anti-tumor immune response leading to regression of the tumor. In humans, the IT delivery of the synthetic TLR4 agonist Glucopyranosyl Lipid A (G100) has showed success in early clinical trials in eliciting Th1 polarized anti-tumor immunity in Merkel cell carcinoma and soft tissue sarcoma, in combination with RT (NCT02501473) (175, 176).

TLR7/8. Stimulation of TLR7/8 with ssRNA, significantly improves DC maturation, Th1 mediated immunity, cross-presentation of TAAs and humoral immune responses.

Imiquimod is an FDA approved small molecule TLR7/8 agonist, formulated as a dermal cream, for HPV mediated external genital warts, superficial basal cell carcinoma and actinic keratosis. Local imiquimod has been used successfully in immunotherapy combinations to treat transplantable mouse models (179, 180), and was tested in a randomized controlled trial (NCT0066872) in patients with nodular and superficial basal cell carcinoma and demonstrated to be superior to excision surgery. Currently imiquimod is tested in more than 100 clinical trials either alone or in combination with classical treatment modalities (150, 175, 176). Topical application of imiquimod resulted in histological regression in melanoma, superficial breast cancer metastases and in anti-tumor effects in T cell and B cell lymphomas (181–189). Promising abscopal effects could be seen after the topical administration of imiquimod in combination with local RT in a breast cancer mouse model. The treatment resulted in complete regression of locally treated tumors and inhibited tumor growth at untreated sites. This anti-tumor response is dependent on CD8+ T cells and an increase of T cell infiltration was noticed in the tumor lesions (149). The established anti-tumor effect could be augmented by pre-treatment with low-dose cyclophosphamide. This resulted in a protection from tumor rechallenge,suggesting that a long-term memory response against the tumor was induced in mice (180).

Another promising lipid-modified imidazoquinoline is 3M-052. It is evaluated as an adjuvant in many vaccine models and showed promising preclinical results in mouse melanoma and prostate tumor models. Moreover, the anti-tumor effect seen in melanoma mouse models was enhanced by concomitant CTLA-4 and PD-L1 blockade (149, 150, 175, 176, 190). Currently, a new TLR7/8 agonist, MEDI9197, is tested in the clinic. In this phase I study this agonist is delivered by IT injection to patients with solid tumors or cutaneous T cell lymphoma in combination with durvalumab and/or palliative radiation (NCT02556463).

TLR9. Bacterial DNA is sensed through the presence of unmethylated CpG motifs by endosomal TLR9. When CpG oligonucleotides were injected IT into human lymphoma lesions objective clinical responses were observed when combined with low-dose limited field RT (NCT00880581) (175, 191–193). Other combinatorial approaches are tested in the clinic in lymphoma patients; such as a phase I/II study combining SD-101, a TLR9 agonist in combination with ipilimumab (NCT02254772), a phase I trial combining anti-OX40 antibody (BMS-986178) together with SD-101 and RT (NCT03410901) and a phase Ib/II trial combining SD-101, ibrutinib and RT (NCT02927964). Treatment is generally well-tolerated, with a dose-related incidence of injection site reactions (149). Raykov et al. demonstrated that the oncolytic parvovirus H-1P enriched for CpG motifs can be used as an anti-tumor vaccine in a rat model for metastatic long cancer (194). Similar effects were observed with a CpG-enriched adenovirus used to treat mice bearing lung cancer and in melanoma models (195).

STING agonist

Foreign (viral or bacterial) DNA in cells, is processed via cGAS into cyclic dinucleotides, which are ligands for the intracytoplasmic sensor STING. Activation of the STING pathway leads to a cascade of events ultimately resulting in the transcription of pro-inflammatory IFNs and other genes associated with the innate immune system. Therefore, it was hypothesized that the use of STING agonists could promote an anti-tumor immune response. This hypothesis is supported by different preclinical studies showing that STING is a key mediator in the induction of a T cell response against tumors. Moreover, this pathway was shown to play a role in mediating the anti-tumor effects of different checkpoint inhibitors (196).

The first reported STING agonists are the anti-cancer flavonoids FAA, DMXAA and CMA. But, cyclic dinucleotides are more similar to the natural ligand cGAMP. IT injection of cyclic dinucleotides unleashes a powerful and often curative anti-tumor immune response in different transplantable tumor mouse models, with the induction of clear abscopal effects (197). A phase I clinical trial evaluating the IT injection of ADU-S100 in patients with (accessible) solid tumors and lymphomas (NCT03172936) (196) is ongoing. Another phase I trial investigates the anti-tumor effects of the combination of ADU-S100 and ipilimumab in patients with advanced solid tumors and lymphomas (NCT02675439).

Recently, it was demonstrated that radiation-mediated cure of immunogenic tumors is dependent on host STING (29). Therefore, the targeting of the cGAS/STING pathway in combination with RT is being investigated in preclinical models (24, 198, 199).

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The major benefit of immunotherapy is the generation of memory $CD8^+$ T cells thereby providing durable protection against metastasis and preventing relapse of the disease. One obvious limitation for *in situ* vaccination is the need to access the tumor for injection. However, modern imaging techniques, such as computed tomography guidance, enable accurate injection of different tumor types even deep within the body. The induction of tumor cell death and DC activation needs to occur simultaneously (in time and place) in order to lead to robust anti-tumor immune responses. By combining RT, OVs or physical therapies with the local delivery of immunomodulatory factors, both can be achieved resulting in potent immune responses. The challenge for *in situ* vaccination is to develop an optimal approach to circumvent local immunosuppression, which is characteristic for tumors, simultaneously resulting in an effective systemic anti-tumor immune response. It is clear that treating a patient with an *in situ* vaccine early in the disease will have the best results since the immune system of patients with metastatic disease will be weaker due to the presence of more immunosuppressive factors. The evaluation of different *in situ* vaccines in early diagnosed patients without evidence of metastatic disease, for example as neoadjuvant therapy prior to surgery, will show the true potential of *in situ* vaccination strategies and combinations for the treatment of cancer patients.

AUTHOR CONTRIBUTIONS

HL, WdM, SdM, and SM wrote sections of the manuscript. MD and KT performed a thorough review of the manuscript adding suggestions for papers to include in the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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REVIEW
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Dendritic Cell Cancer Therapy: Vaccinating the Right Patient at the Right Time

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Immune checkpoint inhibitors propelled the field of oncology with clinical responses in many different tumor types. Superior overall survival over chemotherapy has been reported in various metastatic cancers. Furthermore, prolonged disease-free and overall survival have been reported in the adjuvant treatment of stage III melanoma. Unfortunately, a substantial portion of patients do not obtain a durable response. Therefore, additional strategies for the treatment of cancer are still warranted. One of the numerous options is dendritic cell vaccination, which employs the central role of dendritic cells in activating the innate and adaptive immune system. Over the years, dendritic cell vaccination was shown to be able to induce an immunologic response, to increase the number of tumor infiltrating lymphocytes and to provide overall survival benefit for at least a selection of patients in phase II studies. However, with the success of immune checkpoint inhibition in several malignancies and considering the plethora of other treatment modalities being developed, it is of utmost importance to delineate the position of dendritic cell therapy in the treatment landscape of cancer. In this review, we address some key questions regarding the integration of dendritic cell vaccination in future cancer treatment paradigms.

Keywords: dendritic cell, vaccination, immunotherapy, checkpoint inhibitor, cancer, adjuvant

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INTRODUCTION

Since William Coley made his early contributions to the study of cancer immunotherapy in the 1890s, harnessing the capabilities of the immune system to eliminate cancer cells remained a long-sought dream (1). In the last decade, efforts to realize this dream were finally rewarded with the introduction of immune checkpoint inhibitors (ICI). ICI showed the feasibility of immunotherapy and revolutionized the treatment of cancer. The success of ICI spurred a considerable amount of research activity into the field of immunotherapy. Despite its resounding success, ICI still have two important limitations: they are associated with significant (immune-related) toxicity and a portion of patients does not respond (2–7). Immunotherapy however, encompasses more than ICI alone. Dendritic cell (DC) vaccination is an alternative form of immunotherapy and is a prime candidate to enrich the treatment possibilities for cancer. Considering the fact that the field of immunotherapy is a fast-moving field, it is of utmost importance to delineate the position of DC vaccines in the therapeutic landscape of cancer. In this review, we will explore some important questions regarding this position, with the focus on four malignancies (glioblastoma, melanoma, prostate cancer, and renal cell carcinoma) in which phase III trials with DC vaccines have been performed or are ongoing.

The Evolving Field of Immune Checkpoint Inhibition

Currently, the clinical application of immunotherapy is mainly defined by ICI. ICI target immune checkpoint molecules such as CTLA-4, PD-L1, and PD-1. These molecules have immune response inhibiting functions and are involved in the prevention of autoimmunity and the maintenance of peripheral tolerance. It is well known that tumor cells are able to upregulate the expression of checkpoint molecules, leading to anergy of cytotoxic T-cells in the tumor microenvironment. CTLA-4, PD-L1, and PD-1 have distinct functions; CTLA-4 exerts its inhibitory functions on the initial T-cell activation whereas PD-1 and PD-L1 have roles in the inhibition of the effector functions of T-cells (8, 9). ICI antagonize these molecules and thereby aim to augment the anti-cancer immune response.

In 2010, ipilimumab (a monoclonal antibody targeting CTLA-4) was the first immunotherapeutic agent providing clinical benefit in cancer patients, extending median overall survival (OS) to 10 months (compared to 6.4 months for the control group receiving a gp100 peptide vaccine) in metastatic melanoma (3). With an overall response rate (ORR) of ∼10–20%, ipilimumab was a great improvement compared to the standard of care at the time, but it still offers clinical benefit in only a portion of melanoma patients (10, 11). However, in a substantial portion of responding patients, clinical benefit is durable (5). In 2014, two monoclonal antibodies (pembrolizumab and nivolumab) targeting the PD-1 pathway were also approved for the treatment of metastatic melanoma. Compared to ipilimumab, anti-PD-1 inhibition achieves a higher ORR of ∼40% (4, 5, 12, 13).

After these landmark studies, research into ICI accelerated. With the addition of PD-L1 targeting agents avelumab, atezolimumab, and durvalumab, the field of ICI now encompasses six FDA and EMA-approved monoclonal antibodies (mAb) (14–16). Most of these ICI are approved for the treatment of multiple malignancies (**Table 1**). The number of approved indications of these mAb is likely to grow as they are currently tested in a large number of additional malignancies (17).

Besides PD-1, PD-L1 and CTLA-4, other checkpoint molecules (such as TIM-3 and LAG-3) have shown to inhibit the anti-cancer immune response (18). Several mAb targeting these alternative checkpoint molecules are in various stages of clinical investigation. Therefore, it is expected that the number of clinically available mAb will be further expanded (17). In addition to the treatment of metastatic disease, research is moving toward the application of ICI in the adjuvant treatment of cancer. For example, adjuvant ipilimumab, nivolumab, and pembrolizumab after surgically resected stage III melanoma recently have shown to improve progression-free survival (PFS) and in case of adjuvant ipilimumab, an prolonged OS was seen (19–21).

ICI come with a different toxicity profile compared to other anti-cancer therapeutics, caused by specific immune-related side effects. Monotherapy with anti-PD-1 mAb and anti-CTLA-4 mAb are associated with 10–16% and 30–40% grade 3 or 4 adverse events, respectively (3, 5, 6, 11, 22). In contrast, DC vaccination is associated with little toxicity as grade 3 or 4 adverse events are very uncommon (23–25). In addition, the application of DC vaccination might further improve response rates on ICI.

TABLE 1 | Indications of the six currently approved monoclonal antibodies in the treatment of cancer (as of May 2018).

Monoclonal antibody	Target	FDA/EMA-approved indications
Ipilimumab	CTLA-4	Melanoma
Nivolumab	PD-1	Melanoma, NSCLC, RCC, urothelial carcinoma, MSI-high/dMMR CRC, HCC, Hodgkin's lymphoma, HNSCC
Pembrolizumab	PD-1	Melanoma, NSCLC, HNSCC, urothelial carcinoma, Hodgkin's lymphoma, MSI-high cancer, gastric/gastroesophageal cancer
Avelumab	PD-L1	Merkel cell carcinoma, urothelial carcinoma
Atezolimumab	PD-L1	Urothelial carcinoma, NSCLC
Durvalumab	PD-L1	Urothelial carcinoma, NSCLC
Combined treatment with ipilimumab and nivolumab	CTLA-4/PD-1	Melanoma, RCC

CRC, colorectal cancer; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; HCC, hepatocellular carcinoma; HNSCC, head and neck squamous cell carcinoma; dMMR, DNA mismatch repair deficiency; MSI, microsatellite instability; NSCLC, non-small-cell lung carcinoma; PD-1, programmed cell death protein; PD-L1, programmed death-ligand 1; RCC, renal cell carcinoma.

Dendritic Cell Vaccination

Since their discovery by Steinman in 1973, it became clear that DC are antigen-presenting cells crucial in activating the adaptive immune system (26). DC are spread throughout the body, constantly monitoring their surroundings for antigens and danger signals. Once stimulated by an activating stimulus, they undergo maturation and migrate to lymphoid organs where they activate several effector cells of the immune system, primarily T-cells and B-cells (27).

Through this process, DC are vital for immunosurveillance. Immunosurveillance signifies the crucial role of the immune system in the detection and elimination of both pathogens and cancer cells. However, the development of malignancy is an indolent process in its early stages, therefore, immunosurveillance occasionally fails. At an early stage, tumors sometimes silence an initiated immune response or fail to express the "danger signals" necessary for the activation of the immune system. When the process of immunosurveillance fails, one of the hurdles for the outgrowth of cancer cells is omitted. DC vaccination aims to correct this failure by reversing the ignorance of the immune system to malignant cells. To achieve this, DC are stimulated *ex vivo* with danger signals and loaded with tumor-specific antigen(s) on their major histocompatibility complex molecules with the intent of activating antigen-specific T-cells which selectively eliminate antigen-bearing cancer cells (**Figure 1**). The majority of research groups, including our own, employ treatment schemes with multiple administrations of DC vaccine to induce immunological memory (28).

DC vaccines are produced following some basic principles (**Figure 2**). Natural circulating DC or monocytes are isolated

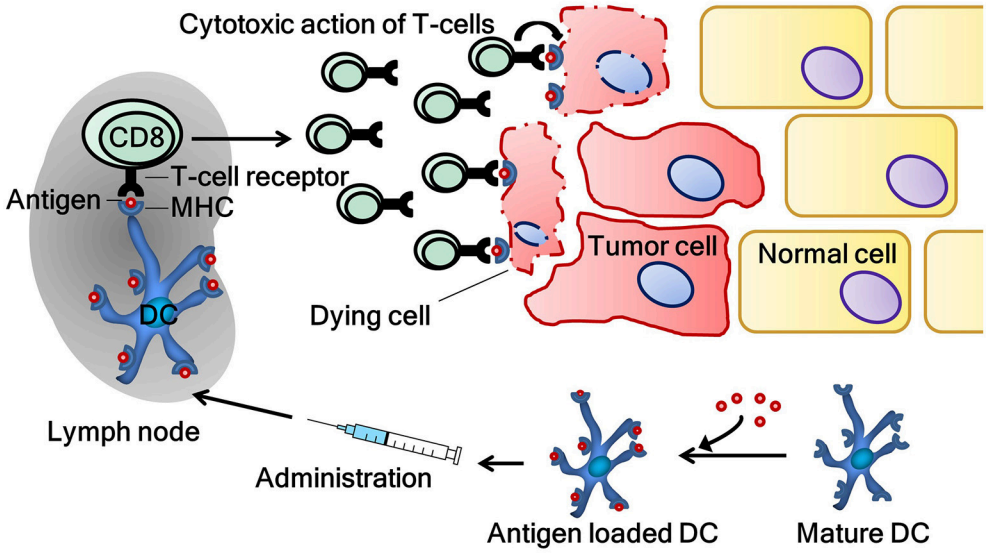

FIGURE 1 | The induction of a tumor-specific immune response by dendritic cell vaccination. Tumor antigen-specific T-cells are activated by dendritic cells, which are *ex vivo* loaded with tumor antigen(s). Activated T-cells subsequently patrol the body in search of their respective antigen. When their target is found, T-cells exert their cytotoxic functions on cancer cells. CD8, cluster of differentiation 8 (cytotoxic T-cell); DC, dendritic cell; MHC, major histocompatibility complex.

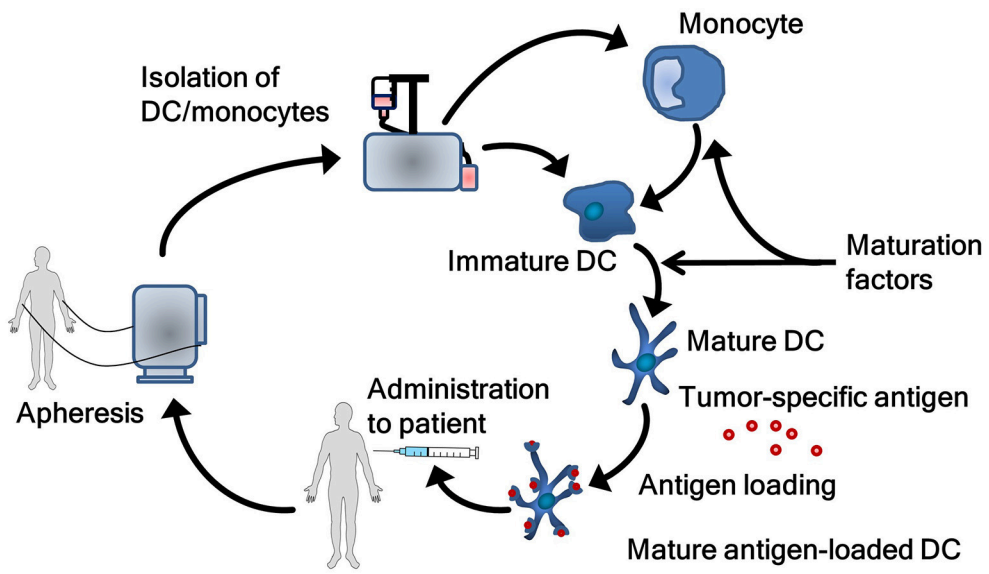

FIGURE 2 | The process of generating dendritic cell vaccines. Autologous dendritic cells or monocytes are obtained via an apheresis procedure. Monocytes first have to be differentiated into dendritic cells. Subsequently, dendritic cells are matured and loaded with tumor antigen. Finally, the dendritic cells are administrated to the patient. DC, dendritic cell.

from autologous peripheral blood mononuclear cells obtained by apheresis. In case of monocytes, *ex vivo* differentiation into DC are required. Both natural circulating DC and monocyte-derived DC are matured as this is essential for effective T-cell activation. Maturation is associated with functional and morphological changes in DC. Following maturation, DC show enhanced expression of major histocompatibility complexes I and II, co-stimulatory molecules and increased capability of cytokine production. These processes are vital, as not or incompletely matured DC can induce tolerance rather than immunity (29). During the process of vaccine manufacturing, DC are loaded with relevant tumor antigen(s) to induce a tumor-specific immune response in the patient. As with the other steps in the process of manufacturing DC, several methods to load DC with antigen exist (30). After quality control, vaccines are administered to the patient.

Despite these basic principles, protocols describing the specific details of DC vaccination manufacturing in trails vary widely. Differences in these protocols cover all aspects of DC vaccination including culture methods, the usage of DC subsets, maturation methods, antigen loading techniques, used antigens and the route of administration. Especially, the subset of DC used, the method of maturation and the choice of antigen(s) are subject of intense research. For example, several groups, including our own, use natural circulating DC instead of monocyte-derived DC. Natural circulating DC do not require extensive culturing which is believed to retain their functionality. Different maturation techniques are also being explored, such as the use of toll-like receptor ligands or electroporation with mRNA-encoding proteins that induce DC maturation (31, 32). Another exciting recent development is the use of neoantigens, which are newly, formed antigens generated from tumor-specific mutated genes, for loading on DC (33). Finally, a more recent development is the recognition that DC, in addition to immune-activating properties, can acquire effector functions (so called killer-DC) following triggering with several differentiating and maturating agents such as interferon (IFN) or lipopolysaccharide (34). Despite these developments, addressing the differences in the generation and production of DC vaccines extensively is beyond the scope of this review.

Regardless of the precise protocol employed, DC vaccination is associated with a very favorable toxicity profile. The majority of side effects reported in various clinical trials were short-lived grade 1 or 2 adverse events, consisting of self-limiting flu like symptoms, fever and local injection site reactions. Treatment-related grade 3 or 4 adverse events following DC vaccination as standalone therapy are uncommon (23, 24).

The goal of DC vaccination is to kill tumor cells by the generation of functional antigen-specific T-cells (23). Despite the challenges associated with measuring the immunological effect of DC vaccination, immunological endpoints are reported in a substantial portion of phase I/II clinical DC vaccination trials using various methods. Several studies even report the generation of antigen-specific T-cells to be positively correlated with survival, strengthening the believe that DC vaccination can result in clinical benefit (25, 35, 36).

Besides the generation of T-cells, intense research is ongoing to find biomarkers, not only for DC vaccination but for immunotherapy in general. Considering ICI treatment, research into predictive biomarkers has revealed several biomarkers predictive for response on ICI (such as mutational burden, PD-L1 expression, and others) (37, 38). Similarly, an example of a predictive biomarker prior to the start of therapy correlated with clinical outcome after DC vaccination is the immune landscape of tumors (39). Up until now, however, biomarkers cannot reliably guide treatment decisions in the clinic for neither ICI nor other forms of immunotherapy, probably owing to the fact that a functional immune response is a complex and multi-step process (40).

The Role of ICI and DC Vaccination in Metastatic Disease

Response rates to DC vaccination vary among cancer types with most studies showing response rates between 10 and 15% (24). Most clinical studies concerning DC vaccination were performed in patients with metastatic disease. Although head-to-head comparisons are not available, ICI achieve superior clinical benefit compared to DC vaccination in most malignancies. In particular for metastatic melanoma and metastatic renal cell carcinoma (RCC), ICI compare favorably in terms of response rates (approximate ORR on anti-PD-1 mAb in RCC: 25%; in melanoma: 40 and 58% when combined with anti-CTLA-4 mAb) (4, 10, 11, 41). ORR in RCC and melanoma patients after treatment with DC vaccines is less, 12 and 9%, respectively (24). Even more important, whereas overall survival benefit for patients with metastatic RCC and metastatic melanoma after ICI treatment is well established, the OS gain for these patients after DC vaccination is less clear (3, 11, 24, 41).

The immunotherapeutic landscape of metastatic castration-resistant prostate cancer (mCRPC) is very different from that of metastatic RCC and metastatic melanoma. Two phase III trials investigating ipilimumab showed, both in pre-docetaxel and post-docetaxel setting, no improvement in OS compared to their control groups (42, 43). Pembrolizumab has shown clinical activity in patients with any type of cancer bearing DNA mismatch repair deficiency (dMMR) and/or microsatellite instability. Individual reports of clinical benefit on anti-PD-1 mAb for patients with dMMR prostate cancer do exist. Unfortunately, dMMR is present in only about 5% of mCRPC patients (44–47). Similar to patients with dMMR, ICI possibly provide benefit in other subgroups of mCRPC patients. For example, nivolumab combined with ipilimumab was tested on patients with an ARV7 mutation which predisposes for a more aggressive form of prostate cancer. In this study, 4 out of 15 patients showed clinical benefit (47). In addition, pembrolizumab has shown some efficacy in a group of patients who progressed after enzalutamide treatment. In a trial of 20 patients, 11 had a partial response or stable disease (45). These patients might be more susceptible to PD-1 antibodies, as PD-1 was shown to be upregulated on DC in patients progressing after enzalutamide (46). After the failure of ipilimumab in prostate cancer patients, a delay in designing new studies with ICI occurred. Currently, ∼35 clinical studies with ICI are enlisted for prostate cancer, usually as combination therapies.

Notably, sipuleucel-T gained approval for the treatment of asymptomatic or minimal symptomatic mCRPC. Sipuleucel-T is manufactured from autologous mononuclear cells obtained via apheresis. These cells are incubated with PA2024, a fusion protein of the tumor antigen prostatic acid phosphatase (PAP) and granulocyte-macrophage colony-stimulating factor (GM-CSF). As DC are not specifically isolated from the apheresis product and the end product contains a variety of cells, sipuleucel-T should strictly speaking not be regarded as a pure DC vaccine. Despite this, sipuleucel-T is generally addressed as a DC based-vaccine and is considered to be the first DC-based therapy approved by the FDA. The approval of sipuleucel-T followed the results of a phase III trial including 512 mCRPC patients. The median survival was prolonged with 4 months compared to placebo (48). Another smaller phase III study confirmed these favorable results (49).

Initial enthusiasm about sipuleucel-T has somewhat subsided in recent years since labor intensive production resulted in

a highly priced cellular product (around $125.000). At the moment, sipuleucel-T is only available in the USA as market authorization was not granted by the EMA. Recently, a Chinese conglomerate (Sanpower) acquired Dendreon (producer of sipuleucel-T) for over $800 million with the intention to extend the market to Asia. Sipuleucel-T enhanced immune responses toward its antigen (PAP/PA2024). A PAP/PA2024-specific immune response (which is defined as the generation of antigen-specific antibodies, antigen-specific T-cell activation and/or antigen-specific T-cell proliferation) was seen in 79% of patients. The immune responses correlated with OS and could be beneficial for the response on subsequent or concomitant immunotherapeutics, a paradigm which will be detailed in the final chapter of this review (50).

In conclusion, in metastatic malignancies such as non-small-cell lung cancer, melanoma, urothelial cancer and RCC, where ICI are particularly effective, it is unlikely DC vaccination will gain a role as monotherapy in widespread metastatic disease due to its less established clinical benefit.

Rationale for DC Vaccination in the Adjuvant Treatment of Cancer

Besides the application of anti-cancer therapeutics in the treatment of metastatic disease, the adjuvant treatment of patients after surgery of local disease is also common practice in oncology. Surgical resection with curative intent aims to excise all tumor burden. However, depending on the type of malignancy, occult residual disease remains in a variable portion of patients and can eventually lead to relapse (51). Adjuvant treatment aims to kill cancer cells, thereby reducing the chance of relapse. With advancing knowledge of the interaction between the immune system and cancer, it becomes increasingly clear that higher tumor load is associated with higher tumor-induced immune suppression. For example, regulatory T-cells (Treg) and myeloid derived suppressor cells (MDSC) attracted by tumor cells induce anergy in T-cells (52). Moreover, several soluble factors secreted by tumor cells, such as TGF-β, IL-10 and VEGF, are recognized to suppress infiltrated effector T-cells (53–55). Also, tumors are able to upregulate indoleamine 2,3-dioxygenase (IDO) which converts tryptophan to kynurenine, inhibiting effector T-cells through a mechanism not completely understood (56). Tumor load-associated immune suppression is generally regarded as the underlying cause of the low clinical response to DC vaccination in metastatic disease (57). Indeed, in our group we detected antigen-specific T-cells in 71% of melanoma patients following adjuvant DC vaccination compared to 23% following vaccination in the metastatic setting (58, 59). In the adjuvant setting, the possibly remaining occult disease represents a low tumor burden, and hence less immune suppression (**Figure 3**). Therefore, DC vaccination may be more successful in the adjuvant compared to the metastatic setting.

There are some additional arguments to consider DC vaccination as an adjuvant treatment option. Besides efficacy, a low toxicity profile is an important hallmark of any adjuvant treatment as a substantial portion of cancer patients receiving adjuvant treatment would not endure a relapse even without this adjuvant therapy. As noted before, DC vaccination is associated with little toxicity, not only compared to chemotherapy but also compared to ICI. In addition, besides a direct clinical benefit for patients, adjuvant DC vaccination might also prove to be beneficial in improving response to subsequent treatment in case of relapse. In theory, tumor-specific T-cells induced by adjuvant DC vaccination might result in an increased tumor-specific immune response when ICI are given at a later moment in the metastatic setting. Indeed, this effect has been observed retrospectively with administration of ipilimumab in patients with relapse after adjuvant DC vaccination for stage III melanoma (60). In addition to ipilimumab, a similar effect was also seen retrospectively in glioblastoma (GBM) patients receiving chemotherapy after DC vaccination (61). These additive effects should be considered when integrating DC vaccines in the therapeutic landscape of cancer. Considering these arguments, the next part will focus on data obtained with DC vaccines in the adjuvant setting.

Adjuvant DC Vaccination in Glioblastoma

Adjuvant DC vaccination has been studied in GBM. In contrast to most malignancies, distant metastases seldom occur in GBM (62). Nonetheless, GBM represents a lethal disease, with patients having a median survival of ∼15 months (63). GBM is commonly treated with maximally safe surgery and adjuvant temozolamide (TMZ) in conjunction with radiotherapy, the so-called Stupp protocol (64). However, even with extensive treatment, residual disease invariably remains, and recurrence is certain. This results from the infiltrative growth and lack of a distinct border between normal brain tissue and tumor. Therefore, DC vaccination in the adjuvant setting after surgery in GBM is different from for example adjuvant DC vaccination in RCC and melanoma in which complete disease control after surgery is possible. In this review, we consider DC vaccination to be adjuvant when it is integrated in treatment protocols after maximally safe surgery in newly diagnosed GBM.

Historically, the central nervous system is considered an immune-privileged site, casting doubt whether GBM could be susceptible to immunotherapy. However, in recent years it has become increasingly clear the central nervous system is subject to active immunosurveillance even with an intact blood-brain barrier (65). Albeit not yet vigorously explored, the research into the treatment of GBM with ICI has not yet resulted in proof of efficacy. Nivolumab is the ICI furthest in clinical development, a phase III trial comparing nivolumab to bevacizumab for the first recurrence after radiotherapy and TMZ is currently ongoing (NCT02017717). Final results are not yet reported in a peer-reviewed journal, but presented results revealed that the primary end-point was not met (median OS in recurrent disease: 9.8 months with nivolumab vs. 10.0 months with bevacizumab) (66). Individual reports of response on anti-PD-1 mAb monotherapy do exist, although these are isolated cases concerning tumors with high mutational load (67–69). With these results in mind and the fact that mutational load and number of tumor infiltrating lymphocytes in GBM are generally low, it is doubtful whether

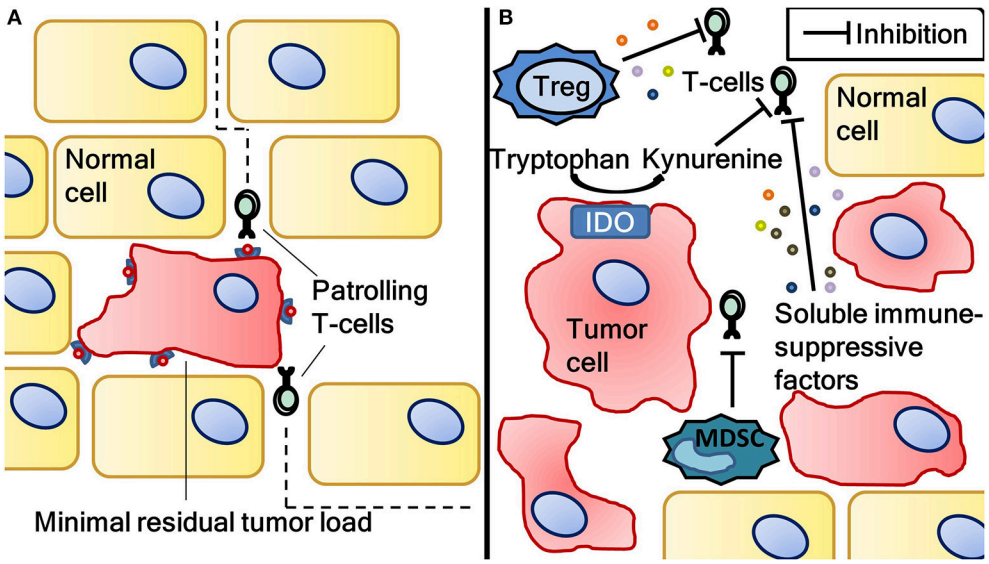

FIGURE 3 | The difference in tumor load-associated immune suppression between minimal residual disease and a situation with high tumor load. Antigen-specific T-cells induced by dendritic cell vaccination eliminating minimal residual disease after surgical resection of cancer **(A)**. Minimal residual disease is associated with less immune suppression as opposed to a situation with more tumor load **(B)**. Tumor load-associated immune suppression is caused by (among other factors) regulatory T-cells, myeloid derived suppressor cells, soluble immune suppressive factors (such as IL-10, TGFβ and VEGF) and indoleamine 2,3-dioxygenase activity. Vaccination-induced T-cells can be rendered anergic by this immune suppression, resulting in inferior clinical results. Therefore, dendritic cell vaccination might be more effective in the adjuvant setting. IDO, indoleamine 2,3-dioxygenase; MDSC, myeloid derived suppressor cells; Treg, regulatory T-cells.

ICI as monotherapy have promise as a future treatment option (70, 71).

Next to monotherapy with ICI, ICI combined with other standard treatment modalities is being investigated in phase III trials. For example, CheckMate 498 (comparing TMZ and radiotherapy to nivolumab and radiotherapy) and the CheckMate 548 (comparing radiotherapy, TMZ, and nivolumab to radiotherapy, TMZ and placebo), both involving nivolumab, are currently ongoing. Similar phase I and II trials combining pembrolizumab or ipilimumab with TMZ and radiotherapy are being performed. Results on such integration of ICI in standard treatment strategies are not yet reported.

Considering DC vaccination studies concerning GBM, DC-based therapy is often integrated into the standard adjuvant treatment for GBM. As of now, the only available phase III trial data involving DC vaccines in GBM are the very recently published interim results of an ongoing clinical study involving a vaccine called DCVax®-L (see also **Table 2**) (72). DCVax®-L is a vaccine manufactured from autologous DC loaded with tumor lysate derived from autologous GBM cells. Unblinded data on 331 patients with newly diagnosed GBM was presented. After surgery, patients were randomized (2:1) to receive either DCVax®-L incorporated into standard of care (TMZ and radiotherapy) or standard of care alone. Due to the study design, which enabled crossover from the standard of care to the vaccination arm upon progression, a total of 86% of patients received vaccination at the time of interim analysis. The authors compare the median OS of 23.1 months for the entire study population with OS data from comparable patients in different trials (which have a reported median OS of 15–17 months), from this comparison they suggest a clinical benefit from their vaccine. The definite results on clinical outcome, including PFS data, are eagerly awaited.

TABLE 2 | Active phase III clinical trials concerning dendritic cell vaccination as adjuvant treatment in various malignancies (as of May 2018).

Disease	Vaccine formulation	Status	Identifier
Melanoma (stage III)	Natural dendritic cell subsets loaded with melanoma-specific peptides	Recruiting	NCT02993315
Uveal melanoma (high risk)	Dendritic cells loaded with autologous tumor RNA	Recruiting	NCT01983748
Glioblastoma (newly diagnosed)	DCVax®-L: dendritic cells loaded with tumor lysate	Active, not recruiting	NCT00045968

Previously, the favorable toxicity profile of DC vaccination was shown in several phase I/II studies showing the safety of adjuvant DC vaccination in GBM (73–78). Important to consider is that in these studies, DC vaccination was often combined with chemotherapy and/or radiotherapy, this combination had little added toxicity compared to chemotherapy and/or radiotherapy without DC vaccination. Despite not being designed for the purpose of assessing clinical outcome, these studies reported favorable median OS compared to their respective control groups ranging from 15 up to 41 months (74, 75, 77, 78). Furthermore, a positive correlation was shown between survival and presence of an immune response after vaccination (61).

Clinical outcome as primary endpoint was reported in several phase II studies. One of the largest studies completed to date involving DC vaccination in GBM, was performed by Ardon et al. and included 77 patients with newly diagnosed GBM (79). There was no control group, all patients received adjuvant DC vaccination integrated in standard treatment with TMZ and radiotherapy after complete resection of their GBM. The study reported favorable median OS of 18.3 months compared to the 14.6 months achieved in the landmark study by Stupp et al (64).

In conclusion, preliminary results on ICI in GBM make it very doubtful monotherapy with ICI will ever gain traction for this indication, results of large trials concerning ICI combined with chemoradiotherapy are pending. For DC vaccination in combination with chemoradiotherapy in GBM, occasionally favorable clinical outcomes have been reported. Due to strict inclusion criteria of these studies, the results are hard to interpret and compare with existing literature. Therefore, these result warrant further research with randomized phase III trials and additional data from the DCVax®-L trial are awaited.

Adjuvant DC Vaccination in RCC and Melanoma

Besides GBM, both RCC and melanoma in certain stages also exhibit high recurrence rates after surgery. For melanoma, the risk of relapse is particularly high when the disease has metastasized to regional lymph nodes (stage III). Melanoma with lymph node metastasis has a 5-year survival rate ranging from 40% (stage IIIC) to 78% (stage IIIA) (80). In RCC, recurrence of disease following surgery is also common, resulting in a declining survival rate with increasing stage (81).

Melanoma and RCC are similar in the sense that both tumors are very chemo-resistant and that their adjuvant treatment strategy in the pre-ICI era was mainly based on cytokine treatment with IL-2 and IFN-α (82, 83). In both cancers, IL-2 and IFN-α provide little clinical benefit and are associated with high toxicity. For melanoma, ipilimumab showed clinical activity in the adjuvant setting with a 5-year recurrence-free survival rate of 41% compared to 30% in the placebo group (hazard ratio for recurrence or death, 0.76; p<0.001). Importantly, 5-year distant metastasis-free survival rate was also improved with 48% compared to 39% (hazard ratio for death or distant metastasis, 0.76; $p = 0.002$) (21). Although these results show efficacy, the application of adjuvant ipilimumab is opposed by its significant toxicity (∼40% of patients experience immune-related grade 3 or 4 adverse events) (21, 84). In addition, both nivolumab and pembrolizumab have shown to increase PFS in the adjuvant setting for melanoma (19, 20). Adjuvant nivolumab was tested against ipilimumab in completely resected stage IIIB, IIIC and IV melanoma. In this study adjuvant nivolumab improved the 1-year PFS rate to 72.3% compared to 61.6% in ipilimumab-treated patients. Similarly, adjuvant pembrolizumab was compared to placebo in stage IIIA, IIIB and IIIC melanoma. The 1-year PFS rates were 75% and 61%, respectively. Despite pending OS data, both the FDA and EMA recently granted approval for adjuvant nivolumab and are considering approval for adjuvant pembrolizumab.

For RCC, adjuvant treatment is also available. Adjuvant sunitinib, a tyrosine kinase inhibitor, for RCC has gained approval by the FDA based on improved PFS (6.8 months vs. 5.6 months for placebo; hazard ratio for recurrence, 0.76; $p = 0.03$). However, utility is limited due to high toxicity and lack of OS gain (85). Based on these considerations, the EMA has, in contrast to the FDA, adopted a negative opinion for the adjuvant application of sunitinib. In contrast to melanoma, for RCC no results on adjuvant ICI have been reported. However, several adjuvant clinical trials are ongoing, including the combination of ipilimumab and nivolumab (NCT03138512); atezolizumab (NCT03024996); pembrolizumab (NCT03142334) and nivolumab (NCT03055013) (82).

In both melanoma and RCC, DC vaccination has also been investigated as adjuvant treatment. Retrospective analysis from our group showed clinical benefit in stage III melanoma patients adjuvantly treated with monocyte-based DC vaccination compared to matched controls. In this study, OS for 78 patients treated with DC vaccines doubled compared to the 209 controls (63.6 months vs. 31.0 months; hazard ratio 0.59; p = 0.018) (58). Markowicz et al. have shown similar results in a prospective study concerning a peptide-loaded DC vaccine. In 22 vaccinated patients the study achieved a 3-year OS of 68% compared to 26% in the 22 patients of the matched historical control group ($p = 0.029$). The primary endpoint however, 3-year PFS rate, was not significantly improved probably due to the small number of patients (vaccinated patients: 41%; controls 15%; $p = 0.108$) (86). No phase III trials currently have been completed on adjuvant DC for melanoma. However, our group is currently conducting a trial which involves the employment of natural circulating DC vaccines in patients with stage IIIB or stage IIIC melanoma (NCT02993315) (**Table 2**).

In RCC, research on DC vaccination is mainly focused on metastatic disease and little data regarding adjuvant DC vaccination is available. However, a phase III trial was performed with adjuvant DC vaccination in various stages of disease. Patients vaccinated with DC loaded with tumor lysate in combination with cytokine-induced killer cells were compared to patients treated with IFN-α. Mainly due to a very heterogeneous study population, no definitive conclusions could be drawn. However, the study showed significant PFS and OS benefit suggesting that further research on adjuvant DC vaccination in RCC is warranted (87).

Currently, too little data is available to claim that DC vaccination is effective in the adjuvant setting. Yet, the above presented data, show favorable clinical results and consistently confirm the limited toxicity in a variety of cancers. More robust prove of efficacy may be under way as several phase III trials on adjuvant DC vaccination are currently being performed (**Table 2**). Whether DC vaccination acquires a definitive role in the adjuvant treatment of cancer will also be dependent on the results of ongoing phase III trials assessing other adjuvant treatments, including trials with ICI (88).

TABLE 3 | Ongoing clinical trials concerning dendritic cell vaccination in combination with clinically approved immune checkpoint inhibitors (ipilimumab, nivolumab, pembrolizumab, avelumab, atezolimumab, and durvalumab) in solid tumors.

Immune checkpoint inhibition (target molecule)	Vaccine formulation	Malignancy	Status	NCT-identifier
Combined Ipilimumab and Nivolumab (CTLA-4/PD-1)	DC with the insertion of the p53 gene	SCLC	Recruiting	NCT03406715
Nivolumab (PD-1)	DC loaded with CMV pp65 mRNA	Recurrent brain tumors	Active, not recruiting	NCT02529072
Nivolumab (PD-1)	DC loaded with NY-ESO-1 peptide	NY-ESO-1^{+} solid tumors	Recruiting	NCT02775292
Nivolumab (PD-1)	DC loaded with autologous tumor lysate	Recurrent glioblastoma	Not yet recruiting	NCT03014804
Pembrolizumab (PD-1)	DC loaded with peptide	Melanoma	Recruiting	NCT03092453
Pembrolizumab (PD-1)	DC-CIK	Solid tumors, NSCLC, Mesothelioma	Recruiting	NCT03190811 NCT03360630 NCT03393858
Nivolumab or Pembrolizumab (PD-1)	DC-CIK	Refractory solid tumors	Recruiting	NCT02886897
Avelumab (PD-L1)	DC/AML fusion vaccine	Colorectal cancer	Not yet recruiting	NCT03152565

CIK, cytokine induced natural killer cells; CMV, cytomegalovirus; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; DC, dendritic cells; NSCLC, non-small-cell lung cancer; SCLC, small-cell lung cancer; PD-1, programmed death-1; PD-L1, programmed death ligand 1.

The Combination of DC Vaccination and Other Modalities for the Treatment of Metastatic Disease

As noted before, the clinical benefit of monotherapy DC vaccination for patients with metastatic disease is probably limited. However, the ultimate role for vaccines may lie in the combination with other modalities. The generation of a cellular immune response upon DC vaccination is commonly reported and may potentiate the effect of other anti-cancer therapeutics (23). Conversely, tumor reduction caused by chemotherapy, radiation therapy or targeted therapy can alleviate tumor-induced immune suppression which hinders efficacy of DC vaccination. However, possible synergies involve more than the mere reduction of tumor load as modalities other than immunotherapy also exhibit immunogenic effects on tumors (**Figure 4**). For example, although chemotherapeutics are associated with lymphodepletion, positive immune modulatory effects are described, including the induction of immunogenic cell death and depletion of Treg and MDSC (89–92). In addition, radiotherapy and different forms of targeted therapy are known to have immunostimulatory properties, i.e., enhanced T-cell infiltration and killing capacity (93–96). Clinical studies combining DC vaccination with chemotherapy, radiotherapy, and/or targeted therapy have been performed. Without extensive elaboration on these studies, the safety of combining DC vaccination with these modalities is confirmed in phase I trials (97–100). Futhermore, ample data exist suggesting efficacy (101, 102). Besides these treatment modalities, the combination of DC vaccination with other forms of immunotherapy intervening in additional steps of the cancer immunity cycle may be of particular interest as it is thought to result in more additive immunogenic effects. For example, it would be very interesting to explore the combination of DC vaccination with chimeric antigen receptor (CAR) T-cell therapy, oncolytic viruses, or other investigational immunotherapies. Here, we will discuss the combination of DC vaccination with the most successful immunotherapeutic agents to date, ICI.

Both ICI and DC vaccination exert their effects primarily through the modulation of the immune system and do so on different steps in the cancer immunity cycle. For response on ICI, tumor-specific T-cells have to be present in the tumor microenvironment, the generation of which may be aided with DC vaccination (103). As introduced before, a higher number of tumor-infiltrating lymphocytes are associated with a better response on ICI. In this respect, especially in tumors with low mutational burden, the addition of DC vaccines could prove to be beneficial (104).

Conversely, T-cells induced by DC vaccination are often hindered by the immune suppressive milieu of tumors. ICI might aid the effector functions of these T-cells by reducing inhibition through PD-1 signaling or by enhancing T-cell activation through the modulation of CTLA-4. The idea that tumor-specific T-cells activated by DC vaccination can be further stimulated with ICI is also supported by pre-clinical data. For example, upregulation of PD-1 on T-cells derived from the blood of vaccinated patients has been shown *in vitro* (105). Subsequent blockade of these upregulated PD-1 molecules could augment T-cell function. In addition, ICI exert several immune augmenting effects besides the direct antagonism of PD-1 and CTLA-4. For example, Treg depletion by anti-PD-1 mAb was shown in a mouse model (106).

In contrast to preclinical data, clinical data on combined treatment with ICI and DC vaccination in humans is scarce. In 2009, Ribas et al. reported safety of combining tremelimumab (CTLA-4 mAb) and DC vaccination in melanoma patients (107). Despite the trial was not designed to assess clinical outcome, 4 out of 16 patients (25%) achieved an objective clinical response. The authors state that clinical benefit was at the higher end of what can be expected from monotherapy tremelimumab. In addition, Wilgenhof et al. showed a promising ORR of 38% in 39 metastatic melanoma patients treated with the combination of ipilimumab

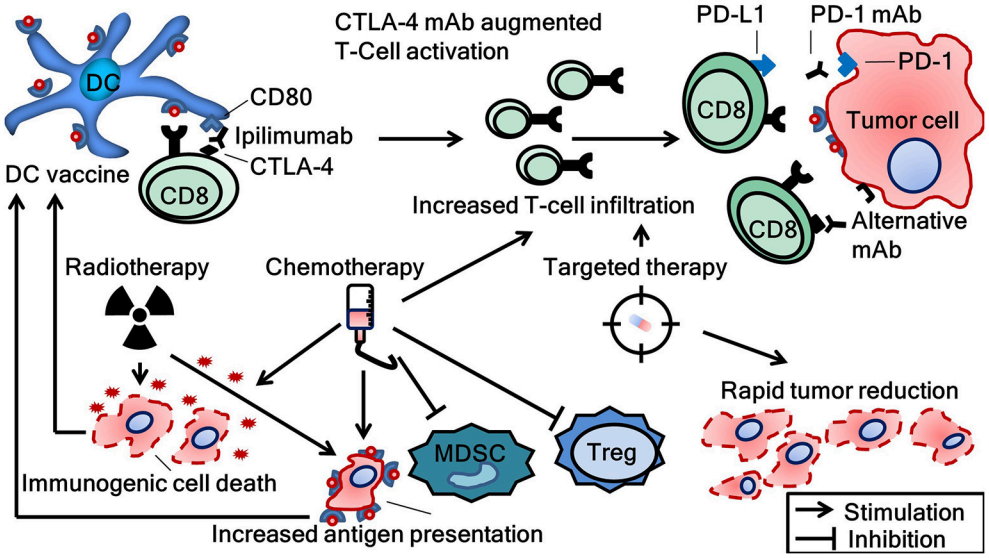

FIGURE 4 | Combinational strategies to achieve synergy between several treatment modalities and dendritic cell vaccination. CTLA-4, cytotoxic T-lymphocyte-associated protein 4; DC, dendritic cell; mAb, monoclonal antibody; CD8, cluster of differentiation number 8 (cytotoxic T-cell); MDSC, myeloid derived suppressor cells; PD-1, programmed cell death protein; PD-L1, programmed death-ligand 1; Treg, regulatory T-cells.

and DC vaccination (108). In 36% of patients grade 3 or 4 adverse events were seen, which is comparable with rates seen in large clinical trials with monotherapy ipilimumab (5, 84). This suggests little added toxicity from the addition of DC vaccines to ICI.

Considering its lower toxicity and better response rates compared to anti-CTLA-4 mAb, anti-PD-1 mAb might be more suitable combinational partners for DC vaccines. As of now, no data is published on the combined anti-PD-1 mAb and DC vaccination. However, several clinical trials investigating combinations of DC vaccination with clinically approved ICI are currently being performed (**Table 3**).

Besides currently approved ICI, DC vaccination can also be combined with ICI targeting alternative immune checkpoints (not -yet- clinically approved mAb). Currently, mAb targeting LAG-3 and TIM-3 are in various stages of clinical development as monotherapy and might be good candidates for combination. LAG-3 mAb for example, were shown to reduce expansion of Treg (109). TIM-3 was shown to be present in conjunction with PD-1 on dysfunctional T-cells after vaccination, suggesting they might form a target for mAb in addition to anti-PD-1 (110). Finally, the combination of multiple ICI and DC vaccination might be a promising strategy, albeit requiring careful considerations concerning the related toxicities (111).

Despite several ongoing clinical trials, an important aspect of combinational strategies, the timing of administration, might be under-investigated. In theory, it would seem logical to first administer DC vaccines to generate tumor-specific T-cells and consequently release immune suppression with anti-PD-1 mAb. Conversely, the timing of administering DC vaccines and ipilimumab may be more complex as both ipilimumab and these vaccines exert their functions in the priming phase of T-cells. Indeed, in a pre-clinical prostate cancer model optimal response on ipilimumab was shown when given on the same day as vaccination (112). Whether the timing of anti-PD-1 mAb and DC vaccination is equally important is not known and forms an interesting subject for further research.

In conclusion, combinational strategies for the treatment of cancer incorporating DC vaccination are a promising field of research. Considering the favorable results on the combination of DC vaccination and anti-CTLA-4 mAb, the results on the currently ongoing combinational clinical trials with anti-PD-1 and anti-PD-L1 mAb are eagerly awaited.

CONCLUSION

Immunotherapy for the treatment of cancer is a fast-moving field. It is important to determine the relative position of DC vaccination to other treatments in this rapidly evolving landscape. Ideally, patients can be selected based on biomarkers predictive for response to therapy. Currently, no predictive biomarkers for DC vaccine response are applied in the clinic to guide treatment decisions but the immune landscape of the tumor might hold promise. Also, few clinically useful predictive biomarkers for ICI are known. With the success of ICI and the lesser clinical benefit of DC vaccination in metastatic disease, it becomes increasingly clear that the future of DC vaccination in extensive metastatic disease as standalone treatment is probably limited. However, the immune-inducing properties of DC vaccination makes it a prime candidate for combination with other anti-cancer modalities, especially ICI. The currently ongoing research on DC vaccination combined with ICI such as anti-PD-1 mAb has to determine whether this combination has a future perspective. The theoretical basis

and the promising clinical data on anti-CTLA-4 mAb combined with DC vaccination does imply this perspective exists. With its highly favorable toxicity profile, another application of DC vaccination might lie in the adjuvant setting. Furthermore, DC vaccination as monotherapy may be more effective in adjuvant setting compared to its application in metastatic setting.

Consequently, for DC vaccination to gain a definitive role in the therapeutic landscape of cancer, research should be focused on well-designed trials in the adjuvant setting, combinational strategies, and patient selection.

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AUTHOR CONTRIBUTIONS

WvW, KB, and IdV conception and design; WvW, KB, MB, GS, IdV, and WG writing, review, and/or revision of the manuscript.

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