DENDRITIC CELL-BASED IMMUNOTHERAPY IN SOLID AND HAEMATOLOGIC TUMORS

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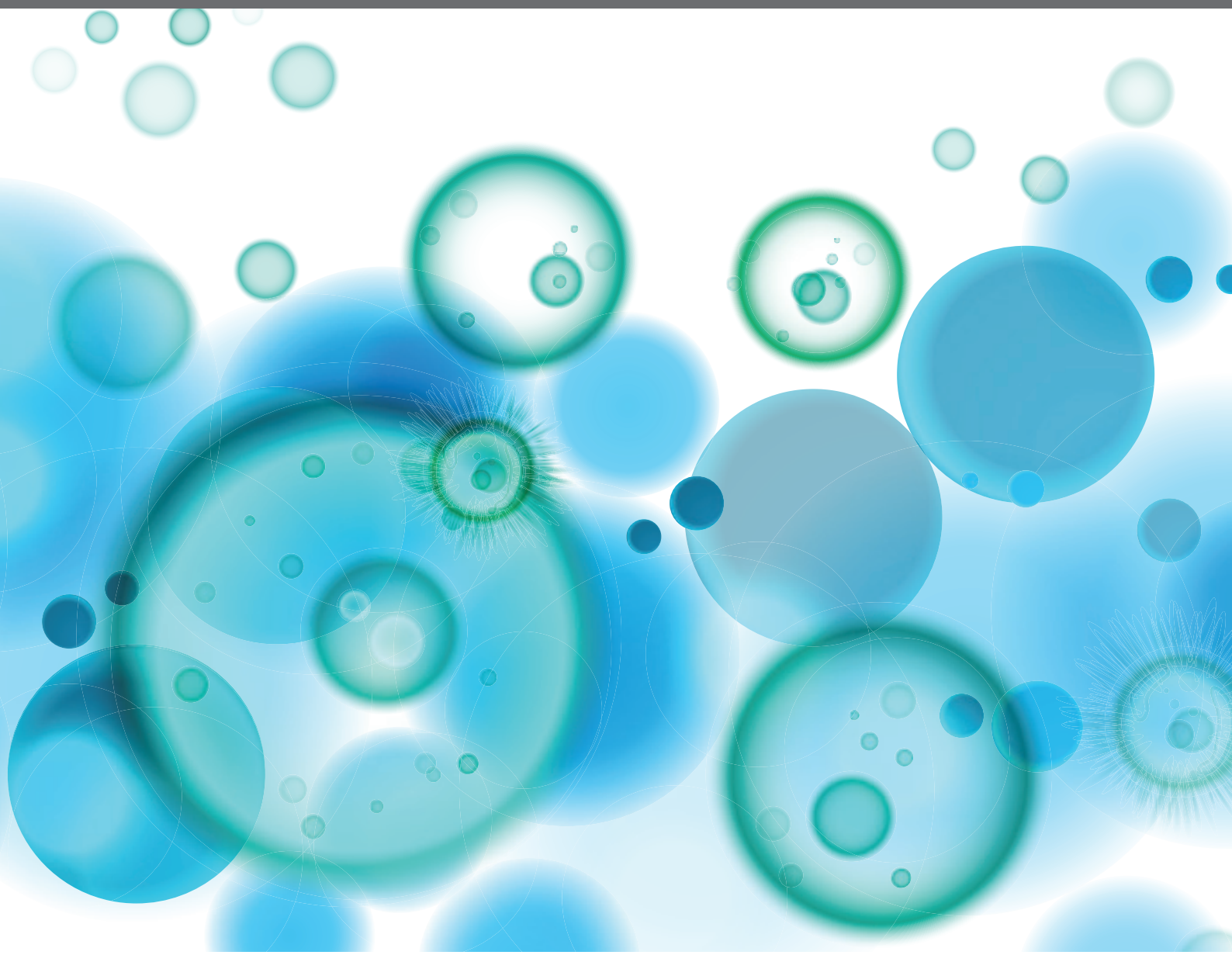

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DENDRITIC CELL-BASED IMMUNOTHERAPY IN SOLID AND HAEMATOLOGIC TUMORS

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EDITORIAL
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Editorial: Dendritic Cell-Based Immunotherapy in Solid and Haematologic Tumors

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Keywords: dendritic cell vaccination, dendritic cell tolerization, anticancer immunotherapeutic combination, immune checkpoint inhibitor, immunogenic cell death

Editorial on the Research Topic

Dendritic Cell-Based Immunotherapy in Solid and Haematologic Tumors

The safety and feasibility of dendritic cell (DC)-based immunotherapies in the treatment of solid and hematologic tumors are well-documented. However, available clinical data indicate that DC-based vaccination as monotherapy provides suboptimal and still unsatisfactory clinical benefits despite the induction and/or strengthening of specific anti-tumor immune responses. In addition to their function as antigen presenting cells, DCs govern the nature of the immune contexture of tumors owing to the intricate interplay they establish with other immune cell populations (1). Accordingly, recent studies indicated an important role of DCs in mediating the clinical response to immune checkpoint inhibitor (ICI) therapy (2–5) and adoptive $CD8^+$ T cell therapy as well (6). In line with these evidences, an emerging area of intense investigation is the development of new and more effective strategies to strengthen the therapeutic efficacy of immune checkpoint blockade with DC-based vaccination. The potential clinical relevance of this combination is supported by convincing preclinical data and clinical trials demonstrating therapeutic synergism and improved efficacy with a retained safety profile (7, 8). This Research Topic of Frontiers in Immunology focuses on the most recent advances in the field of DC-based immunotherapy, a strategy that has recently regained a strong interest as a possible therapeutic complementation of current immunotherapeutic approaches. In this issue, original research by Kodumudi et al. (Brian J. Czerniecki lab) highlighted that the timing and the schedule of ICI treatment and DC-based vaccination are fundamental to reach improved immune response in a preclinical model of $HER2^+$ breast cancer. Indeed, the authors clearly showed a reduction in tumor burden and improved survival benefit only when HER2 peptide-loaded DC vaccination preceded anti-programmed cell death 1 (PD-1) therapy. These results were in line with an increase intratumor infiltration of T lymphocytes whose PD-1 expression was up-regulated by DC vaccination. Interestingly, Bulgarelli et al. (Massimo Guidoboni lab), demonstrated that the ability of DC vaccine to increase the number of tumor infiltrating $CD8^+$ T cells was impaired in metastatic melanoma patients refractory to immune-based therapies, including Ipilimumab (anti CTLA-4 antibody). On the other hand, despite a significant increase of intratumor $CD8^+$ T cell density following DC vaccination, patients previously treated with chemotherapy or radiotherapy, failed to show a concurrent cytotoxic reactivation of T cells. The Authors discussed these results as partially explainable by DC-induced concomitant up-regulation of PD-L1 in cancer cells. Shinde et al. (Lalita Limaye lab) demonstrated that the use of anti-CTLA-4 monoclonal antibody in combination with DC vaccine strengthened the ability of DCs to

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successfully elicit tumor specific immune response in a multiple myeloma model. In this setting, autologous T cells showed signs of exhaustion, which can be rescued with CTLA-4 blockade. These studies clearly illustrate how the tumor immune microenvironment constantly shapes cancer cells and vice versa hence influencing the responsiveness to treatment. Therefore, the most critical challenge of immunotherapy now is the possibility to identify the specific mechanisms involved in the immune escape as well as critical targets responsible for local immunesuppression in each individual patient in order to define the most effective immunotherapeutic combination and personalize the treatment.

Castiello et al. (Cell Factory FaBioCell) thoroughly discussed the possibility to exploit the intratumor inoculation of antigen unloaded DCs, also called *in situ* DC vaccination, in combination with other anticancer therapies particularly with immunogenic pro-apoptotic agents. This strategy of vaccination takes advantage from the ability of inoculated DCs to uptake and process tumor associate antigens (TAAs) and neo-antigens directly released from tumor cells in the surrounding tumor microenvironment (TME). Intratumor injection of activated autologous DCs could efficiently enhance tumor cell elimination especially when used in combination with immunogenic cell death (ICD) inducing therapies (9) or other drugs able to improve cancer cells recognition and phagocytosis by DCs (10). It is well-known that ICD inducers, by promoting the tumor cell release of several molecules belonging to damage-associated molecular pattern (DAMPs), recruit DCs in the TME and improve their activation. In particular, in the present Research Topic, Lamberti et al. (Natalia B. Rumie Vittar lab) described for the first time the involvement of type I IFN pathway in the up-regulation of co-stimulatory signals and tumor-directed chemotaxis in DCs by melanoma cells following exposure to photodynamic therapy. Elucidating the intrinsic determinants of cancer cells undergoing ICD to enhance DC maturation and activation continues to keep the interest high with regard to the opportunity to exploit *ex vivo* ICD to improve DC-based vaccine efficacy.

Another therapeutically important aspect addressed in this special issue is the need to optimize the protocols for *ex vivo* monocyte-derived DC (mo-DC) generation in order to develop *ex vivo* a DC functional phenotype that is as close as possible to that of human DC subsets existing *in vivo*. On these grounds, Zeng et al. (Herbert Schwarz lab) comprehensively described the characteristics and discussed the potential clinical application of mo-DCs differentiated with the use of a CD137 ligand (CD137L) agonist. The authors highlighted the superior capacity of CD137L-DCs to stimulate T cell responses *in vitro* compared to conventional mo-DCs, obtained by exposure to granulocyte-macrophage colony-stimulating factor (GM-CSF) plus interleukin (IL)-4, and envisaged their potential therapeutic use as vaccine in combination with ICI immunotherapy.

The increased knowledge of molecular mechanisms, signaling pathways, epigenetic regulation and metabolic control of DC biology convincingly highlights the huge plasticity of these immune cells and indicates new modalities for *ex vivo* and/or *in vivo* DC manipulation in order to induce pro-inflammatory responses or revert tolerogenic/regulatory phenotypes.

In particular, tumor cells exploit different mechanisms and molecules to promote DC tolerization so as to evade immune surveillance, an aspect discussed in detail by DeVito et al. who gave a comprehensive overview about the important role of DC tolerization not only in tumor-mediated immune evasion, but also in generating immunotherapy resistance. According to these Authors, several lines of evidence highlight the pivotal role of DCs in the responsiveness to different immunotherapeutic approaches, indicating the need to extend ongoing efforts, which are mainly focused on manipulation of the effector phase of the antitumor immune response, also to the priming phase of the immune response by fully exploiting DC potential.

Notably, several molecular pathways involved in DC tolerization are also dysregulated in cancer cells being therefore targets of therapy. However, direct or indirect effects exerted by anticancer agents targeting these pathways on DCs still remain to be investigated. In this respect, Suryawanshi and Manicassamany thoroughly discussed the role of Wnt signaling cascade not only in regulating DC maturation, activation, and antigen presentation, but also in modulating the functions of other immune cells in TME. Interestingly, Fucikova et al. (Radek Spisek lab) debated the opportunity to take advantage of *ex vivo* DC tolerization to develop therapeutic vaccination against autoimmune diseases. In these cases, in fact, it is possible to exploit the ability of tolerogenic DC to stimulate regulatory T cell proliferation. These considerations suggest that a similar approach could be adoptable also in the treatment of lung chronic inflammatory diseases, such as chronic obstructive pulmonary disease or asthma in which an emerging role of DCs in maintaining unresolved inflammation was indicated (11).

All together, the contributions provided by this Research Topic of Frontiers in Immunology critically emphasized the strengths, but also highlighted relevant unresolved questions to be addressed to promote a broader application of DC-based vaccination in the clinical practice for cancer treatment. Moreover, the recent advances in our knowledge of DC tolerization described here clearly indicate DC-based vaccines may also be successfully used for the treatment of diseases characterized by chronic inflammation and dysregulated stimulation of immune responses as in autoimmunity. In conclusion, the central role of DCs in orchestrating immune responses continues to stimulate new strategies to exploit the functions of these immune cells for the development of more effective therapies for an increasing number of clinical settings.

AUTHOR CONTRIBUTIONS

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

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ORIGINAL RESEARCH
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Autologous Hematopoietic Stem Cells Are a Preferred Source to Generate Dendritic Cells for Immunotherapy in Multiple Myeloma Patients

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In multiple myeloma (MM), dendritic cells (DCs), and their precursors are prone to malignant cell-mediated regulation of function leading to low efficacy of DC vaccine. DCs taken directly from MM patient's body or derived from monocytes are fewer in numbers and are also dysfunctional. Here, we investigated the functionality of Hematopoietic stem cell-derived DCs (SC-DCs) from MM patients. Mature-MM-SC-DCs showed all essential functions like antigen uptake, allogenic T cells simulation and migration comparable to those derived from healthy donor (HD) samples. A comparison of Mo-DCs and SC-DCs obtained from the same MM patients' samples revealed that the expression of IL-6 was higher in the precursors of Mo-DCs leading to their impaired migration. In addition, expression of CCR7 which is responsible for DCs migration was found to be lower in MM-Mo-DCs. The chromatin permissiveness as observed by H3K4me^3 histone modification at the Ccr7 promoter in MM-Mo-DCs was significantly lower than those in MM-SC-DCs. Levels of *Zbtb46*- a hall mark DC transcription factor mRNA was also found to be reduced in MM-Mo-DCs. Cytotoxic T cells generated from MM-SC-DCs from autologous naïve T cells exhibited reduced antitumor activity because the T cells were exhausted. Blocking of CTLA-4 on autologous T cells could partially restore T cell proliferation and activation. Thus, a combination of MM-SC-DC vaccine and anti-CTLA-4 antibody may serve as a better candidate for immunotherapy of MM. This study has implications in increasing the efficacy of cancer immunotherapy in MM.

Keywords: multiple myeloma, stem cells, monocytes, dendritic cell vaccine, cytotoxic T cells

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INTRODUCTION

Multiple myeloma (MM) is a hematological malignancy with the second most common propensity with mortality rate of 5.2 to 8 (1). MM is associated with the clonal expansion of malignant plasma cells that suppresses hematopoiesis, often leading to bone lesions, anemia, and renal complications (2, 3). Standard chemotherapy and radiation therapy, followed by autologous stem cell transplantation (AST), is the routine procedure administered to MM patients for treatment.

Although this standard treatment may help patients to achieve remissions but eventually majority of MM patients show relapse of the disease, with incurable progression associated with immune deregulation (4, 5). Reversal of immune suppression and training the effector cells to specifically target malignant plasma cells would help in controlling the progression of the disease and improving the overall survival of patients (6).

Cancer immunotherapy using dendritic cells (DCs) has gained much attention as it has the potential to cure the disease, where other therapies have failed (7). DC vaccines are prepared by differentiation of precursor cells, i.e., monocytes, circulating in the patients' blood, or from differentiation of hematopoietic stem cells (8, 9). MM-associated tumor antigens are loaded onto the DCs so that they can activate a T cell response directed against cells expressing those antigens. The success of the DC vaccine depends on the immunogenicity of the final product (10, 11). The source population from which DCs are generated may differ from each other (12). Many studies, including ours, have suggested that DCs circulating in cancer patient's body and monocyte-derived dendritic cells (Mo-DCs) are functionally impaired (13–15). The precursors of these Mo-DCs secrete higher amounts of IL-6, leading to defective migration of Mo-DCs (16–18). In this study, we assessed the functional properties of DCs derived from a more primitive population, i.e., hematopoietic stem cells (HSCs) from MM patients. For this, we used a two step culture system (19). The first step involves expansion of the HSC pool toward DC precursors, and the second step involves differentiation of these precursors to mature DCs. Apheresis samples obtained from healthy donors (HD) or MM patients at remission were used for the generation of stem cell derived-DCs (SC-DCs).

We compared stem cell derived-DCs from MM patients (MM-SC-DCs) with those from healthy donors (HD-SC-DCs) for their morphology, phenotype and functions. These DCs were also utilized for autologous cytotoxic T cell (CTL) generation. These CTLs were characterized and tested for their targeted cell killing activity.

MATERIALS AND METHODS

Ethical Approval

Written informed consents were obtained from healthy donors and multiple myeloma patients prior to collection of apheresis samples. All experimental procedures and informed consents were approved by the Institutional Ethics Committee (IEC) and Institutional Committee for Stem Cell Research (IC-SCR) of NCCS, and Deenanath Mangeshkar Hospital, in accordance with the Declaration of Helsinki. The study number from the ethics review board is NCCS/IC-SCR/2016-I/2.

Abbreviations: DCs, Dendritic cells; HSCs, hematopoietic stem cells; Mo, monocytes; HD, healthy Donor; MM, multiple myeloma; Mo-DCs, monocytes derived-DCs; SC-DCs, stem cell-derived DCs; HD-Mo-DCs, healthy donor sample monocyte-derived DCs and MM-Mo-DCs, multiple myeloma sample monocyte-derived DCs; HD-SC-DCs, healthy donor sample stem cell-derived DCs; MM-SC-DCs, multiple myeloma sample stem cell-derived DCs; CTLs, Cytotoxic T cells.

Sample Collection

Apheresis samples were collected from healthy donors or MM patients who were administered with GM-CSF for the mobilization of stem cells. Hematopoietic cells-enriched mononuclear cells were collected by using the COBE Spectra Apheresis System (Spectra Cell Separator; Terumo BCT Inc., Tokyo, Japan).

Upon completion of transplantation of the apheresis samples to the patients, 3 to 5 ml of samples leftover in the tubing were procured for this study from the transplantation unit.

a) **Healthy donors**: Eight apheresis samples were collected from healthy donors who were donating cells for allogeneic stem cell transplantation.
b) **Multiple myeloma patients:** Seven apheresis samples were collected from MM patients who were undergoing autologous stem cell transplant. The stage of the disease was diagnosed according to the International Staging System (ISS) for multiple myeloma. MM patients had undergone chemotherapy regimens to achieve remission of the disease. Samples were collected after 3–6 months of chemotherapy, when the patients were in remission.

Dendritic Cell Generation

The details of two step method for generation of DC have been previously reported (19). Briefly, mononuclear cells (MNCs) from HD/MM apheresis samples were seeded for 1 h plastic adherence in tissue culture plates containing IMDM with 1% AB+ plasma in a CO_2 incubator. After incubation, non-adherent cells were enriched for the stem cell population. These cells were expanded for 21 days using a combination of the growth factors, FMS-like tyrosine kinase 3-ligand (FLT3-L), 25 ng/ml; thrombopoietin (TPO), 10 ng/ml and stem cell factor (SCF), 20 ng/ml, to obtain DC precursor cells. Immature DCs were differentiated from the precursor population by using the granulocyte-macrophage colony-stimulating factor (GM-CSF, 50 ng/ml) + interleukin-4 (IL-4, 30 ng/ml) for 3 days, and GM-CSF (50 ng/ml) + Tumor necrosis factor-α (TNFα, 50 ng/ml) for 4 days. Maturation of DCs was induced by the addition of TNF-α, lipopolysaccharide (LPS), and CD40L at concentrations of 100 ng/ml each, for 48 h.

For generation of monocyte-DCs from MM apheresis samples, 10^7 mononuclear cells were seeded/well in six-well plates. The cells were incubated for 1 h at 37°C in 5% CO_2. The non-adherent and the loosely adherent cells were washed off with IMDM. The adherent cells were differentiated as described above. The MM-Mo-DCs and MM-SC-DCs were compared for the expression of IL-6 (Interleukin-6) and Zbtb46 (Zinc Finger And BTB Domain Containing 46), and for anti-trimethyl histone H3K4 modification at CCR7 promoter. All the cultures were maintained in IMDM supplemented with 5% AB^+ plasma supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), and L-glutamine (2 mM) in a 5% CO_2 incubator at 37°C.

Maintenance of Cell Lines

HLA-A2-positive cell lines, U266B1 (Multiple myeloma), MDA-MB-231-luc-D3H2LN (breast cancer), HCT116 (colon

carcinoma), A498 (renal cell carcinoma), and MCF-7 (breast cancer), were used in this study. K562, an HLA null cell line, was used as a non-specific control in *in vitro* CTL assays. All cell lines were maintained according to standard tissue culture practices.

Flow Cytometry

Cells of interest were washed and blocked by PBS containing 1% BSA. For cell surface markers, antibodies against specific markers were added to cell suspensions, with their appropriate isotype controls being added in another tube. Antibody staining was done for 45 min on ice. For intracellular markers, fixation and permeabilization of cells was done prior to antibody staining, using the eBioscience Fix/Perm kit, as per the manufacturer's instructions. Stained cells were washed twice with ice-cold PBS and resuspended in 1% paraformaldehyde. Cells were acquired on FACS Canto II (BD, San Jose, CA, USA). Data were analyzed by FACS Diva (BD), and histogram overlays were prepared using FlowJo (LLC, Ashland Oregon).

Functional Assays for DCs

a) **Antigen uptake:** Antigen uptake by the process of endocytosis was assessed by using FITC-tagged Dextran (Molecular probe). Immature DCs were harvested and incubated with Dextran-FITC (20 μg/ml) either at 4°C (internalization control) or at 37°C (test), for 30 and 60 min. After completion of incubation, cells were extensively washed with ice-cold PBS containing 0.01% sodium azide and 1% BSA. Cells were then fixed with 1% paraformaldehyde (PFA) and acquired and analyzed on BD FACS Canto II.

b) **Chemotaxis:** The migration property of mature DCs was assessed by an *in vitro* migration assay. 2×10^4 cells in 100 μL of cell suspension were loaded on top of inserts (pores size 0.8 μm, BD Falcon). These inserts were placed in tissue culture wells containing IMDM either supplemented with or without rhCCL-19 (500 ng/mL). The culture was incubated for 3 h at 37°C with 5% CO_2. After completion of incubation, migrated cells at the bottom of the well were harvested and counted using a hemocytometer and Trypan blue staining.
To examine the role of autocrine IL-6 on migration of MM-Mo-DCs, anti-IL6 antibody at the concentration of 10 ug/ml was added from day 0 of the MM-Mo-DCs cultures, These MM-Mo-DCs were used for the chemotaxis assay.
To check the specificity of interaction of chemokine receptors (CCR) with CCL-19, in migration assays SC-DCs were pretreated with anti-CCR1/anti-CCR3/anti-CCR5 or anti-CCR7 antibody (10 ug/ml) for 1 h.

c) **Mixed lymphocyte reaction (MLR):** $CD3^+$ T cells were sort-purified from peripheral blood-MNCs of healthy volunteers. T cells were labeled with CFSE dye (Molecular probe) as per the manufacturer's instructions. Co-cultures of mature DCs and labeled T cells in the ratio 1:10 (DC count= 10^4 and T cell count= 10^5) were carried out for 5 days. Labeled T cells without any added DCs were used as controls. CFSE dilution, used as an indication of proliferation of T cells, was assessed by flow cytometry.

d) **ELISA:** IL12p70, IL10, and IFNγ in the culture supernatants was analyzed by using sandwich ELISA. Supernatants either from the *in vitro*-generated DCs or co-cultures of DCs and T cells were collected. The supernatants were then analyzed by ELISA for cytokine content, human-IL-10 and IFN-γ, using the BD Opt EIA ELISA kit (BD Biosciences, USA), and IL12p70 using the eBioscience ELISA kit, as per the manufacturers' instructions.

Real Time PCR

Total RNA was extracted from 10^6 cells using the Trizole reagent as per the manufacturers' instructions. The extracted RNA (1 μg) was reverse transcribed to cDNA using MMLV reverse transcriptase (Sigma Aldrich) and Random hexamers (Invitrogen), as per the instructions. qRT-PCR was performed with specific primers as listed below, using SyBr green with an AB Fast 7,500 platform.

List of primers used for Real time PCR.

No.	Gene name	Sequence (5′ → 3′)
1	Human GAPDH forward	CGGATTTGGTCGTATTG
2	Human GAPDH reverse	GGAAGATGGTGATGGGA
3	Human CCR7 forward	GGTATGCCTGTGTCAAGATG
4	Human CCR7 reverse	GGTTGAGCAGGTAGGTATCG
5	Human IL6 forward	CAATGAGGAGACTTGCCTGG
6	Human IL6 reverse	TGGGTCAGGGGTGGTTATTG
7	Human Zbtb46 forward	AGAGTGCTGGTGATGCCTG
8	Human Zbtb46 reverse	ACAGGTCCGCATTTGAGTC

Chromatin Immunoprecipitation (ChIP)

For the detection of $H3K4me^3$ histone modification on the CCR7 promoter of mature DCs, 10^6 cells were fixed in a 1% formaldehyde solution. Fixed cells were sonicated using a water-bath sonicator, and the assay was performed using the anti-trimethyl histone H3K4 antibody and ChIP assay kit (Milipore) as per the manufacturers' instructions. Quantitation of ChIP DNA (relative enrichment) was analyzed in triplicates by qRT-PCRs (ABI 7,500 fast). A list of the primers used for the CHIP assay is given below.

List of primers used for ChIP-PCR.

No.	Gene name	Sequence (5′ → 3′)
1	Human GAPDH promoter forward	GCCAATCTCAGTCCCTTC
2	Human GAPDH promoter reverse	AAGAAGATGCGGCTGACTGT
3	Human CCR7 promoter forward	TGTATGTGGCAAAAGGG
4	Human CCR7 promoter reverse	CTCAGAAAACACCCAACA

Generation of Anti-tumor CTLs:

Antigen pulsing of immature DCs was carried out by adding Keyhole Leukocyte antigen (KLH, adjuvant) and the target cancer cell lysate at concentrations of 50 and 100 μg/ml for 48 h, respectively. This tumor antigens-pulsed DCs were co-cultured with autologous sort-purified CD3+ CD8+ CD45RA+ naïve T cells. Sorting was performed on the BD Aria III platform;

the gating strategy is given in **Supplementary Figure S6**. The co-cultures were maintained for 2 weeks with the addition of IL-2 (0.1 μg/ml) and IL-7 (5 μg/ml) every alternate day, and with the addition of fresh-pulsed DCs after a week, to provide re-stimulation to the CTLs. The target cancer cells used for CTL generation from HD samples in this study were- MCF7, MDA-MB-231-luc-D3H2LN, A498, HCT116 and U266B1 while U266B1 was used as the target cell line for CTL generation from MM samples.

Functional Assays for CTLs

a) **Assessment of activation by detection of the expression of surface markers:** Expression of CD 69 is the hallmark of T cell activation. Following 5–7 days of incubation, cells from CTL co-cultures were screened for the expression of CD69 using flow cytometry. Cells (2×10^5 per tube) were stained as mentioned earlier. Dual positive cells expressing CD8 and CD69 were analyzed on FACS Canto II from BD.

b) **Intra cytoplasmic staining for granzyme A, B and perforin:** *In vitro*-generated CTLs were tested for the expression of the serine proteases, granzyme A, B, and perforin within the intra cellular compartments. At the end of incubation, cells from the co-cultures were harvested and stimulated by PMA (40 ng/ml) and ionomycin (100 ng/ml), along with Golgi stop- Brefeldin A (1:1000 dilution), for 4 h. After incubation, cells were subjected to surface staining with CD8 for 30 min, followed by fixation and permeabilization. These cells were stained with antibodies against granzyme A, B and perforin for 60 min on ice. Stained cells were acquired and analyzed on FACS Canto II from BD.

c) ***In vitro* CTL assay:** Target cells (10^6/ml) were stained with 10 mM Calcein-AM for 30 min. These labeled target cells were then seeded with the effector cells (CTLs) in U-bottom 96-well microtiter plates, with T: E ratios ranging from 1:1 to 1:40, in triplicates. Target cells without the effector in complete medium (spontaneous release), and target cells in complete medium plus 2% Triton X-100 (maximum release) were used as controls. The HLA-null cell line, K562, was used as a negative control in these experiments. The *in vitro* killing activity was tested only on CTLs that were primed with the individual tumor cell line lysate. Unactivated T cell control could not be included, as the number of naïve T cell population that could be harvested from the available sample volume was limited (From 10^7 MNCs we could get upto 5×10^5 naïve T cells. The *in vitro* CTL assay requires more number of T cells as the ratio of target cells to effector cells gradually increases. Hence we could not use the unactivated naïve T cell control). After incubation at 37°C in 5% CO_2 for 6 h, the supernatant was harvested and transferred into new plates. Fluorescence of the supernatant was measured using a microplate fluorimeter (excitation filter: 485–9 nm; band-pass filter: 530–9 nm) (20). Percent lysis was calculated by using the formula:

Percent lysis = [(test release − spontaneous release)/(maximum release – spontaneous release)] × 100.

CTL Proliferation Monitored by Using Ki67

The Ki67 marker was used to monitor the proliferation of CTLs. Cells from DC-T cell co-cultures were harvested after 72 h of incubation, and stained with Ki-67-PE, and CD8-APC using antibodies. $Ki67^+$ $CD8^+$ dual positive cells were observed in the gated lymphocyte population.

Autologous T Cells Profiling

Autologous $CD3^+$ $CD8^+$ T cells from both healthy donor and MM samples were profiled by staining with CD45RA and CD62L to identify T cell subsets (naïve, terminally differentiated effector cells, effector memory, and central memory T cells). In addition, cell surface staining of CTLA-4 was also performed on $CD3^+$ $CD8^+$ $CD45RA^+$ T cells. The stained cells were acquired and analyzed by FACS.

CTLA-4 Blocking

In some experiments of co-cultures of MM-SC-DCs and autologous naïve T cells, anti-CTLA-4 antibody, or IgG control antibody was added at the beginning. These co-cultures were maintained as described earlier with antibody concentration maintained at 10 μg/ml. After completion of 72 h of incubation, CTLs from the co-culture were assessed for proliferation and activation by monitoring Ki67 expression and CD69 expression by FACS.

Statistical Analysis

Different experimental variables were compared and all the results were expressed as mean ± SEM. "N" represents the number of apheresis samples, i.e., biological replicates, and "n" represents the number of experimental replicates of a single apheresis sample. Statistical analysis was done and graphs were prepared using the Sigma stat software (Version 11). All statistical analyses of experiments between the two groups were evaluated by the one-way repeated measures ANOVA. p-values ≤ 0.05 were considered statistically significant [$p \leq 0.05$(*), $p \leq 0.01$(**), and $p \leq 0.001$(***)].

RESULTS

Patient Population

Age and gender of HD/MM patients and stage of the disease (for MM, at the time of first visit to clinic) are specified in **Supplementary Figure S1A**. In the present study, MM patients were given standard care chemotherapy followed by autologous stem cell transplant which gives faster hematopoietic recovery. Chemotherapy regimes given to individual patients are given in **Supplementary Figure S1B**. Apheresis samples from the MM patients were collected after 6 months of chemotherapy. These samples were used for dendritic cells generation.

Cell Yield, Morphology, and Phenotype of SC-DCs From Healthy Donor and Multiple Myeloma Patients Were Similar

A two-step method was used for the generation of DCs from stem cells of apheresis samples of HD and MM patients as

described in material and methods. HSCs were expanded and terminally differentiated into DCs and were termed as HD-SC-DCs and MM-SC-DCs, respectively. They were systematically compared for morphology, phenotype, and functions. There was only a marginal difference in the absolute number of SC-DCs generated from 10^7 MNCs from HD and MM apheresis samples ($5.3 \pm 1.7 \times 10^6$ and $4.5 \pm 1.2 \times 10^6$, respectively, **Figure 1A**). Phase contrast imaging and Wright's-Giemsa staining of mature SC-DCs from both the sets exhibited characteristic morphology with long dendritic processes (**Figure 1B**). SC-DCs were assessed for the expression of various surface markers associated with mature DCs. As shown in **Figure 1C**, both the SC-DCs have more than 90% expression of HLA-DR, CD54, CD58, CD86, and CD11c. Expression of HLA-ABC and CD80 reached upto 80%, while the expression of CD40 was ∼60% in both the SC-DCs, and the differences were not significant. Expression of CD83 was significantly lower in MM-SC-DCs as compared to HD-SC-DCs, but this difference was not reflected in their mean fluorescence intensity (MFI) values (**Figure 1D**). CD1a expression was around 20% in SC-DCs from HD and MM samples. MFI of all the DC-surface markers were also comparable between the HD and MM samples (**Figure 1D**). Representative histograms plots have been depicted in **Supplementary Figure S2**. Collectively, these results indicate that HD-SC-DCs and MM-SC-DCs exhibited typical mature DC morphology and cell-surface phenotype without any significant differences between them.

Functionality of SC-DCs From HD and MM Source Was Equivalent

Antigen uptake by SC-DCs via receptor-mediated endocytosis was monitored by the uptake of dextran-FITC. HD-SC-DCs and MM-SC-DCs exhibited high and comparable dextran- FITC uptake following both, 30 and 60 min' incubation (**Figure 2A**). Representative histograms of dextran-FITC positive SC-DCs are given in **Supplementary Figure S3**. Mature and functional DCs induced the activation and proliferation of interacting T cells. This function was assessed by *in vitro* co-culturing of SC-DCs from HD or MM samples with allogeneic- $CD3^+$T cells (allo-T cells) from the peripheral blood of unrelated healthy donors. The target T cells were labeled with the dye, CFSE. After 5 days of co-culture, T cells were analyzed for percent CFSE dilution. **Figure 2B** showed that T cells have equivalent cell proliferation rate in HD-SC-DC co-culture ($79.8 \pm 5.8\%$) as well as in MM-SC-DC co-culture ($77.5 \pm 3.5\%$). Representative FACS overlays are given in the **Supplementary Figure S4**. The data suggest that HD-SC-DCs and MM-SC-DCs have similar capacity to induce proliferation in T cells.

IL-12 and IL-10 are two important cytokines secreted by DCs that influence the immune response mounted by T cells. We analyzed the secretion of these two cytokines in SC-DC culture supernatants. It was found that MM-SC-DCs and HD-SC-DCs had secreted similar amounts of IL-12p70 (**Figure 2C**) and IL10 (**Figure 2D**) in their culture supernatant. For assessing the levels of IFNγ, the supernatants of SC-DC, and $CD3^+$ T cell co-cultures were collected and analyzed by ELISA. The levels of IFNγ in the supernatants of $CD3^+$ T cell co-cultures either with MS-SC-DC or HD-SC-DC were also comparable (**Figure 2E**). Next we examined the migration function of SC-DCs from HD and MM samples. Migration of DCs toward a chemokine gradient is an essential functional property for initiation of antigen specific immune response. DCs migration to local lymph node is governed by the chemokine receptor CCR7 and CCL-19 interaction. As seen in **Figure 2F**, MM-SC-DCs showed similar migration toward CCL-19, as compared to HD-SC-DCs. To further confirm this observation, we analyzed CCR7 marker expression on MM-SC-DCs and HD-SC-DCs.

CCR7 expression in SC-DCs of HD and MM samples was equivalent at the transcript as well as protein levels (**Figures 2G,H**). Specificity of CCR7 toward CCL19 was tested by blocking of CCR receptors like CCR1, CCR3, CCR5, and CCR7 while performing *in vitro* migration assay. The migration of SC-DCs was abrogated on blocking of CCR7 only (**Supplementary Figure S5**) highlighting the fact that the DC migration is dependent on CCR7 and CCL-19 interaction. Taken together, these data show that, SC-DCs derived from MM-HSCs have all the functional characteristics similar to those shown by SC-DCs derived from normal healthy HSCs, thus indicating functional equivalence.

MM-Mo-DCs Showed Impaired Migration Than MM-SC-DCs Derived From the Same MM Samples

In many types of cancers including multiple myeloma, autocrine secretion of IL-6 is known to inhibit DCs migration function (14–17). Our earlier studies had revealed that, DCs generated from monocytes of MM samples were impaired in migration function and CCR7 expression (14). In contrast, here we found that these functions were not compromised when DCs were generated from stem cells (MM-SC-DCs), with these showing migration (**Figure 2F**) and associated *Ccr7* expression, equivalent to HD-SC-DCs (**Figures 2G,H**). To gain a deeper insight into this discrepancy, between functions of MM-Mo-DCs and MM-SC-DCs, both types of DCs were generated from the same MM samples' and were then compared.

As seen in **Figure 3A**, migration of MM-Mo-DCs was significantly reduced as compared to those of MM-SC-DCs, from same MM samples. When precursors of MM-DCs were analyzed for autocrine expression of IL-6 at transcript level, it was found to be negligible in SC-DCs, as compared to Mo-DCs (**Figure 3B**). The migration capacity of MM-Mo-DCs was restored when they were differentiated in presence of anti-IL-6 antibody to block the effect of autocrine IL-6 during their differentiation (**Figure 3A**). This observation suggested that higher expression of autocrine- IL6 might be one of the factors contributing to the observed reduced migration of MM-Mo-DCs as compared to MM-SC-DCs.

Epigenetic modification, in the form of DNA methylation and/or posttranslational modifications of histones regulates gene expression at the transcription level by governing the chromatin accessibility. The influence of epigenetic modification on DC

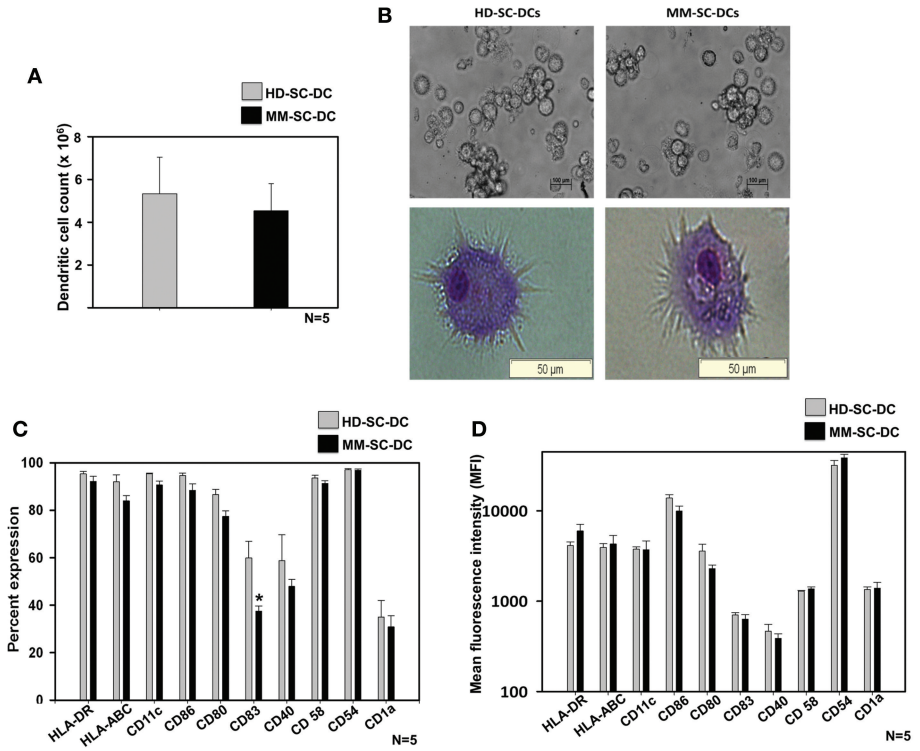

FIGURE 1 | Yield, morphology, and phenotype of SC-DCs derived from HD and MM samples were similar. **(A)** The number of DCs obtained from HD and MM samples were equivalent. **(B)** Representative phase contrast images and Wright's-Giemsa stained cells of HD-SC-DCs (left panel) and MM-SC-DCs (right panel) are depicted. **(C)** The percent expression and **(D)** mean fluorescent intensities (MFI) for the DC-specific surface molecules were similar (except for percent of CD83). Data given are mean ± S.E.M $p \leq 0.5$ (*).

functionality is poorly understood. There are reports suggesting that the migration of DCs is influenced by histone modification at the *CCR7* locus (21, 22). In contrast to MM-SC-DCs, expression of CCR7 at both transcript (**Figure 3C**) and protein levels (**Figure 3D**) was significantly lower in MM-Mo-DCs. We examined the abundance of $H3K4me^3$ at the *CCR7* promoter of Mo-DCs and SC-DCs by ChIP-PCR. The chromatin was isolated from mature SC-DCs and Mo-DCs from same MM samples and ChIP was done using anti-$H3K4me^3$. Real time-PCR revealed that recruitment of $H3K4me^3$ was increased 10-fold at the promoter of the *CCR7* gene in MM-SC-DCs, as compared to MM-Mo-DCs (**Figure 3E**). These data indicated that reduced migration of Mo-SC-DCs might be due to lower CCR7 expression, governed by lower accessibility of this gene for transcription.

The Zbtb46 transcription factor is selectively expressed in the classical dendritic cell (cDCs) lineage and its progenitors. It is required for the development of cDCs (23). This transcription factor is down regulated in DCs and their progenitors when they are exposed to tumor microenvironment, i.e., factors secreted by a tumor (24). When we analyzed the expression level of Zbtb46 in MM-SC-DCs and MM-Mo-DCs obtained from the same MM sample, MM-Mo-DCs showed significantly reduced levels of mRNA for this transcription factor (**Figure 3F**). Thus, prior continuous exposure of precursors of Mo-DCs to a tumor microenvironment may have altered their immunocompetence, leading to their dysfunction.

HD-SC-DCs Could Generate Antitumor Cytotoxic T Lymphocytes

A potent DC vaccine should generate the effector T cells having tumor killing activity, i.e., CTLs. In order to confirm whether SC-DCs from apheresis samples could generate potent autologous CTLs similar to those generated from Cord blood derived SC-DCs (25), initial experiments were done using HD-SC-DCs. We used an HLA-A2 restricted system for the CTL assays. Apheresis samples from healthy donors were screened for expression of the HLA-A2 molecule, and used for CTL generation. Co-cultures of DCs and naïve T cells were set up. Briefly, DCs were generated from these samples and pulsed with individual $HLA\text{-}A2^+$ target cancer cell lines, MDA-MB-231-LUC-D3H2LN, MCF7, A498, or HCT116. Antigen- pulsed HD-SC-DCs were co-cultured with autologous naïve $CD8^+$ T cells. The activation of T lymphocytes induces the expression of cell surface marker CD69. This marker was detected on the cell surface of CTLs using flow cytometry. It was clearly evident that CTLs generated from HD-SC-DCs showed expression of CD69 (**Supplementary Figure S7A**). Intra cytoplasmic staining was performed to detect the presence of proteases granules. We found that CTLs generated form HD-SC-DCs produced granzyme A,

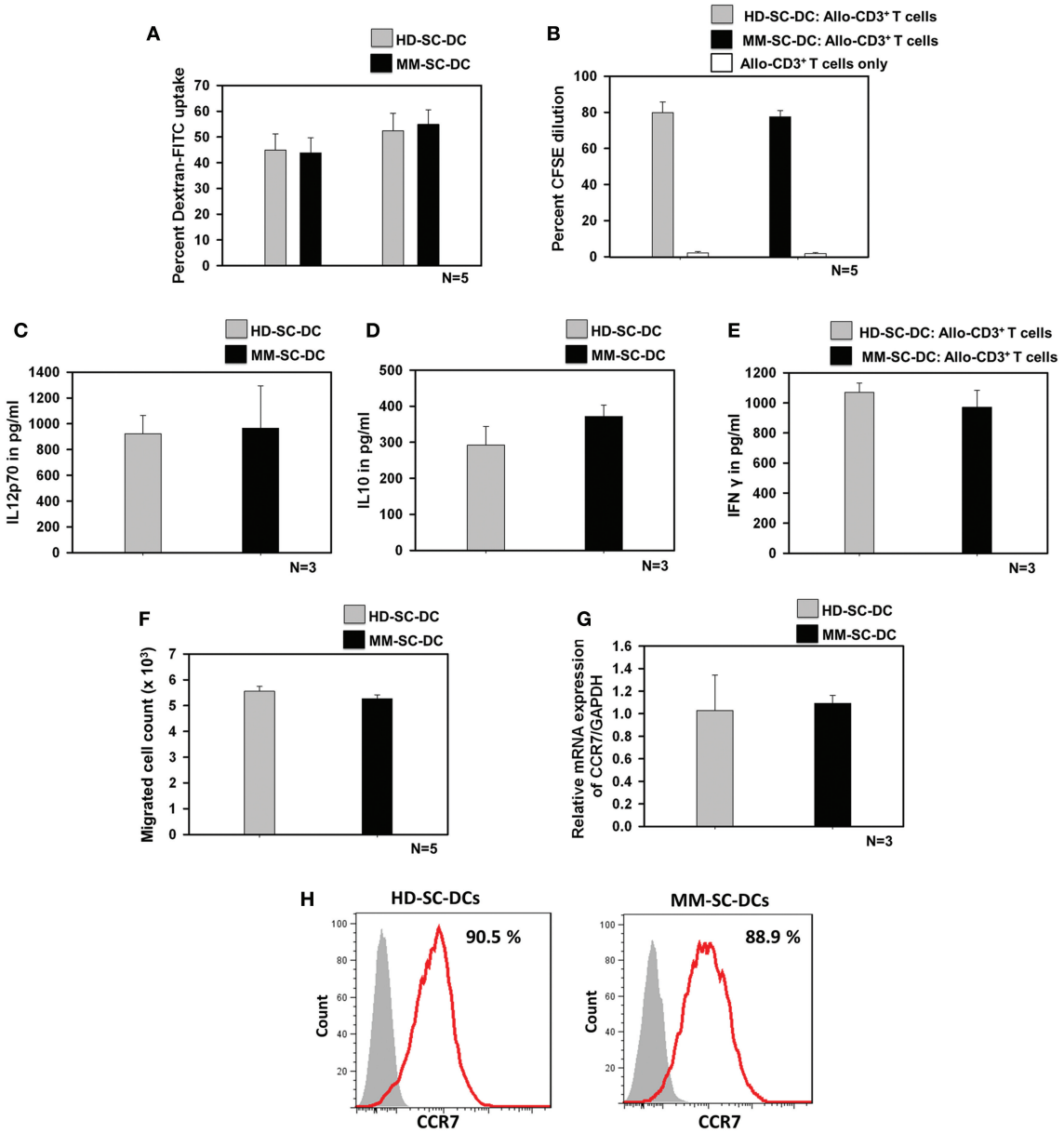

FIGURE 2 | Functional characteristics of HD-SC-DCs and MM SC-DCs were comparable. **(A)** Percent dextran-FITC uptake by HD-SC-DCs and MM-SC-DCs were similar after 30 and 60 min. **(B)** Allogeneic T cells co-cultured with both SC-DCs showed similar proliferation, as observed by CFSE dye dilution in proliferating T cells. **(C)** Secretion of IL-12p70 and **(D)** IL-10 cytokines in culture supernatants of HD-SC-DCs and MM-SC-DCs showed marginal difference. **(E)** Concentration of IFNγ in supernatant of SC-DCs and allo-T cell co-cultures were equivalent, as assessed by sandwich ELISA. **(F)** Migration of SC-DCs toward CCL-19 from HD and MM samples was similar. Expression of CCR7 **(G)** mRNA and **(H)** protein was similar in SC-DCs of HD and MM samples, as observed by RT-PCR and FACS, respectively. Data given are mean ± S.E.M.

granzyme B, and perforin (**Supplementary Figure S7A**). The presence of serine proteases confirms the killing potential of CTLs against their target cells. The expression of these molecules ranged from 69 to 95%. These CTLs also secreted substantial amounts of IFN-γ in the co-culture supernatant (**Supplementary Figure S7B**). The CTLs generated from HD-SC-DCs were able to kill breast cancer triple negative MDA-MB-231-LUC-D3H2LN cells (**Supplementary Figure S8A**), luminal A breast cancer MCF7 cells (**Supplementary Figure S8B**), renal cancer A498 cells (**Supplementary Figure S8C**) and colon cancer HCT-116 cells (**Supplementary Figure S8D**). The specificity of target cell lysis by the CTLs was evident from the fact that, the percent target cell lysis was increased as the target: effector ratio increased from 1:1 to 1:40. Further, negligible cell killing of a non-specific target, i.e., K562 cells (HLA-null) was observed confirming the HLA-restricted killing activity of the CTLs.

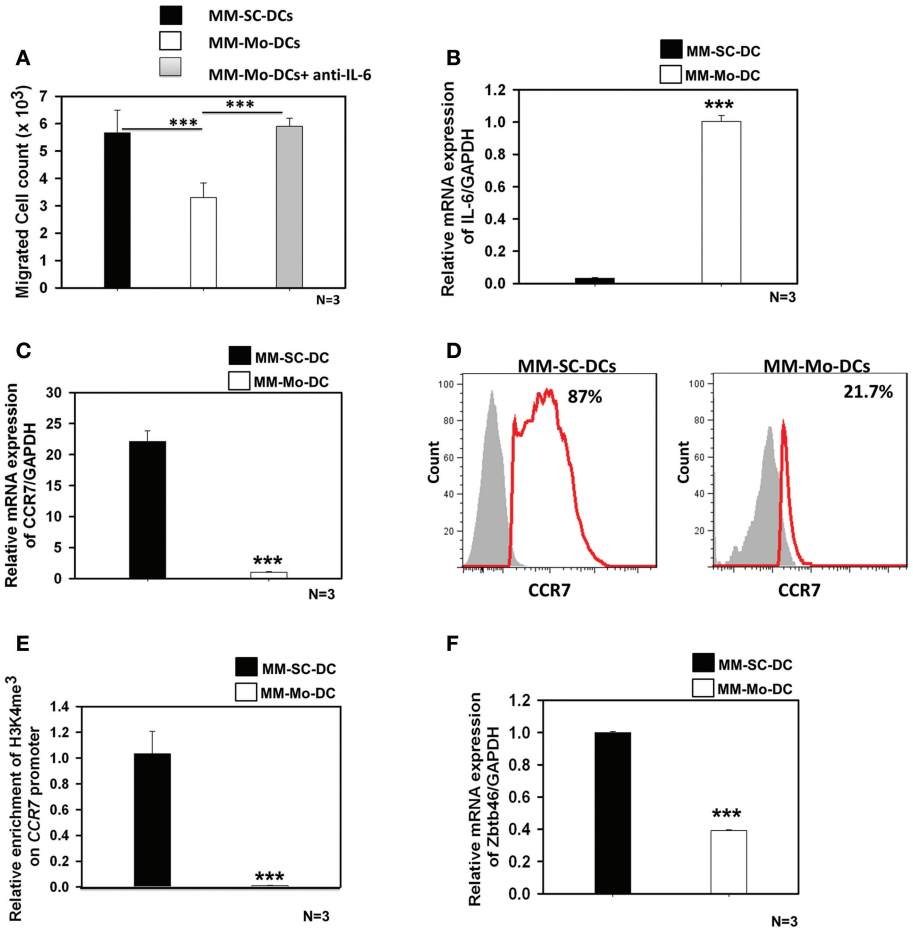

FIGURE 3 | MM-Mo-DCs and MM-SC-DCs generated from the same MM samples showed differences at the molecular level. **(A)** MM-Mo-DCs showed significantly reduced migration toward CCL-19, as opposed to MM-SC-DCs. When MM-Mo-DCs were differentiated in presence of anti-IL6 antibody, their migration capacity was restored. **(B)** Expression of the IL-6 mRNA in precursors of MM-SC-DCs was significantly lower, as compared to that of MM-Mo-DC precursors. Expression of CCR7 at **(C)** the transcript and **(D)** the protein level was significantly lower in MM-Mo-DCs in comparison with MM-SC-DCs as analyzed by Real time PCR and FACS, respectively. **(E)** Relative enrichment of H3K4me^3 at the Ccr7 promoter in MM-SC-DCs was significantly higher, as compared to MM-Mo-DCs. **(F)** Levels of the mRNA of transcription factor Zbtb46 were significantly higher in MM-SC-DCs, as opposed to those in MM-Mo-DCs. Data given are mean ± S.E.M $p \leq 0.001$ (***).

MM-SC-DCs Could Generate Antigen-Specific CTLs, but Were Compromised as Compared to CTLs From HD-SC-DCs

We further studied whether SC-DCs from MM samples could also generate potent CTLs, using the lysate of the HLA-A2$^+$ multiple myeloma cell line, U226B1. CTL generation, characterization and assays for testing their function were performed on HLA-A2 positive HD and MM samples as described earlier. Contrary to our expectations, MM-CTLs generated from MM-SC-DCs were functionally defective and showed signs of exhaustion, as compared to HD-CTLs generated from HD-SC-DCs. The expression of granzyme A (**Figure 4A**), granzyme B (**Figure 4B**), the surface activation marker CD69 (**Figure 4C**), and secretion of IFN-γ (**Figure 4D**) in the culture supernatant were found to be significantly declined in the CTLs from MM samples as compared to the HD samples. Representative dot plot for the expression of CTLs markers are given in **Supplementary Figure S9**. Further, *in vitro* CTL activity against the target U266B1 cell line was significantly reduced in MM samples, as compared to HD-CTLs. Killing of K562 (a non-specific cell line) was negligible, and was not significantly different between HD and MM-CTLs (**Figure 4E**).

Naïve T Cells in MM Showed Exhaustion, Which Could be Rescued Using a CTLA-4 Blocking Antibody

The percent of CD8$^+$ naïve T cell population in the MM samples was significantly lower as compared to that in healthy donor samples (**Figure 5A**). T cell population in apheresis samples were further examined for effector and memory phenotype using CD45RA and CD62L markers. No difference was seen for terminally differentiated effector cells, effector memory, and central memory cells (**Supplementary Figure S10**). To examine

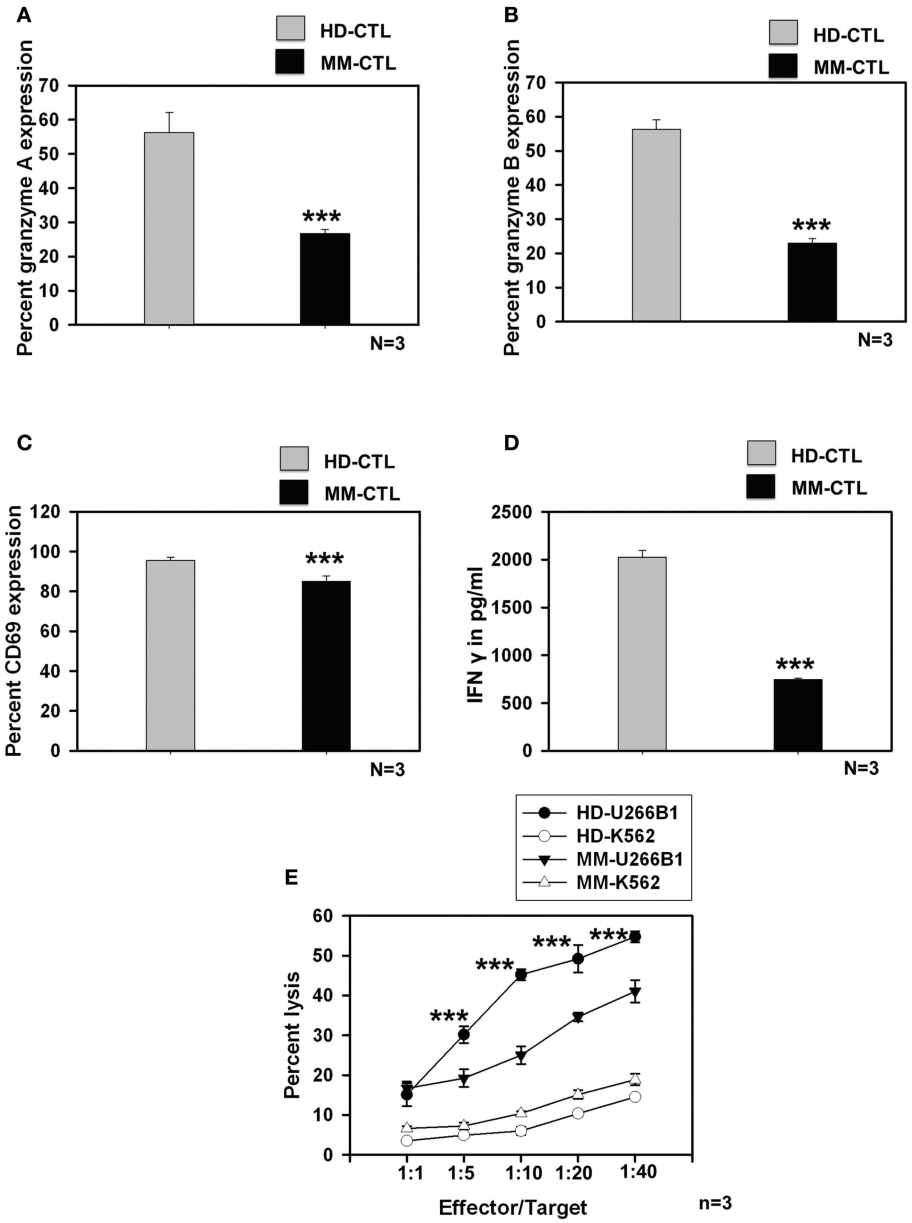

FIGURE 4 | CTLs generated from MM-SC-DCs were compromised, as compared to those from HD-SC-DCs. The percent expression of **(A)** granzyme A **(B)** granzyme B, and **(C)** CD69 was significantly reduced in the MM CTLs, as against that in the HD CTLs. **(D)** The concentration of IFNγ in the SC-DC- CTL co-cultures of MM samples was significantly lower, as compared to those of HD samples. **(E)** The target cell killing activity of MM-CTLs tested against the U266B1 cell line was significantly reduced as compared to HD-CTLs. Killing of non-specific target cell line K562 was negligible in both HD and MM CTLs. Data given are mean ± S.E.M $p \leq 0.001$ (***).

whether functional impairment of MM-CTLs was due to a defect in MM-SC-DCs or autologous T cells obtained from MM samples. Co-cultures of MM-SC-DCs with autologous naïve T cells or allogeneic naïve T cells obtained from HLA-$A2^+$ healthy donor samples were studied. Expression of Ki-67 on T cells was monitored as an indication of initiation of T cell proliferation and activation. It was observed that T cells from an allogeneic source have higher level expression of Ki-67, as compared to T cells from an autologous source (**Figures 5B,C**). The above data clearly indicated that, even though SC-DCs obtained from MM samples were functional and immunocompetent, MM-SC-DC vaccine may not be superior for tumor regression in MM patients, since autologous T cells are exhausted. Exhausted T cells in chronic infections and cancer show expression of the surface molecule, CTLA-4 (cytotoxic T-lymphocyte-associated protein 4, **Supplementary Figures S11A,B**). Binding of CTLA-4

with its ligand initiates a signaling cascade that lowers the effector function of T cells. To test if blocking of CTLA-4 would result in increased proliferation and activation of autologous T cells' we performed MM-SC-DC and autologous T cell co-culture in the presence or absence of anti-CTLA-4 antibody. Expression of Ki-67 and CD69 markers on CTLs was significantly increased in the presence of the CTLA-4 blocking antibody during the co-cultures (**Figures 5D,E**). However, even after blocking of CTLA-4, the percent of Ki-67 positive cells reached only upto 21% which is still lower as compared to Ki-67 positive cells in healthy allo-naïve T cells and MM-SC-DCs co-cultures (upto 58%, **Figure 5C**). Thus, indicating that blocking of CTLA-4 only partially restores the autologous T cell proliferation and activation. In other words, SC-DCs vaccines treatment of MM, may be effective if when used in combination with anti-CTLA-4 antibody.

Taken together our data show that DCs generated from stem cells are more potent than the DCs generated from monocytes of MM patients. Stem cell derived-DCs from apheresis samples from healthy donor could generate functional CTLs. Though the SC-DCs from MM samples were functional, autologous T cells showed signs of exhaustion, which could be partially rescued with the help of the immune check-point inhibitor CTLA-4. Hence, for treatment of MM, the potency of stem cell-derived DC vaccine could be enhanced when they are used in a combination with the checkpoint inhibitor, CTLA-4.

DISCUSSION

In multiple myeloma, activation of an anti-tumor immune response completely eliminates the disease while compromised immunity contributes to its aggression (26). Malignant cells adversely affect immune system leading to its deregulation. Training or modulating the immune system to combat malignant cells with the help of a DC vaccine could be one of the effective solutions to this problem. Therefore, immunogenic DC vaccine preparation is a crucial part of the DC based cancer immunotherapy (27). There are reports of DC vaccines being used for the treatment of MM. However, the efficacy of these vaccines is still below the expectation (28). In order to increase the efficiency some investigators have attempted a combination of DC vaccine with the drug Lenalidomide for treatment of MM (29) and colon cancer (30) in murine model. In yet another study pomalidomide and dexamethasone were combined with DC vaccine and tested in murine MM model (31). Vo et al. (32) have also shown that addition of Lenalidomide in different concentrations *in vitro* on Mo-DCs obtained from multiple myeloma patients improved the functionality of DC.

In our previous study, we had shown that some of the functions of monocytes-derived DCs from MM patients' samples were compromised (15). To evaluate if DCs derived from stem cells of MM patients' samples are similarly impaired, the present study was undertaken. HSCs from apheresis samples were expanded into DC precursors and differentiated into mature DCs using a two step method developed in our lab. It was observed that HD-SC-DCs and MM-SC-DCs were equivalent with respect to morphology, phenotype, and functions. In the phenotype the percent expression of CD83 however was significantly lower in MM-SC-DCs as compared to HD-SC-DCs. CD83 is an important costimulatory molecule expressed on DCs (33). Though the percent values of CD83 were low, the MFI values for CD83 of MM-SC-DCs were comparable with that of HD-SC-DCs (**Figure 1D**).This difference was not seen at transcript level also (**Supplementary Figure S12**). Nonetheless it is worth looking into the mechanism behind low expression of CD83 in MM-SC-DC at protein level.

Cord blood (CB) being an easily available source of HSCs, CB-HSCs derived-DCs are well-studied. The anti-tumor activity of stem cell-derived DCs from cord blood or mobilized peripheral blood were tested in Phase I and II clinical trials with promising outcomes of generation of tumor-specific immunity against metastatic melanoma (9, 34, 35). Titzer et al. (36) reported that stem cell derived-DCs can induce clinically-relevant humoral and cellular idiotype-specific immune responses in advanced MM patients. Currently, a two-arm phase I randomized trial is underway (NCT01995708) to test the efficacy of autologous stem cell-derived DCs transfected with mRNA encoding TAAs, for the treatment of multiple myeloma patients. These studies strongly suggest that, stem cells could be used to generate large numbers of functional DCs for cancer immunotherapy.

To gain deeper insights into the observed differences in the functionality of Mo-DCs and SC-DCs from MM patients, a paired sample study was performed, which revealed that the precursor of MM-Mo-DCs had higher autocrine IL-6 secretions as compared to MM-SC-DCs. It is well-documented that high expression levels of IL-6 in DCs are responsible for defects in migration in other types of cancers including, cervical cancer (17), breast cancer (18), and ovarian cancer (37). Blocking of IL-6 during differentiation of MM-Mo-DCs resulted in the gain of migration function. Migration in MM-Mo-DCs was significantly lower when all four sets were compared (**Supplementary Figure S13**). DCs that can initiate an antitumor immune response are defined by chemokine receptor CCR7, the transcriptional factor Zbtb46, and the Flt3L and Kit receptors (38). In MM-Mo-DCs, expression of CCR7 was reduced as compared to MM-SC-DCs. Lower abundance of $H3K4me^3$ at the CCR7 promoter might also be responsible for lower CCR7 expression and its associated migration in MM-Mo-DCs as compared to MM-SC-DCs. The signature DC transcription factor, Zbtb46, was also lower in the MM-Mo-DCs, as opposed to MM-SC-DCs, suggesting that the mature DC population may vary in its intrinsic properties if these cells are derived from two distinct precursor populations from the same patient. Thus, our findings suggest that SC-DCs are potential candidates for use as cancer vaccines in MM. Though here our study is limited to migration function of SC-DCs, still further molecular characterization of other functions like antigen processing and presentation remains to be looked into.

Previous studies have reported that tumor cells and their microenvironment convert myeloid cells into immunosuppressive cells (39, 40). Peripheral blood monocytes which are taken directly from MM patients' body have been exposed to a tumor regulatory microenvironment. Mo-DCs obtained from these monocytes were therefore found to be

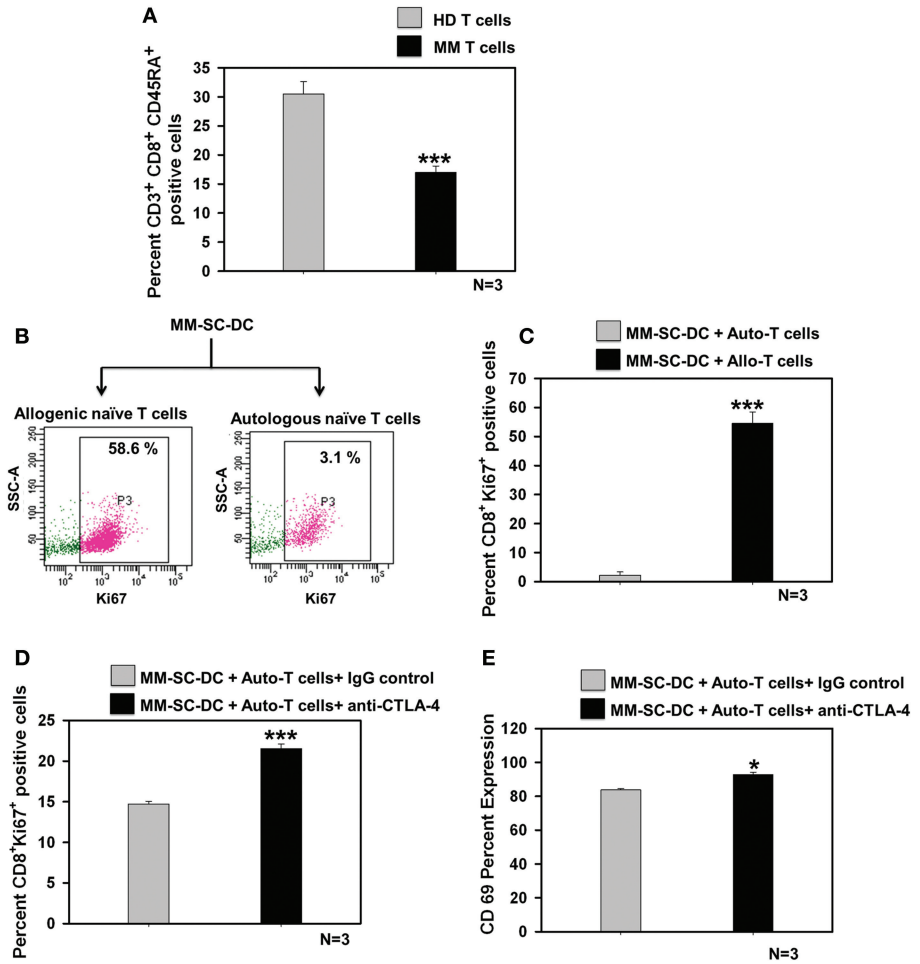

FIGURE 5 | Exhaustion of naïve T cells of MM could be rescued by blocking the CTLA-4 molecule. **(A)** Percent of naïve T cells in the MM samples was significantly lower as compared to HD samples. **(B)** Representative FACS profile of Ki-67 positive cells and **(C)** Data from three different samples of MM-SC DCs and allo/auto- T cell co-cultures is depicted. Percent Ki-67 positive cells were significantly lower in MM-SC-DCs co-cultured with autologous T cells as compared to co-cultures with allogeneic T cells. **(D)** Blocking of CTLA-4 molecule in the co-cultures of MM-SC-DCs and autologous T cells enhanced Ki-67 positive population as compared to co-cultures where IgG isotype antibody was added. **(E)** T cells activation in co-cultures of MM-SC-DCs and autologous naïve T cells was significantly increased after blocking of CTLA-4 receptor. Data given are mean ± S.E.M, $p \leq 0.05$ (*) $p \leq 0.001$ (***).

impaired. For generation of SC-DCs, HSCs were cultured in the presence of FLT3-L, SCF and TPO, which are known to promote the expansion of DC-precursors (41). These precursor cells were then differentiated into mature and functional DCs as seen by up regulation of costimulatory molecules in all four groups (**Supplementary Figure S14**). In cancer patients, presence of tumor cells or tumor-derived cytokines, or other factors, leads to defective differentiation, and functions of DCs (42). However, this was not the case for *ex vivo*–generated SC-DCs, since a tumor-associated microenvironment was completely absent during expansion and differentiation of the DCs precursors. Exposure to prolonged culture conditions and absence of tumor microenvironment during *in vitro* differentiation may have contributed to similarities observed in functional characteristics of MM-SC-DCs to HD-SC-DCs. Reports also suggest that DCs obtained from *in-vitro* differentiation of stem cells are more immunocompetent than the peripheral blood circulating DCs or monocyte-derived DCs of cancer patients (43, 44). We were also able to obtain functional DCs from stem cells, but not from monocytes of same MM samples. The ability of SC-DCs from MM patients for their anti-tumor response under *in vivo* situation is worth studying using humanized mouse model of multiple myeloma.

Generally for studying antitumor CTL generation from DC vaccine, a HLA-A2 restricted system is employed by various investigators (45–47). Similarly here we have focused on $CD8^+$ T cells as we are using HLA-A2 restricted system. We have screened both healthy and MM samples for HLA-A2 positivity and used U266B1 cell line which is not only HLA-A2 positive but also a model cell line for MM. Peptide antigen generated from MUC1 antigens are known to be cross presented by DCs in the form of a complex with HLA-$A2^+$ (48). MUC 1 is highly expressed in

U266B1 (49). Cytotoxicity assay in our study provides evidence that the killing of target cancer cell lines is HLA dependent. However, alloreactivity and tumor antigens dependent killing by the CTLs primed by tumor antigen pulsed SC-DCs remains to be verified.

$CD4^+$ T cells also have a major role to play in $CD8^+$ T cells stimulation. It is worth looking into how MM-SC-DCs contribute to anti- tumor CD4 generation from naïve CD3 cells. These studies form a part of our future work plan.

Our data indicated that CTLs primed from HD-SC-DCs of apheresis samples from healthy donors exhibited efficient killing of cancer cell lines *in vitro*. However, CTLs from MM-SC-DCs exhibited reduced killing activity due to exhaustion of autologous T cells. Recently, Leone et al. (50) have shown that BM of MM patients, have a $CD8^+$ T cell population that expresses Foxp3, produces IL-10 and TGF-β, and exerts pro-tumor activity. Antigen specific T cell activation requires T cell receptor (TCR) engagement with the antigen/MHC complex followed by costimulatory signal. After antigen recognition, co-stimulatory molecules on APCs interact with their binding partner on T cells and initiate T cell proliferation and activation. Prolonged antigen exposure in chronic diseases is responsible for T cell exhaustion. Such T cells express the surface marker, CTLA-4, which arrests T cell activation. Cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) and CD28 are the two receptors on T cell. Both of these receptors bind to co-stimulatory molecules B7.1 and B7.2 on APCs. CD28 acts as an activator of T cell proliferation by binding to B7 and promoting IL-2 production and thus initiating T cell activation. Whereas, CTLA-4 act as the negative regulator, it inhibits T cell activation by inhibiting IL-2 production and cell cycle progression of T cells (51). CTLA-4 signal is crucial for Foxp3+ Treg development (52, 53).There are reports that Treg cells promote tumor progression. T_{reg} cells hamper immune surveillance against cancer and prevent the development of effective antitumor immunity in tumor-bearing patients, and promote tumor progression. Therefore, for successful cancer immunotherapy, it is required that T regulatory cells should be kept suppressed in the tumor microenvironment (54).

We studied the effect of blocking of CTLA-4 on T cell proliferation and it was observed that it partially restored the proliferation in T cells and their activation. However, the T cell phenotype analysis that we have performed in the study is limited to CD45RA and CD62L. More detailed study that defines MM associated memory T cell exhaustion using CD45RA and CCR7 needs to be undertaken. In addition it is also important to further characterize the T cells by staining with key markers such as PD-1, TIM-3, TIGIT, and LAG-3 for demonstration of T cell exhaustion.

Blocking of CTLA-4 using antibodies has been reported to reactivate effector function of T cells from their exhausted state (55, 56). Previously, metastatic melanoma and ovarian carcinoma patients were vaccinated with DC vaccine or GM-CSF secreting tumor cells (57). In these patients, the T cell- mediated anti-tumor response against melanoma, and ovarian carcinoma was increased after the administration of an anti-CTLA-4 antibody. In other words, their data showed that, the efficiency of CTLs generated by a cellular vaccine was increased by administrating the cancer patients with anti-CTLA-4 antibody. In a preclinical study using mouse model, it was shown that check point inhibitors like CTLA-4 and PD-1 activate $CD103^+$ DCs and increase IL-12 secretion by them, leading to an increase in the anti-tumor immune response (58). Vo et al. (59) have used a dual combination of lenalidomide and programmed death (PD)-1 blockade to enhance efficacy of DC vaccine in MM in murine model.

A combination of a DC vaccine with a CTLA-4 blocking antibody (ipilimumab) was studied in a Phase II clinical trial by Wilgenhof et al. (60). In the cohort, 51% of the patients achieved complete or partial remission of melanoma and the overall response rate was 38%. Currently, numerous clinical studies on the effect of the combination of DC vaccines with immune check point inhibitors on cancer patients are underway (www.clinicaltrials.gov). We also observed in present study that activation of T cells and their proliferation was partially regained by blocking the CTLA-4 receptor in MM-SC-DCs and autologous T cells co-cultures. In conclusion, our findings demonstrated that combining MM-SC-DCs with a check point inhibitor could be a preferred DC-vaccine strategy for MM cancer immunotherapy.

Many clinical trials with DC-based cancer treatment modalities have shown that their clinical efficacy still needs improvement. Our findings would be helpful in enhancing the efficacy and feasibility of personalized cancer immunotherapy using DC vaccines for multiple myeloma.

ETHICS STATEMENT

Written informed consents were obtained from healthy donors and multiple myeloma patients prior to collection of apheresis samples. All experimental procedures and informed consents were approved by the Institutional Ethics Committee (IEC) and Institutional Committee for Stem Cell Research (IC-SCR) of NCCS, and Deenanath Mangeshkar Hospital, in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

PS carried out the experiments and analyzed the data. PS and LL were involved in the interpretation of the data. MS contributed to epigenetic study design. SM provided clinical samples. PS, LL, and VK wrote the manuscript. LL conceived and designed the study. All authors reviewed the data, and approved submission of the 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.2019.01079/full#supplementary-material

Supplementary Figure S1 | Healthy donors and multiple myeloma patients' characteristics. **(A)** Details of age and sex of the healthy donors, and stage of the disease at the time of the first visit (for MM patients) is depicted for 8 HD and 7 MM samples. **(B)** chemotherapy regimens of 7 MM samples is tabulated.

Supplementary Figure S2 | Cell surface marker expression on HD-SC-DCs and MM-SC-DCs was comparable. Representatives histogram of DC lineage surface marker (HLA-DR, HLA-ABC, and CD58 in green color; CD86, CD80, CD83, and CD54 in orange color; CD11c, CD40, and CD1a in red color lines) along with their appropriate isotype controls (Gray color filled) are depicted.

Supplementary Figure S3 | MM-SC-DCs and HD-SC-DCs show equivalent dextran-FITC uptake. A representative FACS histogram overlay of dextran- FITC uptake profile from HD-SC-DCs and MM-SC-DCs is depicted. Green line shows uptake at 37°C while the blue line shows uptake at 4°C. Gray shaded histogram shows unstained control cells.

Supplementary Figure S4 | Proliferation of allogeneic T cells in co-cultures of SC-DCs from HD and MM samples. A representative FACS profile of T cells after 5 days of co-cultures with SC-DCs from both the groups is depicted. Dark green filled histogram represents CD3+ allogeneic T cells only while light green filled histogram represents SC-DCs –CD3+ allo-T cells co-cultures.

Supplementary Figure S5 | CCR7 and CCL-19 interaction is essential for DC migration. *In vitro* migration of HD-SC-DCs and MM-SC-DCs toward CCL-19 was significantly reduced only when the mature DCs were treated with anti-CCR7 antibody. Whereas, blocking of other receptors such as CCR1, CCR3, and CCR5 did not show any significant reduction in DCs migration. Data given are mean ± S.E.M $p \leq 0.001$ (***).

Supplementary Figure S6 | Sorting of naïve T cells from HD/MM samples. Representative FACS profile and gating strategy for sorting of naïve T cells from apheresis samples of MM patients are shown.

Supplementary Figure S7 | Characterization of CTLs from HD-SC-DCs primed against MDA-MB-231-luc-D3H2LN **(A)** Representative FACS profile for the expression of granzyme A, granzyme B, perforin, and CD69 in CTLs generated from HD samples is depicted. **(B)** Levels of IFNγ in the CTLs obtained from three different HD samples were similar.

Supplementary Figure S8 | CTLs generated from healthy donor samples showed killing effect against different cancer cell lines *in vitro*. HD-SC-CTLs could be primed against the desired cancer cell lines and had very specific target-killing activity for **(A)** MDA-MB-231-LUC-D3H2LN **(B)** MCF-7 **(C)** A498, and **(D)** HCT-116. The CTLs showed very negligible killing of K562, which was a non-specific target cell line. Data given are mean ± S.E.M.

Supplementary Figure S9 | MM-CTLs have reduced expression of serine proteases and activation marker as compared to HD-CTLs: Representative FACS dot plot with isotype control for **(A)** granzyme A **(B)** granzyme B, and **(C)** CD69 in CTLs of both HD samples and MM samples are shown.

Supplementary Figure S10 | Analysis of CD8+ T cell subtypes. Naïve effector and memory CD3+CD8+ T cells were analyzed based on marker expression of CD45RA and CD62L. The data shown are for Naïve T cells ($CD45RA^{+}CD62L^{+}$) terminally differentiated effector memory cells ($CD45RA^{+}CD62L^{-}$) memory cells ($CD45RA^{-}CD62L^{-}$) and central memory cells ($CD45RA^{-}CD62L^{+}$) from three different HD and MM samples. Only naïve CD8+ T cells were significantly reduced in MM samples. Data given are mean ± S.E.M $p \leq 0.01$ (**).

Supplementary Figure S11 | Naïve T cells from MM samples had higher expression of CTLA-4. **(A)** Dot plot showing the representative FACS profiles of naïve T cells from MM and HD samples showing CTLA-4 expression are given. **(B)** Cumulative data from three different samples of HD and MM naïve T cells for CTLA-4 basal expression is given. Data given are mean ± S.E.M $p \leq 0.001$ (***).

Supplementary Figure S12 | Expression of costimulatory molecules CD40, CD80, CD83 and CD86 on HD-Mo-DCs, MM-Mo-DCs, HD-SC-DCs and MM-SC-DCs is depicted.

Supplementary Figure S13 | MM-Mo-DCs showed significantly lower migration toward CCL-19 as compared to HD-Mo-DCs, HD-SC-DCs and MM-SC-DCs.

Supplementary Figure S14 | Percent expression of costimulatory molecules CD40, CD80, CD83 and CD86 on immature and mature HD-Mo-DCs, MM-Mo-DCs, HD-SC-DCs and MM-SC-DCs is given.

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Sequential Anti-PD1 Therapy Following Dendritic Cell Vaccination Improves Survival in a HER2 Mammary Carcinoma Model and Identifies a Critical Role for CD4 T Cells in Mediating the Response

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Patients with metastatic HER2 breast cancer (MBC) often become resistant to HER 2 targeted therapy and have recurrence of disease. The Panacea trial suggested that HER2 MBC patients were more likely to respond to checkpoint therapy if TIL were present or if tumor expressed PD-L1. We assessed whether type I polarized dendritic cells (DC1) could improve checkpoint therapy in a preclinical model of HER2$^+$ breast cancer. TUBO bearing mice were vaccinated with either MHC class I or class II HER2 peptide pulsed DC1 (class I or class II HER2-DC1) concurrently or sequentially with administration of anti-PD-1 or anti-PDL1. Infiltration of tumors by immune cells, induction of anti-HER2 immunity and response to therapy was evaluated. Class I or class II HER2-DC1 vaccinated mice generated anti-HER2 CD8 or CD4+ T cell immune responses and demonstrated delayed tumor growth. Combining both MHC class I and II HER2-pulsed DC1 did not further result in inhibition of tumor growth or enhanced survival compared to individual administration. Interestingly class II HER2-DC1 led to both increased CD4 and CD8 T cells in the tumor microenvironment while class I peptides typically resulted in only increased CD8 T cells. Anti-PD-1 but not anti-PD-L1 administered sequentially with class I or class II HER2-DC1 vaccine could improve the efficacy of HER2-DC1 vaccine as measured by tumor growth, survival, infiltration of tumors by T cells and increase in systemic anti-HER2 immune responses. Depletion of CD4+ T cells abrogated the anti-tumor efficacy of combination therapy with class II HER2-DC1 and anti-PD-1, suggesting that tumor regression was CD4 dependent. Since class II HER2-DC1 was as effective as class I, we combined class II HER2-DC1 vaccine with anti-rat neu antibodies and anti-PD-1 therapy. Combination therapy demonstrated further delay in tumor growth, and enhanced survival compared to control mice. In summary, Class II HER2-DC1 drives both a CD4 and CD8 T cell tumor infiltration that leads to increased survival, and in

combination with anti-HER2 therapy and checkpoint blockade can improve survival in preclinical models of HER2 positive breast cancer and warrants exploration in patients with HER2 MBC.

Keywords: breast cancer, dendritic cells, PD-1, PD-L1, HER2, immune checkpoints, CD4 T cells, Th1

INTRODUCTION

Breast cancer is the most commonly diagnosed tumor and a major cause of cancer death among women (1). A subset of breast cancers present expression/amplification of the HER2 protein/oncogene which correlates with increased recurrence rates and poor survival (2–4). HER2-targeted treatments have led to meaningful improvement in clinical outcomes for patients with breast tumors driven by HER2. For example, the HER2-targeted antibodies, trastuzumab and pertuzumab, combined with docetaxel improved the median overall survival (mOS) of patients with HER2-positive ($HER2^+$) metastatic breast cancer (MBC) to 56.5 months compared to 20.3 months for patients receiving chemotherapy alone (5, 6). In the second line setting, treatment with the antibody drug conjugate ado-trastuzumab (T-DM1) improved the mOS in patients with trastuzumab-resistant $HER2^+$ MBC from 15.9–25.9 to 22.7–29.9 months when compared to chemotherapy or to treatment with small tyrosine kinase inhibitor (lapatinib) (7). Taken together these data support the clinical validity of the HER2 antigen as a valid predictive biomarker of clinical benefit for treatment with HER2-targeted therapies even after disease progression with approved targeted agents. Notwithstanding the recent advances, HER2+ MBC will eventually acquire resistance to HER2-targeted therapies and disease progression will ensue. Therefore, alternative or other combinatorial approaches are needed to overcome resistance to HER2-targeted treatment and improve clinical outcomes.

The presence of tumor infiltrating lymphocyte (TIL) in $HER2^+$ breast cancer is consistently associated with improved prognosis and better survival (8–10). Trastuzumab treatment of breast cancer patients with the presence of TIL have improved survival and complete response to neoadjuvant therapy (9–12). HER2 antibody treatment has been reported in preclinical studies to induce adaptive and innate immune responses and to increase infiltration of immune cells into the tumor microenvironment (13).

Several co-inhibitory immune checkpoint signals such as programmed death 1 (PD-1) receptor/PD-ligand 1 (PD-L1) have been shown to inhibit anti-tumor immune responses (14, 15). Binding of PD-L1 to its receptor PD-1 on the surface of T cells can induce TIL exhaustion and evade anti-tumor immunity (16). Preclinical studies combining antibodies against PD-1 improved the immune-mediated effects of anti-HER2 monoclonal antibody therapy (17). These data provide a strong rationale for the use of immune checkpoint inhibitors as a combinatorial approach in $HER2^+$ breast cancer. The phase 1b/2 KEYNOTE-014/PANACEA trial evaluated the efficacy of pembrolizumab (anti-PD-1 antibody) in combination with trastuzumab in $HER2^+$ MBC patients that progressed after previous HER2 targeted therapies. Fifteen percent of the patients that were PD-L1 positive achieved an overall response and no overall response was observed in the PD-L1 negative cohort (18). Another study in a phase 1 trial evaluated trastuzumab in combination with durvalumab (anti-PD-L1) in metastatic $HER2^+$ breast cancer patients and no impact on objective responses was observed and all the patients enrolled in the trials had lower than 1% PD-L1 expression (19). Overall, these studies suggest that checkpoint inhibitors combined with anti-HER2 therapy have minimal impact. Developing strategies that can increase T cell infiltration in tumors may improve the efficacy of these therapies.

Vaccine strategies using dendritic cells to activate the immune system and generate Th1 immune responses have been extensively studied (20, 21). It has been well-documented that Th1 cytokine, IFN-γ can induce PD-L1 expression on tumor cells (22). In preclinical models of various cancer types with increased levels of immune checkpoint molecules expression on TIL, immune checkpoint blockade in combination with vaccine strategies has shown a superior response compared to monotherapy (23, 24). The role of $CD8^+$ T cells to generate anti-tumor immunity in $HER2^+$ breast cancers has been shown in various clinical trials (25) and has had minimal clinical impact (26, 27). However, the role and prognostic value of $CD4^+$ T cells has not been extensively studied in breast cancer. Previous findings from our lab have shown that anti-HER2 $CD4^+$ T helper cell (Th1) immunity plays a crucial role in cancer therapy and peripheral loss of the Th1 response correlates with poor treatment response and prognosis (28). Administration of class II HER2 peptide-pulsed Type I polarized dendritic cell (DC1) vaccine induced a strong anti-HER2 immune response with pathologic complete response rate (pCR) in $HER2^+$ DCIS patients (29–31). Very little is known about the role of anti-HER2 $CD4^+$ Th1 immune responses in combination with immune checkpoint therapy. Based on these preliminary findings, we hypothesized that HER2 peptide pulsed DC1 vaccine could prime an anti-HER2 response and generate anti-HER2 Th1 immune responses leading to the conversion of "cold" to "hot" tumors and thus improve the efficiency of immune checkpoint antibody therapy. The goal of this study was to investigate the anti-tumor efficacy of HER2 peptide pulsed DC1 vaccine in combination

Abbreviations: BM, Bone marrow; BSA, bovine serum albumin; Class I HER2-DC1, Class I HER2 pulsed DC1 vaccine; Class II HER2-DC1, Class II HER2 peptide pulsed DC1; DAPI, 4′6-diamindino-2-phenylindole; DC1, Type I polarized dendritic cells; FMO, Fluorescent minus one; iDC, Immature DC; LPS, lipopolysaccharide; MBC, Metastatic HER2 breast cancer; mOS, Median overall survival; NSCLC, Non- small cell lung carcinoma; PD-1, Programmed death 1 receptor; PD-L1, Programmed cell death 1 ligand 1; PVDF, Polyvinylidene difluoride; rHER2, rat HER2 oncogene/peptide; s.c., Subcutaneously; T-DM1, Ado-trastuzumab/ado-trastuzumab emtansine; Th1, T helper cell; TIL, Tumor infiltrating lymphocyte.

with PD-1/PD-L1 blockade and HER2 targeted therapy in a preclinical model of HER2$^+$ breast cancer.

MATERIALS AND METHODS

Animals

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee at the University of South Florida (#A4100-01). Mice were euthanized by CO_2 inhalation according to the American Veterinary Medical Association Guidelines. Mice were observed daily and euthanized if a solitary subcutaneous tumor exceeded the end point. All efforts were made to minimize suffering. Female Balb/C mice (6–8 weeks old) were purchased from Charles river. Mice were housed at the Animal Research Facility of the H. Lee Moffitt Cancer Center and Research Institute.

Tumor Cell Lines

TUBO breast cancer cell line (kind gift from Dr. Wei Zen Wei, Wayne State University) was cloned from a spontaneous mammary tumor in BALB/c mice transgenic for the rat Her-2/neu gene (BALB-neuT) (32) and was maintained by serial *in vitro* passages in complete medium (CM). Complete media consisted of RPMI 1640 (Fisher Scientific, Cat. No. MT-10-040-CM) supplemented with 10% heat-inactivated FBS (Fisher Scientific, Cat. No. MT35010CV), 0.1 mM nonessential amino acids (Fisher Scientific, Cat. No. 25025CI), 1 mM sodium pyruvate (Fisher Scientific, Cat. No. 25000CI), 2 mM fresh L-glutamine (Fisher Scientific, Cat. No. 25005CI), 100 mg/ml streptomycin and 100 U/mL penicillin (Fisher Scientific, Cat. No. MT-30-002-CI), 50 mg/mL gentamicin (Gibco, Cat. No. 15750060), 0.5 mg/mL fungizone (Gibco, Cat. No. 15290018) (all purchased from Life Technologies, Rockville, MD), and 0.05 mM 2-ME (Gibco, Cat. No. 21985023).

DC Generation

Bone marrow (BM) cells were harvested from femurs and tibias of Balb/C mice as described previously (33). Briefly, BM cells were flushed into a cell suspension in RPMI 1640, and RBCs were lysed using ACK lysing buffer. Cells were cultured with rFLT3L (VWR Peprotech, Cat. No. 10778-670) at 25 ng/mL and rmIL-6 (R&D Systems, Cat. No. 406-ML-025) at 30 ng/mL in T75 flasks and incubated for 6 days at 37°C and 5% CO2. The BM cells were then harvested, washed with RPMI 1640 and cultured with 50 ng/mL of rmGM-CSF (R&D Systems, Cat. No. 415-ML-050) and 10 ng/mL of rmIL-4 (R&D Systems, Cat. No. 404-ML-050) overnight, followed by DC1 maturation for 6–8 hours (h) with DC1 polarizing signals: CPG/ODN1826 (InVivoGen, Cat. No. tlrl-1826), a TLR 9 agonist at 10 ng/mL and lipopolysaccharide (LPS) (Millipore Sigma, Cat. No. L4391), a TLR-4 agonist at 20 ng/mL as described previously (33). When used for vaccination, DC1 cells were pulsed with multi-epitope peptides from the rat HER2/neu (rHER2/neu) oncogene at the concentration of 10 μg/ml of each peptide individually overnight; p5 (ELAAWCRWGFLLALLPPGIAG), p435 (IRGRILHDGAYSLTLQGLGIH), and p1209 (SPPHPSPAFSPAFDNLYYWDQ) and were pooled for class II HER2-DC1 vaccine studies (34). DC1 were pulsed with class I rat HER2/neu peptide p66 (TYVPANASL) for class I HER2-DC1 vaccine studies (35). All the peptides were synthesized from Bachem Americas, Inc. DC maturation was confirmed in a subset of samples at 24 h post addition of LPS and CPG by FACS analysis of cell surface markers, MHC class II (I Ad), CD80, CD86, and CD40 (FITC anti-mouse I-Ad (Clone 39-10-8, Biolegend, Cat. No. 115006); PE anti-mouse CD80 (Clone 16-10A1, Biolegend, Cat. No. 104708) anti-mouse CD40; PE anti-mouse CD86 (Clone GL-1, Biolegend, Cat. No. 105008); PE anti-mouse CD40 (Clone 3/23, Biolegend, Cat. No. 124610). IL-12 (p70) secretion by DC1 in culture supernatants was measured by standard IL-12 (p70) ELISA from R& D systems (Cat. No. M1270).

Monoclonal Antibodies

The monoclonal antibodies anti-PD-1 (clone RMP1-14, Cat. No. BE0146) and anti-PDL-1 (clone 10F.9G2, Cat. No. BE0101) were purchased from BioXCell (West Lebanon, NH). InVivoMAb rat IgG2a isotype (BioXCell, Cat. No. BE0089) was used as control. Anti-HER2 mouse monoclonal antibody 7.9.5 was a kind gift from Dr. Mark Greene, University of Pennsylvania and clone 7.16.4 was purchased from BioXCell (Cat. No. BE0277).

Immunofluorescence Staining for HER2

TUBO cells were grown to 80% confluence on sterile round glass coverslips in a six well tissue culture plate. Cells were washed three times with PBS and fixed in 4% (wt/vol) paraformaldehyde (Fisher Scientific, Cat. No. 50-980-487) for 15 min. Next, cells were permeabilized with 0.2% Triton X (Sigma Aldrich, Cat. No. T8787) for 10 min and washed three times with PBS. After washing, cells attached to cover slips were incubated with 5% (wt/vol) bovine serum albumin (BSA) (Fisher Scientific, Cat. No. BP1605) in PBS for 1 h at room temperature. Cells were then incubated with monoclonal primary anti-HER2/ErbB2 antibody (Cell Signaling Technology, Beverly, MA, Cat. No. 2165) over night at 4°C followed by incubation with Alexa Fluor 594-conjugated anti-rabbit secondary antibody (Cell Signaling Technology, Beverly, MA, Cat. No. 8889) for 1 h at room temperature in the dark. After being washed with PBS three times, coverslips were mounted using VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories, Cat. No. H-1200). The stained coverslips were examined and imaged using a Zeiss Apotome.2 fluorescence microscope (Carl Zeiss Inc., Thornwood, NY).

Western Blot Analysis

Total protein was isolated from TUBO and 4T1 cells for Western blot analysis. Briefly, cells were lysed with 1X RIPA buffer (EMD Millipore^{TM}, Cat. No. 20-188) containing protease inhibitor (Millipore Sigma, Cat. No. P8340) and phosphatase inhibitor (ThermoScientific Pierce, Cat. No. A32957), for 20 min at 4°C. The cell lysate was centrifuged at 15,000 rpm for 20 min and the supernatant containing total protein was collected

and stored at −80°C until further use. Protein concentration was measured by Bradford protein assay (Bio-Rad, Hercules, CA, Cta. No. 5000006). For Western blotting, 20 μg of each protein sample was resolved in a 4–12% SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Sigma, Cat. No. IPVH00010) using eBlot® L1 wet transfer system (GenScript, Piscataway, NJ). Membranes were blocked with 5% bovine serum albumin (BSA)/TBS-T for 1 h at room temperature, followed by overnight incubation with primary monoclonal anti-HER2/ErbB2 antibody (Cell Signaling Technology®, Cat. No. 2165) (1:1,000 dilution in 5% BSA/TBS-T) at 4°C. The next day, membranes were washed three times, 10 min each wash with TBS-T, and was probed with goat anti-rabbit IgG (H + L)-HRP conjugated secondary antibody (Cell Signaling Technology®, Cat. No. 7074; 1:5,000 dilution in 5% non-fat dry milk/TBS-T) for 1 h at room temperature and detected using the ECL western blotting detection system (Thermo Scientific Pierce, Cat. No. 32106). β-actin was used as endogenous control for all Western blot data analyses.

In vivo Treatments

A total of 2.5×10^5 TUBO cells were injected subcutaneously (s.c.) in female Balb/C mice. Seven days later when tumors were palpable, mice were treated with six doses of class I or class II HER2-DC1 vaccine subcutaneously either once, twice or three times weekly. For combination therapy, mice received either class I or class II HER2-DC1 vaccine (1×10^6 cells/mouse/subcutaneous injection/ 100 μl) with 150 μg of monoclonal antibody (isotype control or anti-PD-1 or anti-PD-L1) intraperitoneally twice a week concurrently or DC1 vaccination given first, followed by checkpoint antibodies. Mice continued to receive checkpoint antibody treatment twice a week until the tumor reached a size of 2 cm in diameter. Tumor size was measured and recorded every 2–3 days. Six mice per group were used and each experiment was performed three times. For functional analysis, mice were euthanized at day 28 after tumor injection. Tumors and splenocytes were harvested for *in vitro* assays.

CD4 T Cells Depletion

Anti-CD4 antibody (InVivoMab clone GK1.5 purchased from BioXCell, Cat. No. BE0003-1) was used to deplete CD4 T cells in the experimental mice. Three days before the TUBO injection, Balb/c mice were administered intraperitoneally with 300 μg of anti-CD4 antibody and continued with two injections per week until the end point. When tumors were palpable around days 7–10, mice were treated with multi-epitope class II HER2-DC1 vaccine subcutaneously twice a week. Another group of TUBO bearing mice without CD4 depletion received class II HER2-DC1 vaccine twice a week for total of six doses. Mice treated with or without CD4 depleting antibody were randomized into two groups to receive a follow up treatment with anti-PD1 antibody twice a week until the end point. Tumor size was measured and recorded twice a week.

HER2 Blockade in Combination With Class II HER2-DC1 and Anti-PD-1 Therapy

For *in vivo* treatments of HER2 targeted therapy in combination with HER2-DC1 vaccine and anti-PD-1 antibody, Balb/C mice were injected with 3×10^4 TUBO cells/50 μl in mammary fat pad per mouse. On day 12 after TUBO cells injection and when tumors were palpable, mice were randomized in four groups: (1) untreated, (2) anti-PD-1 therapy, (3) class II HER2-DC1 vaccine, and (4) combination therapy with anti-HER2, anti-PD-1, and HER2-DC1. For combination treatments, mice received anti-HER2 antibodies (clone 7.16.4 and 7.6.5) (50 μg/clone/mouse) on day 12. One week after anti-HER2 antibody treatment, mice received HER2-DC1 vaccine subcutaneously twice a week concurrently with combined 7.16.4 and 7.6.5 antibodies given once a week for 3 weeks. Upon completion of the combination treatment, follow up with anti-PD-1 antibody was given twice a week until the end point. Tumor growth was monitored twice a week and tumor volume was calculated following the formula: $(L \times W^2)/2 = mm^3$.

Flow Cytometry

On day 28 after tumor injection, spleens and/or tumors were harvested under sterile conditions. Single-cell suspensions were prepared, and red blood cells were lysed using ACK lysis buffer. Tumor cell suspensions were prepared from solid tumors by enzymatic digestion in HBSS (Fisher Scientific, Cat. No. MT-21-022-CM) containing 1 mg/ml collagenase (Cat. No. C9891 and C-5138), 0.1 mg/ml DNase I (Cat. No. DN25), and 2.5 U/ml of hyaluronidase (Cat. No. H-6254-1G) (all purchased from Millipore Sigma) with constant stirring for 2 h at room temperature as described previously (36). For analysis of immune cell populations, 1×10^6 cells (tumor digest suspension) were incubated for 30 min with Live/ Dead Zombie near IR (Biolegend, Cat. No. 423106) for 30 min in 1X PBS at room temperature in dark. After washing cells with 1X PBS, cells were stained with anti-mouse CD3 Alexa 488 (Clone 17A2, Biolegend, Cat. No. 100210), anti-mouse CD4 BV805 (Clone GK1.5, BD Biosciences, Cat. No. 564922), and anti-mouse CD8 pacific Blue (Clone 53–6.7, BD Bioscience, Cat No. 558106) and anti-mouse PD-1 BV605 (Clone 29F.1.A12, Biolegend, Cat. No. 135220) for 20 min on ice in staining buffer for surface expression analysis, according to the manufacturer's instructions (all antibodies were purchased from BD Biosciences). Samples were analyzed using an LSRII (BD Biosciences) cytometer and FACS data was analyzed using FlowJo software (Tree Star).

Functional Assays

To examine antigen specificity following HER2-DC1 vaccination in TUBO bearing mice, 2×10^6 splenocytes from control and treatment groups were cultured with 2 μg/ml of class I (p66) peptide, control peptide, or no peptide (complete media only) or multi-epitope class II rat HER2/neu peptides (p5, p435, p1209) individually for 3–4 days. Culture supernatants were collected to measure IFN-γ secretion using a standard quantikine IFN-γ ELISA (R&D systems, Cat. No. SMIF00) according to manufacturer's recommendations.

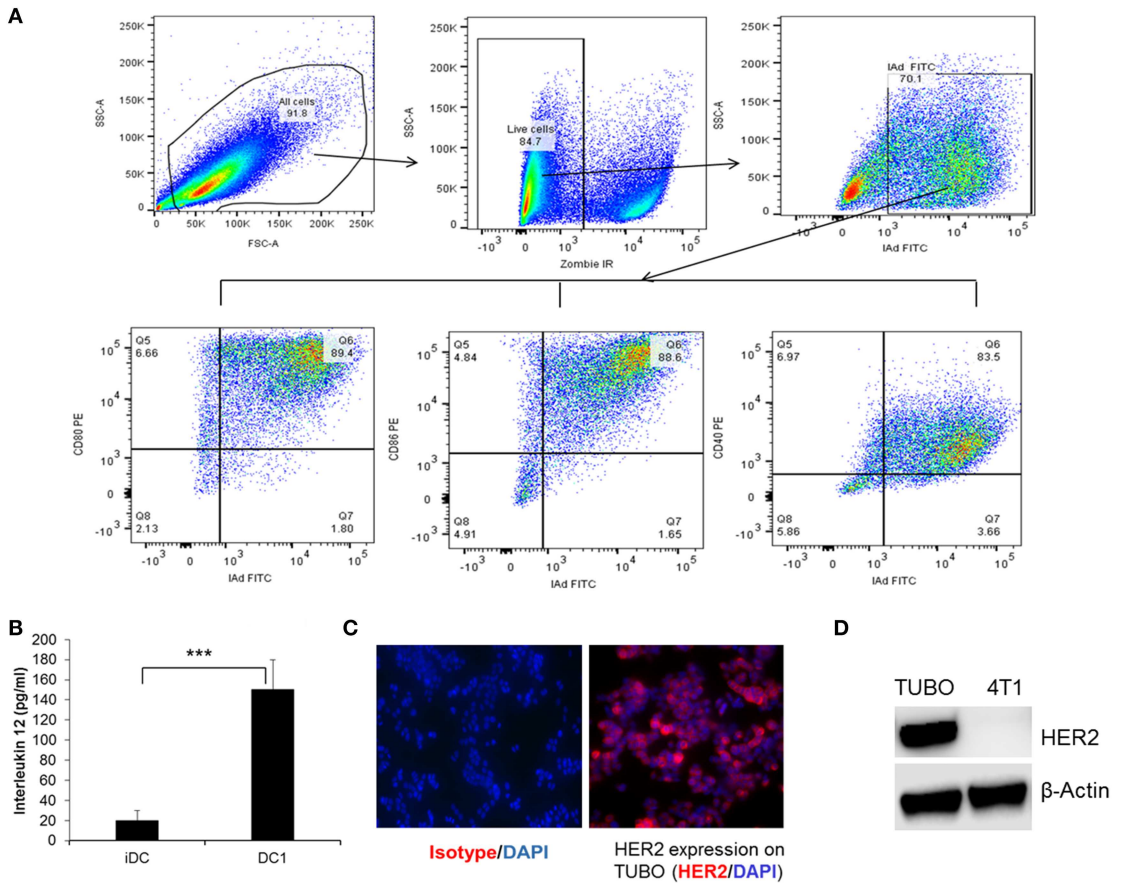

FIGURE 1 | Immunophenotyping and function of type I polarized DC1 from Balb/C mice. **(A)** DC collected after maturation with CpG and LPS were stained for MHC class II (IAd), CD80, CD86, and CD40 and data were acquired on LSRII flow cytometer and analyzed by FlowJo software. Flow gating strategy and representative flow dot plot of DC1 staining for MHC class II, CD80, CD86, and CD40. **(B)** Culture supernatants were collected before and after DC maturation and measured for IL-12 secretion using standard ELISA. **(C)** HER2 expression on TUBO cells using immunofluorescence staining. **(D)** Western blot analysis of HER2 protein expression on tumor cells. *P*-values were determined by Student *t*-test. ***$p < 0.001$.

Statistical Analysis

The Mann–Whitney test (unpaired) or the Student's *t*-test was used to compare results between two treatment groups. All statistical analyses of data were performed using GraphPad Prism software. Statistical significance was achieved at $p < 0.05$.

RESULTS

DC1 From Balb/C Mice Secrete IL-12 and Express CD80, CD86, and CD40

To examine the phenotype and the maturation status of DC generation from bone marrow of Balb/C mice, DC1 were collected following maturation and stained for the expression of cell surface markers, class II (I-Ad), CD80, CD86, and CD40 and data was acquired on flow cytometer as described in the Materials and Methods section. Functional status of DC1 was measured by IL-12 production 24 h after addition of final maturation signals. Culture supernatants from immature DC (iDC) were used as control. Flow gating strategy is shown in **Figure 1A**. Cells were gated on live population followed by gating on MHC class II (IAd) positive cells for CD80, CD86, and CD40 expression. Addition of CPG and LPS resulted in a higher percentage of DC maturation surface markers CD80, CD86, and CD40 (**Figure 1A**) with higher levels of IL-12 production compared to iDC (**Figure 1B**, $p < 0.001$). To examine the anti-tumor efficacy of DC1 vaccine in a $HER2^+$ breast tumor model, we utilized the TUBO cell line which was derived from a spontaneous mammary tumor in Balb/c mice transgenic for the rat Her-2/neu gene (BALB-neuT). As shown in **Figure 1C**, we confirmed the surface expression of HER2 on TUBO cells by immunofluorescence. HER2 protein expression was also confirmed by western blot, along with 4T1, a triple negative cell line (negative for ER, PR, and HER2), as negative control (**Figure 1D**). Overall, our data suggests that DC1 we generated from bone marrow of Balb/C mice secrete high levels of IL-12, and express mature DC phenotype.

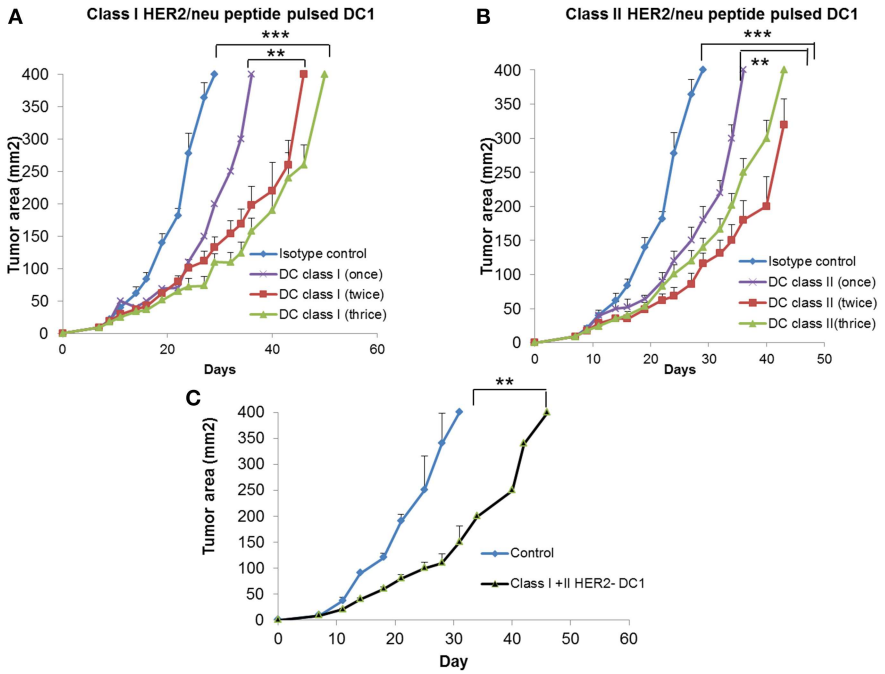

FIGURE 2 | Vaccination with DC1 pulsed with class I or class II HER2 peptides delays tumor growth in TUBO bearing mice. **(A)** DC1 was generated as described in methods section and pulsed with class I rHER2 peptide p66 (class I HER2-DC1). Balb/C mice were injected with 2.5×10^5 TUBO cells subcutaneously on the right flank on day 0. When tumors were palpable on day 7, mice received class I HER2-DC1 vaccine subcutaneously on the left flank once, twice or three times a week for a total of six doses. $N = 8$ mice/group was used for these studies. Tumor area was measured 2–3 days a week. **(B)** Balb/C mice received tumor cells and HER2-DC1 vaccine as described above. DC1 were pulsed with 10 μg/ml of each peptides from the rat HER2 (rHER2) oncogene; p5 (ELAAWCRWGFLLALLPPGIAG), p435 (IRGRILHDGAYSLTLQGLGIH), and p1209 (SPPHPSPAFSPAFDNLYYWDQ) and p66 (TYVPANASL). **(C)** Balb/C mice received tumor cells as described above and received both class I and class II pulsed HER2-DC1 vaccine. Data shown are the representative from three independent experiments and are shown as mean number ± SEM $^{***}p < 0.001$, $^{**}p < 0.01$ using Student *t*-test.

Class I or Class II HER2 Peptide- DC1 Vaccine Delay Tumor Growth and Induce Anti-HER2 Th1 Immune Response With Increased T Cell Infiltration in TUBO Bearing Mice

To examine the anti-tumor efficacy of DC1 vaccine, we utilized the HER2 positive TUBO model. Balb/C mice were injected with TUBO cells (2.5×10^5 cells/mouse/s.c.,) on day 0. Starting on day 7 when tumors were palpable, TUBO bearing mice were treated with either class I HER2 pulsed DC1 vaccine (Class I HER2-DC1, 1×10^6 DC1/mouse/100 μl /s.c.,) or class II HER2 peptide pulsed DC1 (Class II HER2-DC1). Treatment groups included TUBO bearing mice with no treatment, HER2-DC1 vaccine given once a week, twice or three times a week for total of up to six injections. TUBO bearing mice receiving class I or class II pulsed HER2-DC1 vaccine showed significantly delayed tumor growth compared to control mice (**Figures 2A,B**; $p < 0.001$) irrespective of whether HER2-DC1 vaccine was given once, twice or three times weekly. However, TUBO bearing mice receiving HER2-DC1 vaccine twice or three times a week had reduced tumor burden compared to the mice receiving weekly dose of class I or class II HER2-DC1 vaccine (**Figures 2A,B**). Although there was a significant delay in tumor growth in mice receiving HER2-DC1 vaccine given three times a week, as shown in **Figure 2A**, toxicity was observed (weight loss, hunched and sudden death) in this group. There was none observed in mice receiving once or twice weekly class I HER2-DC1 vaccine.

Since we observed anti-tumor effects of class I and class II HER2-DC1 vaccines shown in **Figures 2A,B**, we examined whether combining both class I and class II pulsed HER2-DC1 vaccine could have a synergistic effect in delaying tumor growth. As shown in **Figure 2C**, combined class I and class II pulsed HER2-DC1 vaccine given twice a week significantly delayed tumor growth in TUBO bearing mice but there was no additive benefit in reducing tumor burden (**Figure 2C**) compared to class I or class II HER2-DC1 alone as shown in **Figures 2A,B**.

HER2-DC1 Vaccine Generates Anti-HER2 Th1 Immune Responses in TUBO Bearing Mice

Next, we evaluated whether vaccination with HER2-DC1 could generate strong anti-HER2 Th1 immune responses in TUBO bearing mice. Spleens were harvested 1 week after the last DC1 vaccination and splenocytes were cultured with class I or class II peptides as described in the Materials and Methods section.

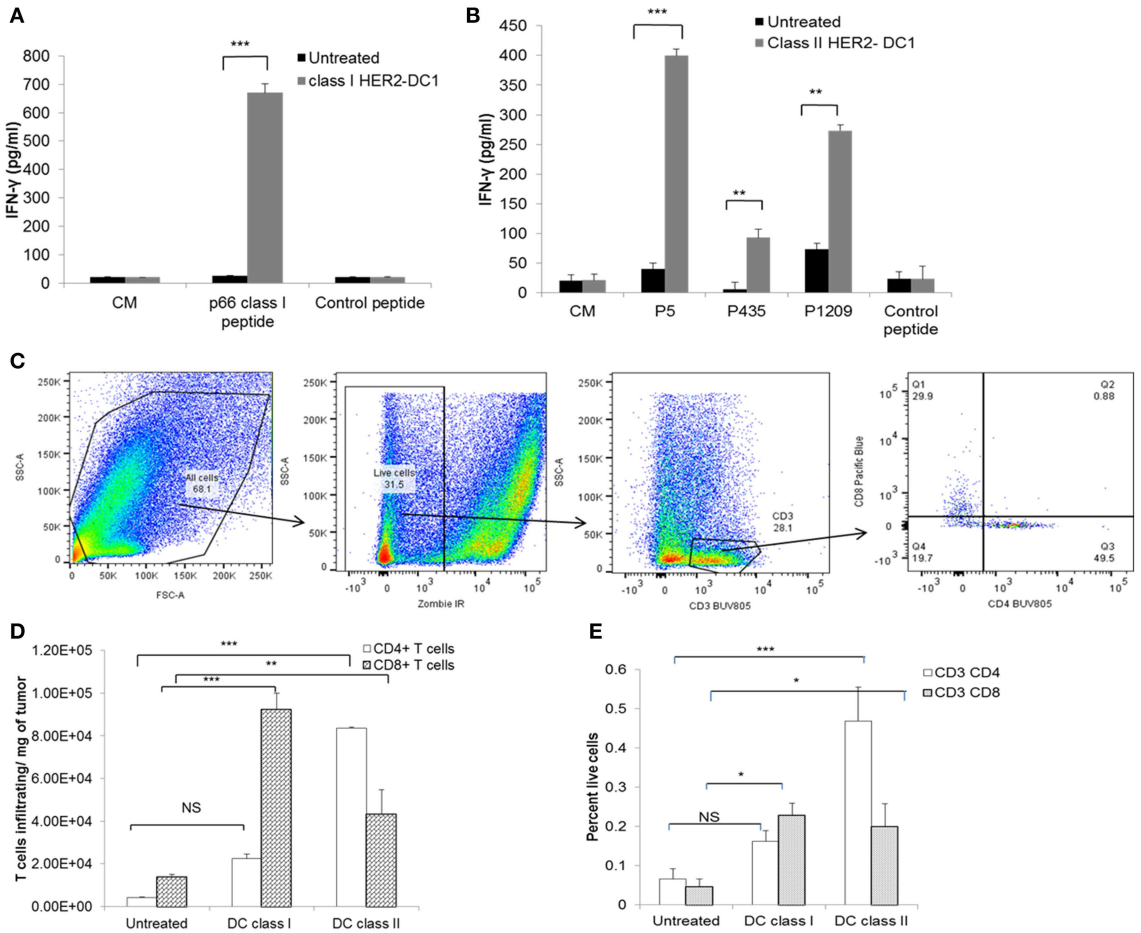

FIGURE 3 | Vaccination with HER2-DC1 enhances anti-HER2 Th1 immune response and increases T cell infiltration in TUBO bearing mice. **(A)** Splenocytes from class I HER2-DC1 vaccinated mice were re-stimulated with p66 rHER2 peptide. Culture supernatants were collected after 3 days and IFN-γ secretion was measured by standard IFN-γ ELISA. **(B)** Splenocytes from class II HER2-DC1 vaccinated mice were re-stimulated with (class II) rat HER2 peptides (rHER2); p5 (ELAAWCRWGFLLALLPPGIAG), p435 (IRGRILHDGAYSLTLQGLGIH), and p1209 (SPPHPSPAFSPAFDNLYYWDQ) individually and IFN-γ secretion was measured by standard IFN-γ ELISA. **(C)** Flow gating strategy and analysis of T cell infiltration in tumors by flow cytometry. Tumors were harvested on day 30 and processed as described in Materials and Methods section. Single cell suspension was stained for live/dead near IR, CD3, CD4, and CD8. Data were acquired on an LSRII flow cytometer and analyzed by FlowJo software. **(D)** Bar graphs represent T cell infiltration gated on live cells per mg of tumor and **(E)** percent T cells of all live cells within the tumor single cell suspension;. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ using Student *t*-test.

Re-stimulation of splenocytes from class I HER2-DC1 vaccinated mice with p66 (class I) peptide had significantly increased IFN-γ secretion compared to splenocytes from untreated mice (**Figure 3A**, $p < 0.001$). Similarly, re-stimulation of splenocytes from the class II HER2 peptide pulsed DC1 group had higher levels of IFN-γ production in response to HER2 peptides, p5, p1209, and p435 peptides (**Figure 3B**, $p < 0.001$). These findings suggest that class I and class II HER2-DC1 vaccine can generate anti-HER2 $CD8^+$ and $CD4^+$ Th1 specific immune responses and can delay tumor growth in $HER2^+$ TUBO bearing mice. We next examined whether vaccination with HER2-DC1 vaccine could improve T cell infiltration within the tumor. Tumors were excised from control and treatment groups and single cell suspensions were prepared and stained for cell surface markers (Live/Dead Zombie near IR, CD3, CD4, and CD8 gated on live cells) as described in Materials and Methods section. The flow gating strategy to identify $CD4^+$ and $CD8^+$ tumor infiltrating lymphocytes is shown in **Figure 3C**. Class I (p66) HER2-DC1 vaccine in TUBO bearing mice led to a significant increase in tumor infiltrating $CD8^+$ T cells but not $CD4^+$ T cells, while class II HER2-DC1 vaccine significantly increased both CD4 and $CD8^+$ T cell infiltration compared to untreated controls (**Figures 3D,E**, $p < 0.001$). **Figure 3D** represents the T cell infiltration per milligram of tumor and **Figure 3E** shows percent of T cells of all live cells from tumor digest suspension. This data suggests that HER2-DC1 vaccine enhances T cell infiltration within the tumor in TUBO bearing mice.

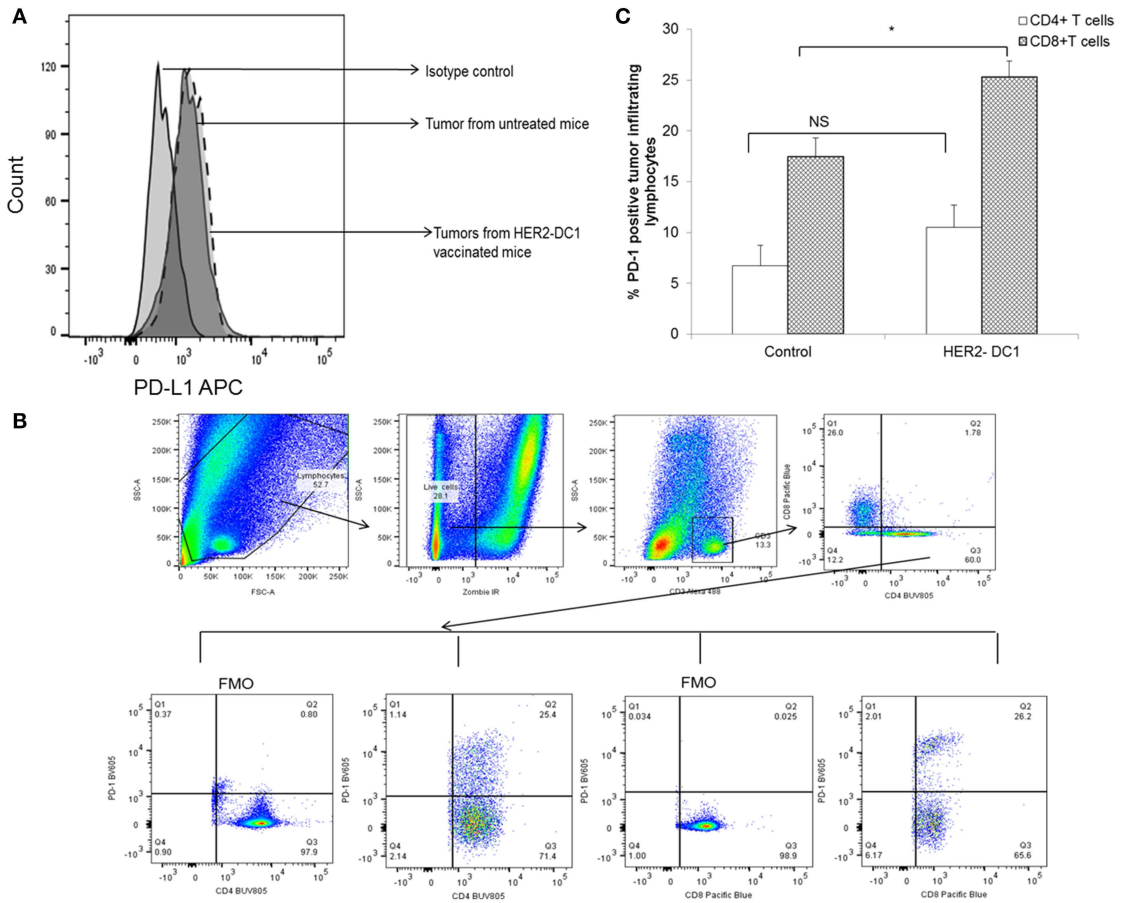

FIGURE 4 | PD-1 expression on TIL and PD-L1 expression on tumors of TUBO bearing mice. **(A)** Tumors were harvested 1 week after the last HER2-DC1 treatment and single suspensions were prepared as described in Materials and Methods section. Single cell tumor digest suspensions were stained for PD-L1 expression and data were acquired on a LSR-II and analyzed using Flowjo software. **(B)** Flow gating strategy of PD-1 expression on tumor infiltrating T cells. **(C)** Tumors were harvested as described in Materials and Methods section and flow staining was performed. Bar graphs show the percent PD-1 expression on CD4 and CD8+ TIL $^*p < 0.05$, NS, Not significant using Student *t*-test.

TIL From HER2-DC1 Vaccinated Mice Express Higher Levels of PD-1 Checkpoint Receptor

Inhibitory receptors such as PD-1 expressed on T cells and their ligands such as PD-L1 expressed on tumor cells have been shown to contribute to immune mediated suppression. We investigated whether HER2-DC1 has any effect in modulating the expression of PD-L1 on tumor cells and PD-1 receptor expression on TIL. Tumors were harvested from experimental mice, a single cell suspension was prepared and flow staining was performed as described in Materials and Methods section. As shown in **Figure 4A**, PD-L1 expression was observed in tumors from untreated mice and HER2-DC1 vaccinated tumor-bearing mice. We did not observe any difference in the expression levels of PD-L1 between tumors from control and HER2-DC1 vaccinated mice. We evaluated the expression of PD-1 on tumor infiltrating lymphocytes. Flow gating strategy is shown in **Figure 4B**. Cells were gated on live population followed by gating on CD3 positive cells. PD-1 expression on CD4 and CD8 cells was analyzed on samples by gating on Fluorescent minus one (FMO) controls. We observed increased PD-1 expression on $CD8^+$ T cells infiltrating within the tumor following HER2-DC1 vaccination compared to control mice (**Figure 4C**; $p < 0.05$). In contrast to $CD8^+$ T cells, there was only a modest but not statistically significant increase in PD-1 expression on $CD4^+$ T cells within the tumor following HER2-DC1 vaccination compared to untreated control (**Figure 4C**). This data suggests that blockade of immune checkpoints in combination with HER2-DC1 vaccine may improve the anti-tumor immune responses in the preclinical model of HER2 positive TUBO breast cancer.

Class I HER2-DC1 Vaccine in Combination PD-1/PD-L1 Blockade

The presence of TILs has been associated with a favorable prognosis in $HER2^+$ breast cancer and to potentially predict responders to immune checkpoint blockade (37, 38). Targeting

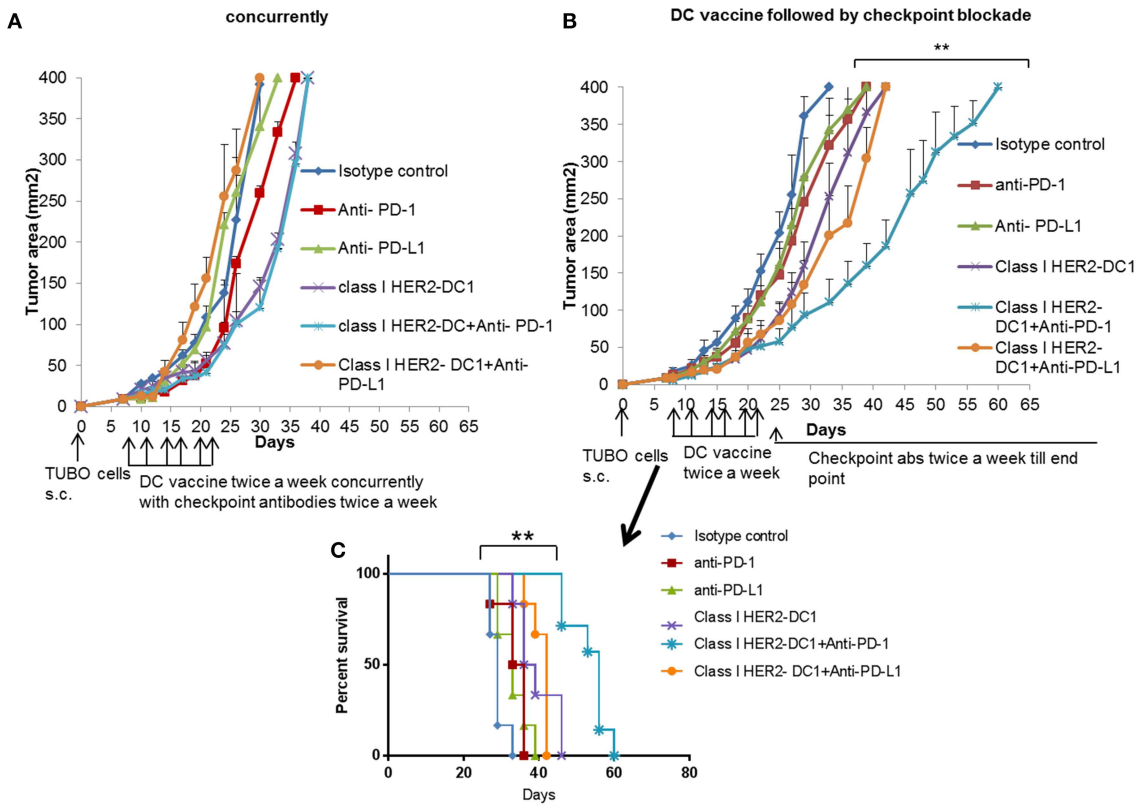

FIGURE 5 | Optimal scheduling of checkpoint antibodies is critical in improving the efficacy of class I HER2-DC1 vaccine in TUBO bearing mice. **(A)** Balb/C mice were injected with 2.5 × 10^5 TUBO cells subcutaneously on the right flank on day 0. When tumors were palpable on day 7, mice received class I HER2-DC1 vaccine subcutaneously on the left flank twice a week for a total of six doses concurrently with intraperitoneal injection of 150 μg/mouse/200 μl of anti-PD-1 or anti-PD-L1 antibody. **(B)** Balb/C mice were injected with 2.5 × 10^5 TUBO cells subcutaneously on the right flank on day 0. When tumors were palpable on day 7, mice received class I HER2-DC1 vaccine (1 × 10^6 DC1/mouse/100 μl) subcutaneously on the left flank twice a week for a total of six doses. Anti-PD-1 or anti-PD-L1 antibody therapy (150 mg/200 ml/mouse/intraperitoneally) twice a week was started after the last injection of HER2-DC1 and continued until the end point. $N = 8$ mice/group was used for these studies and the line graph shown is the representative of triplicate experiments. **(C)** Survival curve. Data shown are the representative from three independent experiments and are shown as the mean number ± SEM. *P*-values were determined by unpaired student *t*-test **(A,B)** or a log-rank test **(C)**. $^{**}p < 0.01$.

the PD-1 pathway with pembrolizumab in combination with trastuzumab has shown efficacy in HER2 positive trastuzumab resistant patients. The overall response was encouraging in PD-L1 positive cohort. However, there was no overall response in the PD-L1 negative cohort (18). Since we observed increased PD-1 expression on TILs and PD-L1 expression on tumors cells following HER2-DC1 vaccination in TUBO bearing mice, we investigated whether blockade of immune checkpoints, PD-1 or PD-L1 in combination with HER2-DC1 vaccine would enhance the anti-tumor immune response in TUBO bearing mice. Balb/C mice were injected with TUBO cells on day 0. On day 7, when tumors were palpable, two different treatment regimens were followed to examine the efficacy of combination therapy. One group of mice received checkpoint monoclonal antibodies (anti-PD-1 or anti-PD-L1) in combination with class I HER2-DC1 concurrently. Another group of mice received class I HER2-DC1 vaccine twice a week and at the completion of sixth HER2-DC1 vaccine, mice received anti-PD-1 or anti-PD-L1 antibody therapy twice a week until the end point. In addition other treatment groups received either HER2-DC1 alone, anti-PD-1, or anti-PD-L1 antibody as monotherapy. Control mice received isotype control antibody as described in Materials and Methods section. As shown in **Figure 5A**, TUBO- bearing mice that received Class I (p66) HER2 peptide pulsed DC1 concurrently with intraperitoneal injection of anti-PD-1 or anti-PD-L1 monoclonal antibodies had no significant delay in the tumor growth compared to mice treated with class I (p66) HER2-DC1 alone. However, TUBO bearing mice that received Class I (p66) HER2 peptide pulsed DC1 vaccine followed by treatment with anti-PD-1 monoclonal checkpoint antibodies had a significant delay in tumor growth compared to the mice that received DC1 or checkpoint antibodies alone (**Figure 5B**). Importantly, TUBO bearing mice that received Class I (p66) HER2 peptide pulsed DC1 in combination with anti-PD-1 antibody had significant delay in tumor growth and doubled the survival rate in TUBO bearing mice, compared to mice that received single treatment or no treatment (**Figures 5B,C**, $p < 0.01$). However, sequential combination of Class I (p66)

HER2-DC1 with anti-PD-L1 did not have an impact on delaying tumor growth or survival benefit compared to DC1 alone (**Figures 5B,C**).

Class II HER2 Peptides Pulsed DC1 in Combination With Anti-PD-1 Antibody Therapy

The role of $CD8^+$ T cells in improving immune checkpoint blockade has been shown previously (39, 40). However, to the best of our knowledge, the role of $CD4^+$ helper T cells in facilitating and mediating anti-tumor immune responses in combination with checkpoint blockade has not been studied. To address this, we evaluated therapeutic efficacy of class II HER2-DC1 vaccine in combination with anti-PD-1 antibody therapy. Balb/C mice were injected with TUBO cells on day 0. On day 7, when tumors were palpable, mice received class II HER2-DC1 twice a week for 3 weeks followed by anti-PD-1 antibody therapy twice a week until the end point. Addition of anti-PD1 antibody delayed tumor growth in class II HER2-DC1 vaccinated mice compared to HER2-DC1 alone with survival rate tripled (**Figures 6A,B**). We also evaluated anti-tumor efficacy of class II HER2-DC1 vaccine in combination with anti-PD-L1 antibodies. We did not observe any additional therapeutic benefit of class II HER2-DC1 vaccine when combined with anti-PD-L1 antibody (Data not shown). These results suggest that checkpoint inhibitors given concurrently with HER2- DC1 vaccine do not have any additive benefit, while administration of anti-PD-1 antibody following generation of anti-HER2 Th1 immune response has an impact on both tumor growth and survival. Overall, these findings suggest that optimal scheduling of immune checkpoints is critical in enhancing the efficacy of HER2-DC1 vaccine.

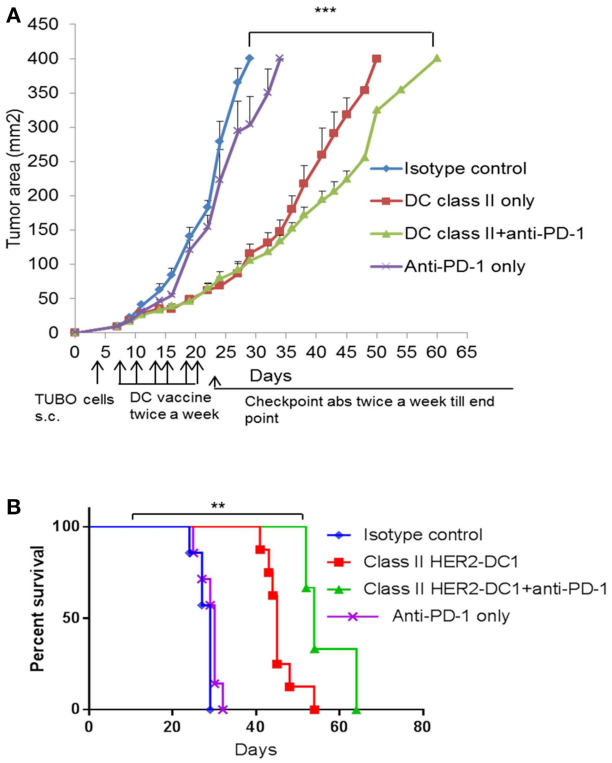

FIGURE 6 | Anti-tumor efficacy of class II HER2 peptides pulsed DC1 in combination with anti-PD-1 antibody therapy. **(A)** Balb/C mice were injected with 2.5×10^5 TUBO cells subcutaneously on the right flank on day 0. When tumors were palpable on day 7, mice received class II HER2 peptides pulsed DC1 vaccine (1×10^6 DC1/mouse/100 μl) subcutaneously on the left flank twice a week for a total of six doses followed by intraperitoneal injection of 150 μg/mouse/200 μl of anti-PD-1 antibody twice a week until the end point. **(B)** Survival curve. Data shown are representative from three independent experiments and are shown as the mean number ± SEM. *P*-values were determined by unpaired student *t*-test **(A)** or a log-rank test **(B)**. $^{***}p < 0.001$, $^{**}p < 0.01$.

Class I and Class II HER2-DC1 Vaccine in Combination With Anti-PD-1 Antibody Therapy Improves T Cell Infiltration, Function, and Specificity

The effect of PD-1 blockade in combination with HER2-DC1 on T cell infiltration, function and specificity was examined. Balb/C mice were injected with TUBO cells on day 0. On day 7, when tumors were palpable, mice received class I or class II HER2-DC1 twice a week for 3 weeks followed by anti-PD-1 antibody therapy. Spleens and tumors were collected from experimental mice on day 35 to examine the T cell infiltration, function and antigen specificity as described in Materials and Methods section and figure legends. Administration of anti-PD-1 antibody in combination with class I HER2-DC1 vaccination increased CD8+ T cell infiltration in tumors (per milligram of tumor) compared to tumor bearing mice that received class I HER2-DC1 only (**Figure 7A**, $p < 0.01$). We did not observe any changes in CD4+ T cell infiltration per milligram of tumor in mice that received class I HER2-DC1 alone or in combination with anti-PD-1 antibody therapy. Administration of class II HER2-DC1 in combination with anti-PD-1 antibody therapy significantly increased both CD4 and $CD8^+$ T cell infiltration per milligram of tumor ($p < 0.01$) compared to HER2-DC1 group alone and untreated controls as shown in **Figure 7B**. Data shown in **Figure 7C** represent the percent T cells of all live cells from tumor digest suspension.

Splenocytes from mice that received treatments were re-stimulated with p66 class I HER2 peptide. Irrelevant OVA peptides served as negative controls and did not show any non-specific reactivity. Higher levels of IFN-γ production were observed in class I HER2-DC1 in combination with anti-PD-1 therapy compared to class I HER2-DC1 alone group (**Figure 7D**, $p < 0.01$). Similar results were observed in mice that received class II HER2-DC1 vaccine when re-stimulated with relevant peptides. Cumulative response to p5, p1209, and p435 peptides is shown in **Figure 7E**. There was a slight trend toward increased IFN-γ levels between the class II HER2-DC1 alone group and the combination of class II HER2-DC1 and anti-PD-1 antibody treated group in response to re-stimulation with peptides. This data suggest that addition of anti-PD-1 antibody therapy with

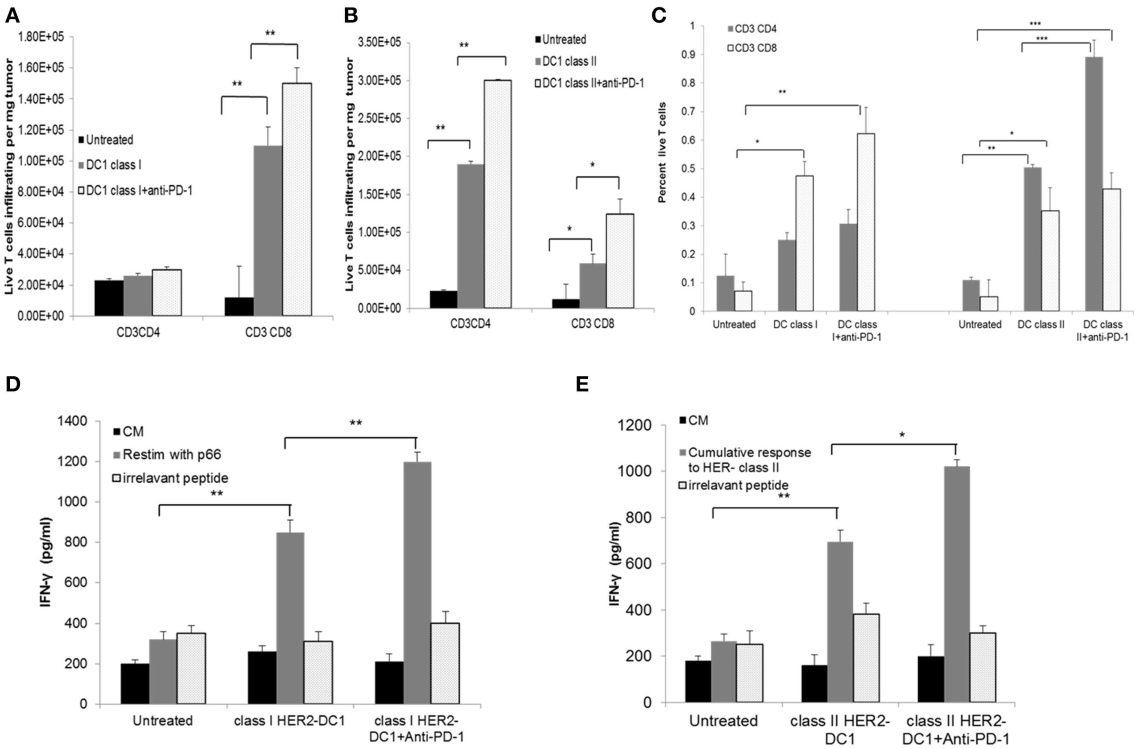

FIGURE 7 | HER2-DC1 vaccine in combination with anti-PD-1 antibody therapy improves T cell infiltration, function, and specificity. **(A,B)** Tumors were collected and single cell suspensions were prepared and stained for CD3, CD4, and CD8 as described in methods. Bar graph shows T cell infiltration per mg of tumor. **(C)** Percent T cells of all live cells within the tumor single cell suspension. **(D,E)** Splenocytes were re-stimulated with class I, class II HER2 peptides or irrelevant OT-I or OT-II peptides. Culture supernatants were collected after 72 h and IFN-γ was measured by standard ELISA. *P*-values were determined by unpaired student *t*-test ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

HER2-DC1 vaccine significantly increases T cell infiltration, enhances tumor specificity and function.

Effect of Combination Therapy With Class II HER2-DC1 and PD-1 Blockade Is Mediated by CD4$^+$ T Cells

Our findings suggest that TUBO bearing mice treated with class II HER2-DC1 vaccine can drive both CD4 and CD8$^+$ T cell infiltration. To determine the role of CD4$^+$ T cells in mediating tumor delay in TUBO tumors, mice were treated with CD4 depleting antibody on day −3 followed by injection of TUBO cells on day 0. When tumors were palpable, mice received either class II HER2-DC1 alone for 6 weeks or followed by anti-PD-1 antibody twice a week until the end point. Mice that received combination treatment with class II HER2-DC1 and anti-PD-1 antibodies in the absence of CD4 T cell depleting antibodies served as positive control. Control mice received isotype antibody control. As shown in **Figure 8A**, treatment with combination class II HER2-DC1 and anti-PD-1 antibody significantly delayed tumor growth compared to single treatments alone ($p < 0.03$) while combination therapy of class II HER2-DC1 with anti-PD-1 antibody further delayed tumor growth and survival (**Figures 8A,B**; $p < 0.002$). Depletion of CD4$^+$ T cells led not only to the loss of anti-tumor effects mediated by the combination therapy with class II HER2-DC1 and anti-PD-1 but also to more rapid tumor growth and diminished survival. Nevertheless, class II HER2-DC1 vaccine failed to delay tumor growth in the absence of CD4$^+$ T cells. Similar results were also observed when class II HER2-DC1 vaccine was combined with anti-PD1 antibody in the absence of CD4$^+$ T cells (**Figure 8A**). This data strongly support a critical role of class II HER2-DC1 vaccine in mediating CD4$^+$ Th1 immune responses.

Synergistic Effect of HER2 Targeted Therapy in Combination With Class II HER2-DC1 Vaccine and PD-1 Blockade Delays Tumor Growth and Enhance Survival Rate

In a PANACEA trial, Pembrolizumab plus trastuzumab showed a clinical benefit in patients with PD-L1-positive, trastuzumab-resistant, advanced, HER2$^+$ breast cancer, and in patients with increased TIL. However, the trial did not show any clinical benefit in the PD-L1 negative cohort (18). This trial suggests the importance of immune mechanism in trastuzumab resistance populations, PD-L1 expression on tumors, and TIL infiltration. Since we observed increased TIL infiltration following HER-2

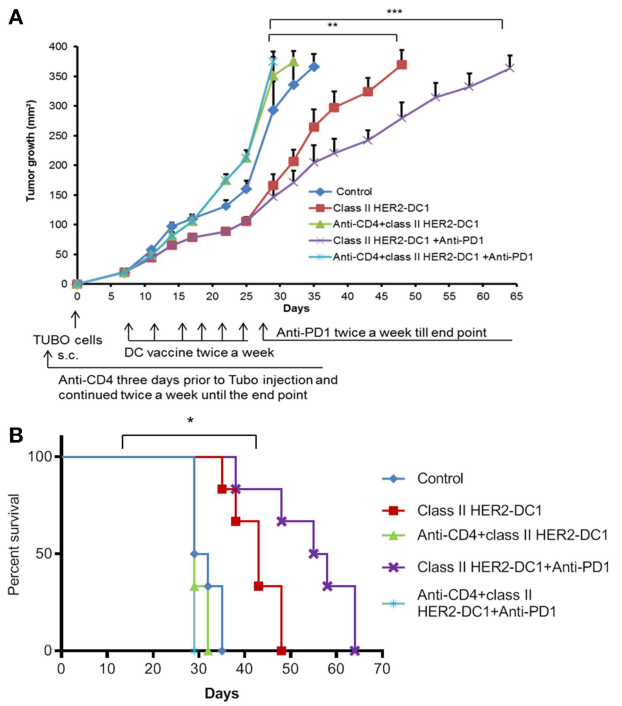

FIGURE 8 | Depletion of CD4+ T cells abrogates the anti-tumor efficacy of HER2-DC1 vaccine and anti-PD1 antibody therapy. **(A)** Balb/c mice were injected s.c. with TUBO cells and treated with class II HER2-DC1 vaccine (1×10^6 DC1/mouse/100 μl, twice a week for 3 weeks) with or without anti-PD1 antibody (150 mg/200 ml/mouse/intraperitoneally twice a week until the end point). CD4 depleting antibody (clone GK1.5) was administered 3 days prior to TUBO induction and continued twice a week until the end point. Tumor growth was monitored periodically. **(B)** Survival curve. Data shown are representative from three independent experiments and are shown as the mean number ± SEM. *P*-values were determined by unpaired student *t*-test **(A)** or a log-rank test **(B)**. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

DC1 vaccination and higher levels of PD-1 expression on TIL, we investigated whether combination with anti-HER2 antibodies, class II HER2-DC1 vaccine, and anti-PD-1 therapy have an impact on tumor burden in the TUBO breast cancer model. TUBO cells (30,000 cells) were injected in the mammary fat pad on day 0 and when tumors were palpable, mice received anti-HER2 antibody once a week for 3 weeks or in combination with class II HER2-DC1 given twice a week for 3 weeks. Upon completion of combination treatment, anti-PD-1 antibody was given twice a week until the end point as described in Materials and Methods section. The treatment schema is outlined in **Figure 9A**. As shown in **Figure 9B**, blockade of HER2 overexpression using anti-rat neu antibodies (clones 7.16.4 and 7.9.5) significantly reduced tumor burden when combined with class II HER2-DC1 and anti-PD-1 antibody (**Figure 9**; $p < 0.001$). The class II HER2-DC1 vaccine treatment alone significantly delayed tumor growth compared to TUBO bearing control mice and doubled the survival rate. We observed no significant effect in delaying tumor growth in TUBO bearing mice treated with anti-PD-1 antibody alone while anti-rat neu antibody had minimal effect in delaying tumor growth. The combination of class II HER2-DC1 alone with anti-rat neu antibodies 7.16.4 and 7.6.5 did not have any significant delay in tumor growth compared to class II HER2-DC1 alone. Interestingly, the addition of anti-PD-1 antibody with monoclonal anti-rat neu antibodies 7.16.4 and 7.6.5 and class II HER2-DC1 vaccine not only significantly delayed the tumor growth but also enhanced and quadrupled the survival rate from control mice (**Figures 9B,C**). This data suggest that addition of HER2 targeted therapy to the combination of HER2-DC1 vaccine and anti-PD-1 antibody reduces tumor burden and improves survival rate in an orthotopic HER2 positive breast cancer model.

DISCUSSION

HER2 overexpression/amplification accounts for about 25% of breast cancers and is associated with aggressive disease and poor clinical prognosis. As previously discussed, the humanized monoclonal antibodies (trastuzumab and pertuzumab) directed against HER2 in combination with chemotherapy have been demonstrated to be clinically effective for the treatment of patients with $HER2^+$ MBC (41). Other therapeutic options for these patients include combinations of Lapatinib (an oral tyrosine kinase inhibitor), T-DM1, and chemotherapies (42). It should be emphasized however that patients with $HER2^+$ MBC will eventually face disease progression while receiving treatment with currently approved HER2-targeted therapies. Development of new treatments is an obvious unmet clinical need not only for patients with *de novo* MBC but also for the subset of patients with $HER2^+$ resectable breast cancer who will have MBC recurrence despite multimodality treatment (43, 44). Therefore, effective therapy to overcome resistance and improve the clinical response in metastatic patients is warranted.

In this study, for the first time we describe the anti-tumor efficacy of MHC class I HER2 peptide p66 and class II HER2 peptides (p5, p435, and p1209) pulsed DC1 vaccine alone or in combination with immune checkpoint blockade and HER2-targeted therapy in a preclinical model of HER2 over expressing breast cancer. Vaccination with class I HER2-DC1 or class II HER2-DC1 generated anti-HER2 Th1 immune responses and delayed tumor growth. In contrast, there was no enhanced anti-tumor efficacy in mice vaccinated with both class I and class II HER2 peptide pulsed DC1 compared to mice vaccinated with class I or class II HER2-DC1 alone. While class I HER2-DC1 enhanced only $CD8^+$ T cell infiltration but not $CD4^+$ T cells, class II HER2-DC1 vaccination induced both CD4 and $CD8^+$ T cell infiltration, suggesting that vaccination with class II HER2-DC1 may be sufficient in generating anti-tumor immunity and HER2-specific immune responses. Re-stimulation of splenocytes from vaccinated mice with class II HER2 peptides led to increased IFN-γ production, suggesting the critical role of the anti-HER2 $CD4^+$ Th1 immune response in mediating reduction in tumor burden with enhanced survival benefit. IFN-γ is recognized as a key cytokine in mediating $CD4^+$ and $CD8^+$ Th1 immune responses (45, 46). We have previously shown that TLR-4 activated DC1 inhibit $CD4^+CD25^+FoxP3^+$ regulatory T cells and converts them in to Th1 like effector cells. These

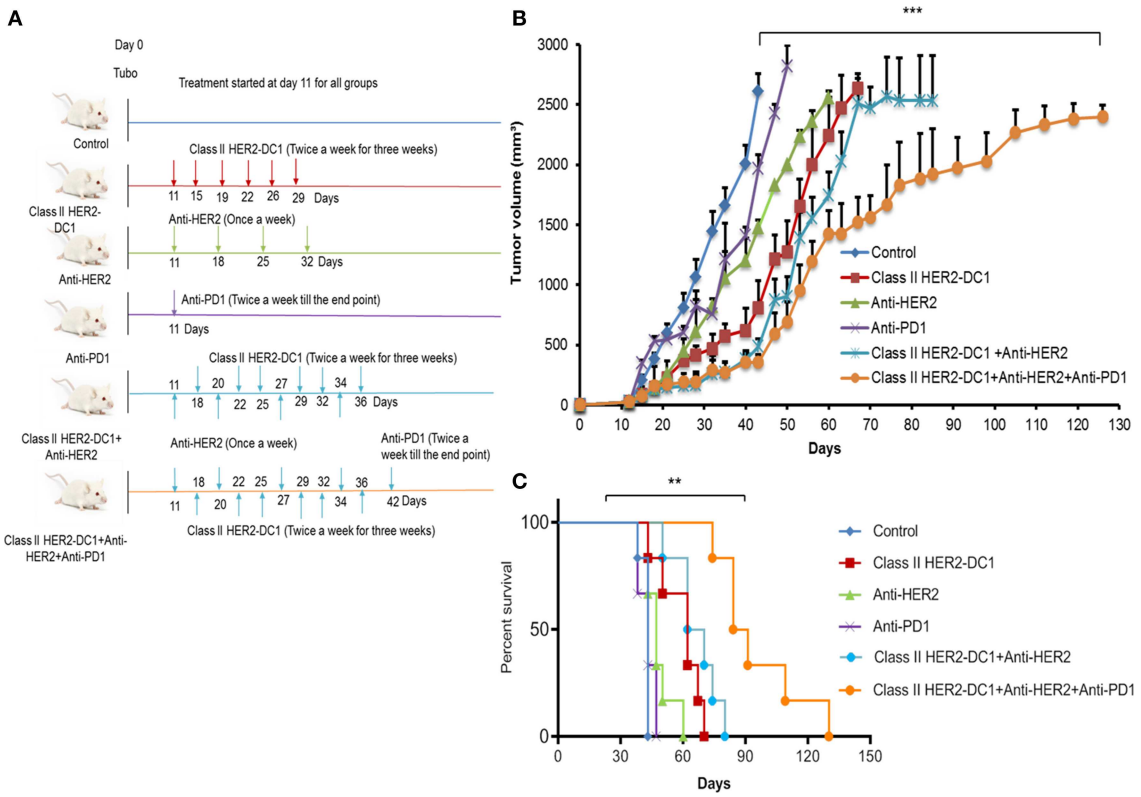

FIGURE 9 | HER2 targeted therapy in combination with HER2-DC1 vaccine and anti-PD-1 therapy delays tumor growth and improves survival. **(A)** The treatment schema. **(B)** Balb/C mice were injected with 3×10^4 TUBO cells into the mammary fat pad to create orthotopic primary tumors. TUBO bearing mice with orthotopic primary tumor were treated with class II HER2 peptide pulsed DC1 vaccine (1×10^6 DC1/mouse/100 μl, twice a week for 3 weeks), anti-PD-1 antibody (two times per week) or the combination of HER2 targeted monoclonal antibodies 7.16.4 and 7.6.5 (once in a week) and class II HER2 peptide pulsed DC1 vaccine followed up with anti-PD-1 antibody twice a week. **(C)** Survival curve. Data shown are representative from three independent experiments and are shown as the mean number ± SEM. *P*-values were determined by unpaired student *t*-test **(B)** or a log-rank test **(C)**. ***$p < 0.001$, **$p < 0.01$.

Th1 like effector cells co-expressed T-bet and increased IFN-γ production. This suggests the critical role of DC1 in mediating $CD4^+$ Th1 immune responses (47). A recent study from our lab reported that IFN-γ eliminates HER2 expressing breast cancer cells through JAK-STAT-1 dependent induction of senescence and apoptosis (48). Our preclinical findings corroborate the clinical trial data showing that treatment of HER2 positive invasive breast cancer patients with HER2 peptide-pulsed DC1 vaccine resulted in successful restoration of anti-HER2 Th1 immune response with improved pCR (28, 31, 49).

Various clinical findings have shown that HER2 expressing breast cancer cells in the tumor microenvironment utilize the PD-1/PD-L1 dominant immune checkpoint pathway to down regulate anti-tumor immune cells function and evade immune cells mediated tumor eradication (50, 51). Importantly, PD-L1, a ligand for PD-1 is constitutively expressed on HER2 overexpressing breast cancer (52). Despite the reports that monoclonal antibodies that directly inhibit PD-1 and PD-L1 have been a successful treatment options for advanced melanoma, non-small cell lung carcinoma (NSCLC), and some patients with high mutational burden like HNPCC colorectal cancer, limited success rate was noted in breast cancer clinical trials (53). The combination of trastuzumab and pembrolizumab in HER2 resistant advanced breast cancer patients had modest clinical benefit only in the PD-L1 positive cohort (18) or in those with at least some TIL infiltration and no benefit was seen in the PD-L1 negative cohort.

Our preclinical findings suggest that both class I and class II HER2-DC1 increased infiltration of TIL which express high levels of PD-1 receptor. Class I HER2-DC1 vaccine given sequentially in combination with anti-PD-1 antibody therapy had an impact in reducing tumor burden with improved survival benefit. In contrast, HER2-DC1 vaccine and anti-PD-1 antibody given concurrently did not generate any synergistic effect. These results corroborate with a recent study indicating that in MMTV-PyMT mammary cancer model, concurrent treatment with anti-PD1 antibody and anti-OX-40 diminished the therapeutic efficacy. However, sequential treatment with anti-PD-1 and anti-OX-40 resulted in improved therapeutic efficacy and was associated with worse outcomes and increased T cell apoptosis (54). This supports our data suggesting that sequence and timing of checkpoint blockade is critical for combinatorial

strategies. The optimal impact of checkpoint antibody treatment on tumor growth and resistance also likely depends in part on the tumor burden. A recent study demonstrated the anti-tumor efficacy of anti-CTLA-4 and anti-PD-1 in the low tumor burden state in pre-clinical melanoma model as well as in melanoma patients. Their data suggest that in the low tumor burden setting, combination therapy induced higher levels of IFN-γ receptor on activated tumor specific T cells which were more susceptible to apoptosis than naïve T cells. In this setting, combination therapy induced deletion of tumor-specific T cells and altered the T cell repertoire compared to the high tumor burden setting. The authors suggest that there is a less exhausted immune status in the low tumor burden state. In addition, they suggest that in the setting of high tumor burden, duration of antigen exposure and antigen loads could alter or reprogram the exhaustion status of T cell profile (55). We believe that in our model, one possible reason for the difference in response to checkpoint therapy could be that mice receiving concurrent HER2-DC1 and anti-PD-1 antibody treatment in the low tumor burden setting made anti-PD-1 therapy less effective. While in the mice receiving sequential treatment, where anti-PD-1 therapy was given in a setting of higher tumor burden, treatment was more effective. Altogether our results indicate that the effect of checkpoint blockade largely depends on optimal scheduling to be successful with other immunotherapeutic strategies.

Class II HER2-DC1, when combined with anti-PD-1 antibody, quadrupled the survival rate with increased anti-HER2 $CD4^+$ Th1 immune response and increased $CD4^+$ and $CD8^+$ T cells infiltration within the tumor. Interestingly, depletion of $CD4^+$ T cells completely abrogated the anti-tumor efficacy of the class II HER2-DC1 alone or in combination with anti-PD-1 therapy suggesting the crucial role of $CD4^+$ T cells. A recent study indicates that NSCLC patients with highly dysfunctional CD4 immunity had no objective response to PD-1/PD-L1 blockade therapy. However, in patients with non-dysfunctional CD4 responses there was a response rate of about 50% to PD-1/PD-L1 blockade therapy. More importantly, CD8 immunity was recovered only in patients with functional CD4 immunity (56). These data support our findings that boosting the anti-HER2 $CD4^+$ Th1 immune responses prior to immune checkpoint blockade will be beneficial in breast cancer patients.

In contrast, no effect on delaying tumor growth was observed when class I or class II HER2-DC1 vaccine was combined with anti-PD-L1 antibody. In the clinical setting, Avelumab, a PD-L1- antibody, has been shown to have only modest responses in breast cancer subtypes in the JAVELIN study (57). It is well-known that IFN-γ upregulates PD-L1 expression (45). Despite the fact that $HER2^+$ TUBO cells expressed higher levels of PD-L1, we were unable to observe synergy with anti-PD-L1 antibody and HER2-DC1 vaccine. Further studies are warranted on the optimal dosing and scheduling of anti-PD-L1 antibody and to validate its efficacy in combination with HER2-DC1 vaccine.

Our data suggests that addition of anti-HER2 antibodies further enhanced the efficacy of class II HER2-DC1 vaccine in combination with anti-PD-1 antibody, with a prolonged survival advantage. In a HER2 overexpressing and trastuzumab resistant preclinical breast cancer model, targeted treatment with ado-trastuzumab emtansine (T-DM1) in combination with anti-PD1 antibody or anti-CTLA-4 antibody showed enhanced anti-tumor efficacy, T cells trafficking in to the tumor and Th1 cell polarization (58). Preclinical studies combining IFN-γ and anti-HER2 antibody have been shown to induce a synergistic effect in reducing HER2 expressing orthotopic mammary tumor growth *in vivo* (14). Taken together, this study highlights the critical role of $CD4^+$ T cells and the use of class II HER2-DC1 vaccine in combination with immune checkpoint blockade and HER2 targeted therapy in facilitating and mediating anti-HER2 Th1 immune responses. This combinatorial approach could be directly translated to clinical settings.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

KK and BC conceived and designed the experiments and supervised the work. KK, CS, GR, AB, YJ, HZ, and LL performed the experiments. KK, CS, GR, and BC analyzed data and contributed to data analysis. KK, BC, and RC wrote the manuscript. GR, CS, DW, APB, SA, AB, LL, HZ, MG, RC, and YJ edited the manuscript.

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In situ Vaccination by Direct Dendritic Cell Inoculation: The Coming of Age of an Old Idea?

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For more than 25 years, dendritic cell (DC) based vaccination has flashily held promises to represent a therapeutic approach for cancer treatment. While the vast majority of studies has focused on the use of antigen loaded DC, the intratumoral delivery of unloaded DC aiming at *in situ* vaccination has gained much less attention. Such approach grounds on the ability of inoculated DC to internalize and process antigens directly released by tumor (usually in combination with cell-death-inducing agents) to activate broad patient-specific antitumor T cell response. In this review, we highlight the recent studies in both solid and hematological tumors showing promising clinical results and discuss the main pitfalls and advantages of this approach for endogenous cancer vaccination. Lastly, we discuss how *in situ* vaccination by DC inoculation may fit with current immunotherapy approaches to expand and prolong patient response.

Keywords: dendritic cell (DC), *in situ* vaccination, cancer immunotherapy, checkpoint inhibitor combination therapy, intratumor administration, monocyte derived dendritic cells (MoDC)

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INTRODUCTION

Since the discovery that monocytes, cultured with GM-CSF and IL-4, differentiate into dendritic cells (DC) (1), the idea to use *ex vivo* generated DC to vaccinate cancer patients against tumor antigens has been largely explored (2, 3). Many different protocols have been developed for DC differentiation and/or maturation (4), but there is still a strong need to characterize the relationship between *ex vivo* derived DC and the several *in vivo* circulating DC subsets for which many information are now available in terms of phenotype and functionality (5). Over the last 25 years, hundreds of clinical trials have been performed mostly without showing consistent clinical responses, despite some encouraging results, especially in recent years (6–8). The vast majority of these studies have used mature IL-4-conditioned-DC loaded *ex vivo* with tumor antigens. However, antigen selection has represented one of the major limitations of DC vaccines and it is now widely accepted that broad patient-specific antigen repertoire, using patient tumor lysate or mutanome-derived peptides, represents the most promising DC antigen source (7–10).

An alternative to *ex vivo* antigen loading is represented by the so-called *in situ* vaccination. *In situ* vaccination aims at stimulating DC in the tumor to capture and process antigens released by the tumor and present them to immune cells upon migration to draining lymph node. This approach is receiving renewed interest because of the necessity to expand the antigenic repertoire of T cell responses in the checkpoint blockade therapy era (11–16). Several approaches are being evaluated in early trials, mostly using DC activators directly inoculated within the tumor (13, 16). However, given the low number of pre-existing DC at tumor site, combination therapy with

stimulator of hematopoietic differentiation of DC, such as Flt3L, seems to be required for efficient DC activation (17–19).

One way to overcome low intratumoral DC number and ensure a better control of DC phenotype is represented by intratumoral inoculation of *ex vivo* generated DC (*it*DC) aimed at an *in situ* vaccination. First attempts of *it*DC date 20 years back (20–22). Since the initial studies, many promising observations were collected on feasibility and efficacy of *it*DC (23–28), even though they did not get under the spot into the mainstream DC vaccine field. However, recent clinical results (29–31), together with increased interest in *in situ* vaccination to enforce current immunotherapies, highlight *it*DC as a powerful approach that can be rapidly implemented in current checkpoint blockade therapies. In this review, we will present the main results collected in pre-clinical and clinical use of intratumoral delivery of DC and discuss their potential use in combination with current immunotherapy.

INTRATUMOR INJECTION OF DC: A PLATFORM FOR ENDOGENOUS VACCINATION

As professional antigen processing cells, DCs are characterized by the ability to internalize, process and present antigens and potently interact with T cells, thus inducing their activation (32). However, tumors develop several "escape mechanisms" to exclude or reduce immune recognition of tumor-associated antigens, including DC exclusion from tumor microenvironment (33) and inhibition of DC activity (34). Within such an immunosuppressive environment, the injection of *ex vivo* cultured DC represents a valuable approach to overcome some tumor escape mechanisms, process antigens released in necrotic or apoptotic tumor mileu and activate immune response against tumor-associated antigens (35). *it*DC can be potentially applied to almost any tumor type: the only pre-requisite is the possibility

TABLE 1 | Major clinical trials testing *it*DC.

DC type	Maturation status	Clinical setting	Tumor pre-conditioning	Major findings	References
IL4-DC	Immature	Metastatic melanoma and breast carcinoma	–	Regressing lesions showed lymphocytes infiltration and reactivity against heat shock proteins	(22)
IL-12 transduced IL4-DC	Immature and mature	Advanced metastatic digestive carcinomas	–	IL8 retains DC at tumor site	(27, 36)
IL4-DC	Immature	Refractory hepatoma	Radiotherapy	Systemic antitumor immune response, NK cytotoxicity	(37)
IL4-DC	Immature	Glioma	–	Increased overall survival in patients receiving *it*DC vs. intra dermal DC	(28)
IL4-DC	Immature	Melanoma	±Hyperthermia	Systemic antitumor immune response, enhanced by local hyperthermia	(26)
IL4-DC	Mature	Inoperable pancreatic cancer	Gemcitabine same day	Systemic antitumor immune response and clinical response in combination with lymphokine activated killer cells stimulated with anti-CD3	(38)
IL4-DC	Immature	Prostate	Radiotherapy, hormone therapy	Treatment feasibility, T cells infiltration at tumor site (limited), systemic antitumor immune response (limited)	(39)
IL4-DC	Mature	Esophageal cancer	Chemotherapy	DC are retained at tumor site	(40)
IL4-DC	Immature	Soft tissue sarcoma	Radiotherapy	T cells infiltration at tumor site correlated with antitumor immune response,	(41)
IL4-DC	Immature	Follicular lymphoma	Rituximab and radiotherapy. (GM-CSF given same day)	Systemic antitumor immune response correlated with clinical response	(29)
IFN-DC	Partially mature	Melanoma	Chemotherapy	Systemic antitumor immune response	(42)
Allogeneic IL4-DC	Mature	Metastatic renal cell carcinoma	–	Inflammation at tumor site	(43)
CCL21-transduced IL4-DC	Immature	Non-small cells lung cancer	–	Systemic antitumor immune response, T cells infiltration and increased PD1 expression at tumor site	(31)
GM-CSF DC	Partially mature	Unrespectable, locally advanced, or metastatic solid tumors	–	Increased production of specific cytokines by DC correlated with clinical efficacy	(35)
IFN-DC	Partially mature	Follicular lymphoma	Rituximab	Systemic antitumor immune response; abscopal effect	(30)

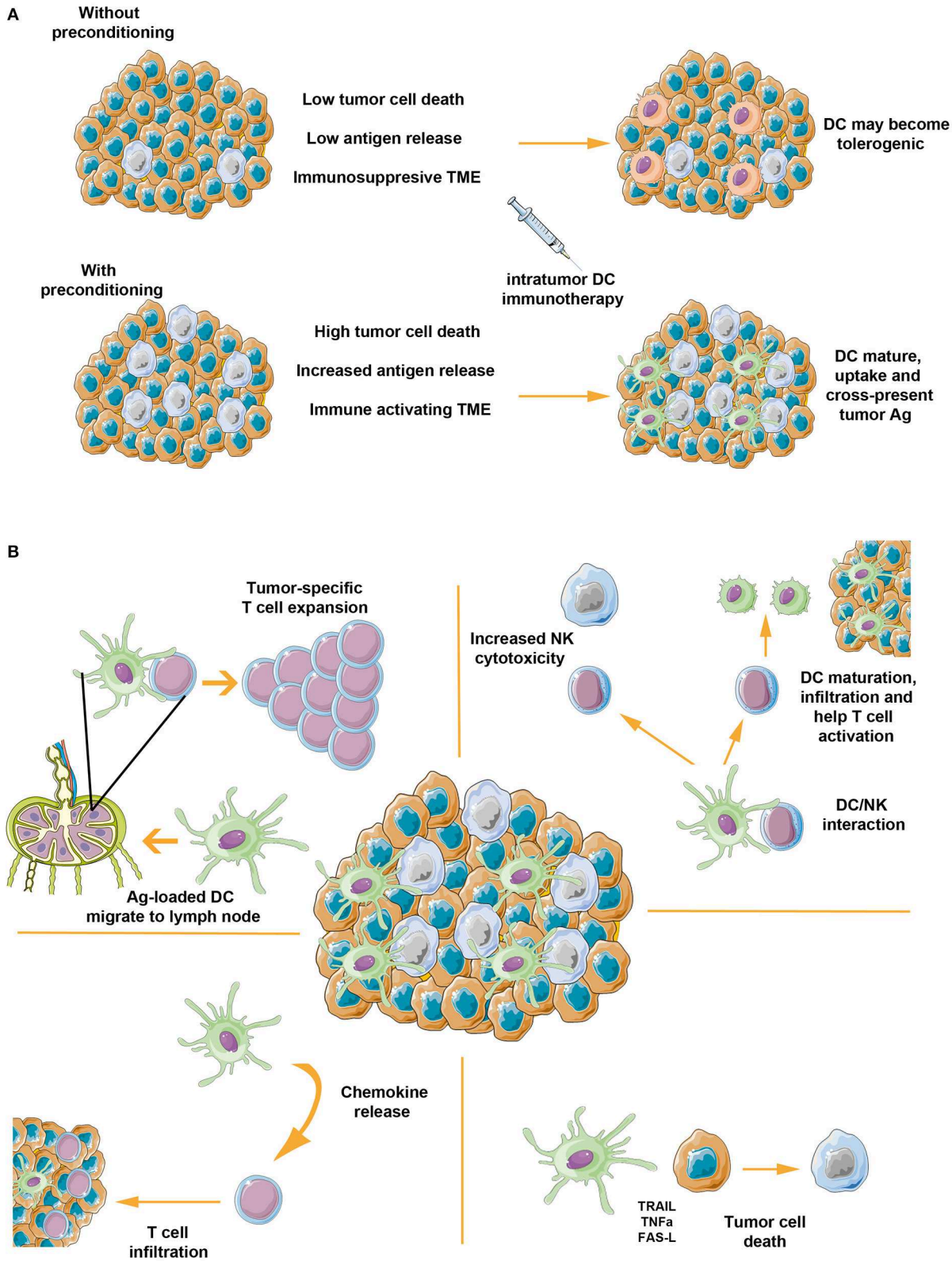

FIGURE 1 | Intratumor inoculation of DC: the importance of preparing tumor microenvironment and its multiple ways of action. **(A)** In the absence of any treatment, tumors are characterized by low levels of basal apoptotic/necrotic cells and an immunosuppressive microenvironment. Within this setting, *it*DC might become

(Continued)

FIGURE 1 | tolerogenic, thus increasing tumor immunosuppressive features and eventually causing a detrimental effect. Tumor preconditioning with immunogenic cell death agents, instead, can enhance tumor cells apoptosis, resulting in increased release of tumor-associated antigens and immune activating signals. In this scenario, intratumor inoculated DC sense proinflammatory and immune activating signals, process tumor antigens, and activate antitumor response. **(B)** *it*DC can activate immune response by acting on several mechanisms. After loading tumor released antigens, mature DC migrate to draining lymph node where they interact with T cells and lead to increased clonality and richness of antitumor T cell responses. The DC interaction with intratumor NK cells can activate their cytotoxic activity, which in turn can activate a positive feedback on DC themselves by boosting their maturation, their infiltration and favoring DC/$CD4^+$ T cell interactions. *it*DC can also increase infiltration of T cells by secreting chemokines and exert direct cytotoxic effect, resulting in increased tumor cell death and, more importantly, increased release of tumor antigens. The figure was made using the Servier Medical ART set by Servier.

to directly inoculate DC in the tumor. In fact, as summarized in **Table 1**, *it*DC trials have been performed against pancreas (27, 38, 44), liver (27, 37), colorectal (27), prostate (39), esophagus (40), brain (28), skin (26, 42), lung (31, 35), bile duct (27, 35), breast, ovarian, bladder, neuroendocrine (35), renal (43), and hematological tumors (29, 30), and soft tissue sarcoma (41). With the exception of melanoma (which is clearly accessible), the inoculation of DC was guided by ultrasound, computed tomography scan, or endoscopic ultrasound. Only in the setting of a brain tumor was an intraventricular catheter used (28).

Even though basal tumor apoptosis/necrosis can be exploited (27, 35, 44), *it*DC vaccination strongly benefits from tumor pre-treatment with death-inducing agents, because of the increased release of tumor antigens (**Figure 1A**) (24, 26). Among the pre-conditioning regimens used, the ones causing immunogenic cell death are clearly preferred, because they couple the release of tumor antigens with DC activating signals (45–47). However, as shown by Teitz-Tennenbaum, radiotherapy (RT), inducing calreticulin exposure and other activating signals (48, 49), stimulates DC processing ability, homing to lymph node, and their ability to stimulate T cells even when RT was not inducing tumor cell death (50). This point indicates that *it*DC can strongly benefit, not only from tumor pre-conditioning with immunogenic cell death treatments, but also with regimens that simply increase immunogenicity of tumors, thus enlarging the range of possible agents that can be used.

Among the several pre-conditioning regimens, RT represents the broadest applicable one considering also the ease of adding *it*DC into already well-established RT regimens (24, 37, 41). However, clinical trials have also been performed using local hyperthermia (26), systemic chemotherapy (40), and tumor-targeting monoclonal antibodies (29, 30) (**Table 1**). While an ideal pre-condition approach should be tailored to tumor type, the use of tumor-targeting monoclonal antibodies raises some fascinating advantages and synergies. First, considering that DC are endowed with antibody-dependent cell-mediated cytotoxicity (51), the two treatments could directly synergize. In fact, we have shown that direct cytotoxic activity of DC against the lymphoma cell line Karpas-422 was increased after rituximab pre-treatment (30). Second, it has been shown that, for a successful monoclonal therapy, an NK-DC crosstalk needs to be mounted, where NK cell activation leads to increased cross-presentation and maturation of DC, thus resulting in antitumor T cell activation (52, 53). Therefore, *it*DC might boost such crosstalk, leading to increased NK cell activity and stronger adaptive antitumor immune responses. Third, combining monoclonal antibody with *it*DC can potentially lead to *in situ* vaccination targeted against clinically relevant, rare cells within the tumor, such as cancer stem cells. In fact, even though monoclonal antibodies recognizing cancer stem cells have not yet shown promising results (54), the possibility to directly target CSC with monoclonal antibodies, in combination with *it*DC to activate T cell immunity against CSC, may hold great promises and deserves future testing.

*it*DC, a 360-Degree Immunotherapy

Even though *it*DC based therapy is principally aimed at direct *in situ* vaccination, several complementary immunotherapy effects can also result (**Figure 1B**). As clearly shown by pre-clinical studies on *it*DC, NK cells can be directly targeted and activated by *it*DC (55, 56). In fact, depletion of NK cells led to impaired efficacy of *it*DC. This is not surprising in light of the tight crosstalk existing between DC and NK cells (57, 58). On one side, DC can potently activate NK cell cytotoxicity against tumor cells through secreted cytokines and cell-to-cell contact (59, 60). This, in turn, stimulates NK cells to secrete CCL5, XCL1, and Flt3L in the tumor (61, 62), thus promoting natural DC infiltration and additional cross-priming of tumor-associated antigen (63). On the other side, NK cells can strongly enhance DC maturation and IL-12 production, stimulate CD4 T cell response and, through IFN-γ, help DC-driven Th1 polarization (64, 65). In line with this crosstalk, it has been observed that high levels of NK cells after DC vaccination correlated with clinical response in acute myeloid leukemia (66) and advanced hepatoma patients (37).

Another complementary effect of *it*DC that should be taken into account is the ability of DC (especially upon maturation) to secrete several chemokines that can favor the infiltration of T cells and endogenous DC in the tumor microenvironment. This possibility has been recently tested in renal cell carcinoma by injecting allogeneic DC, therefore, excluding any direct vaccination effect but rather potentiating inflammatory-related signals due to cell allogeneity (43). Notably, the authors observed a high level of T cell infiltration and induction of tumor specific T cell responses in three out of 11 evaluable patients. Even more interestingly, despite clinical responses not being registered, an unexpected response consisting in high infiltration of T cells was observed in patients subsequently treated with tyrosine kinase inhibitors, thus suggesting a synergistic effect of the allo-*it*DC with tyrosine kinase inhibitors, possibly mediated by their effect against Treg and MDSC (43, 67). This approach has been additionally tested in advanced hepatocellular carcinoma patients where induction of tumor-specific immune activation in a substantial number of patients was observed (68). Alternative to the use of allogeneic cells, another approach to boost *it*DC ability to inflame the tumor and/or stimulate immune cells has been

tested by genetically modifying DC for constitutive expression of activating factors. DC transduced for the expression of IL-7, IL-12, IL-15, IFN-α, and CCL21 have all been tested in pre-clinical models of *it*DC showing encouraging results (20, 21, 25, 55, 69, 70), even though clinical experience with IL-12 transduced DC showed limited success (27, 36).

Lastly, despite being usually neglected, DC are also characterized by direct tumoricidal activity, which, in the context of *it*DC, might result in additional tumor cell death and more importantly in increased release of tumor antigens and damage associated molecular patterns, thus potentiating immune reactivation. In fact, *ex vivo* generated DC, circulating conventional DC and plasmacytoid DC exert direct cytotoxicity against tumor cells (51). This ability has been demonstrated against a large variety of cancer cell lines and can be mediated by both cell-to-cell signals and secreted factors. While TRAIL is the major signal by which DC exert their tumoricidal activity (71), TNFα, FAS-L, caspase-8, IFN-γ, and Granzyme B can also play a role (51).

The Ideal DC Phenotype for *it*DC Immunotherapy: Lessons Learned

Conversely to the classical antigen-loaded DC vaccination approach, for which many different protocols for DC differentiation and maturation have been developed and compared (4, 72, 73), minimal discussion has been raised regarding the phenotype of DC to be used for intratumoral inoculation. Initial studies focused on the use of immature DC to take advantage of enhanced phagocytic and antigen processing ability of these cells over the mature counterparts (21, 25, 74). However, in absence of strong DC activating stimuli (i.e., when tumor pre-conditioning is not performed or not inducing strong immunogenic cell death), immature DC can have a detrimental effect exerting more immunosuppressive rather than immunostimulatory activity (75–78). Therefore, a semi-mature phenotype may be preferable to couple phagocytic activity with the predefined immunostimulatory mature phenotype (35). However, additional immunotherapy effects of *it*DC (see above) should also be taken into account.

While other protocols to generate semi-mature DC have been developed (79, 80), we opted for DC differentiated in the presence of IFN-α instead of IL-4 (30, 42). These cells (named as IFN-DC) have been discovered by our group almost 20 years ago and are characterized by a partially mature phenotype and are endowed with a high migratory behavior and immunostimulatory ability (81–83). They have been shown to be more efficient than conventional IL-4-DC in internalizing tumor antigens and in the cross-priming of $CD8^+$ T cells, thus promoting anti-tumor immune responses (84, 85). Moreover, it has been shown that IFN-DC can promote efficient NK cell activation, increase expression of cytotoxicity receptors, and stimulate extensive IFN-γ production by NK cells (86). Interestingly, in two different clinical trials, we observed induction of long-term T cell immune response against tumor associated antigens upon *it*DC immunotherapy using IFN-DC (30, 42).

The Coming of Age of *it*DC: Clinical and Immunological Responses in Recent Trials

While initial attempts of *it*DC showed limited success, recent trials have convincingly shown not only safety and feasibility of *it*DC immunotherapy, but also clear-cut clinical and immunological responses in a high percentage of patients (**Table 1**). In two studies in follicular lymphoma patients, *it*DC in combination with low-dose rituximab alone (30) or in combination with low-dose rituximab, plus local radiotherapy, and GM-CSF (29), showed induction of 50 and 36%, respectively, of objective clinical responses in treated and untreated lesions. Notably, in both studies', induction of both CD8 and CD4 antitumor specific responses were collected and the magnitude of immune activation appeared to correlate with clinical response. Despite several differences between the two trials (type of DC used, pre-conditioning regimen, treatment schedule), these two studies clearly indicate that follicular lymphoma is particularly suited for *it*DC and that this immunotherapy approach is worth being tested in phase II-III clinical trials.

In another interesting clinical study, Lee et al. used CCL21 transduced DC in NSCLC (31). Despite minimal clinical effects being recorded, induction of T cell responses against tumor associated antigens were observed in 6/16 patients and, in four patients, induction of humoral response was reported. However, more noteworthy is the observation that, with only two DC inoculations, an increase in CD8 T cell infiltration was observed in 56% of patients and that this was correlated with increased expression of checkpoint inhibitors (31). Similar results were collected by another study using activated DC in several tumor types, showing that increased PD-L1 expression in the majority of patients usually paired by T cell infiltration (35). Thus, altogether both studies suggest that *it*DC itself increased PD-L1 expression as a result of antigen recognition and CD8 T cell infiltration at the tumor site, clearly pointing to synergies that can result by combining *it*DC and checkpoint blockade.

*it*DC for Checkpoint Blockade Immunotherapy: Arming T Cells While Preparing the Battlefield

Checkpoint blockade is revolutionizing cancer therapy with impressive long-term responses in a large variety of tumors. However, the majority of patients still do not benefit from this therapy because of either primary or secondary resistance (87). Several factors have been identified playing a role behind response to checkpoint blockade: tumor mutation burden (88), PDL1 expression (89, 90), T cell inflamed microenvironment (91), T cell repertoire richness and clonality (92), HLA-I diversity (93), intestinal microbiota (94, 95), and specific mutations have all been identified as potential markers with prognostic or predictive value in checkpoint blockade therapy (87). Additionally, cross-priming and CXCL9/10 secretion mediated

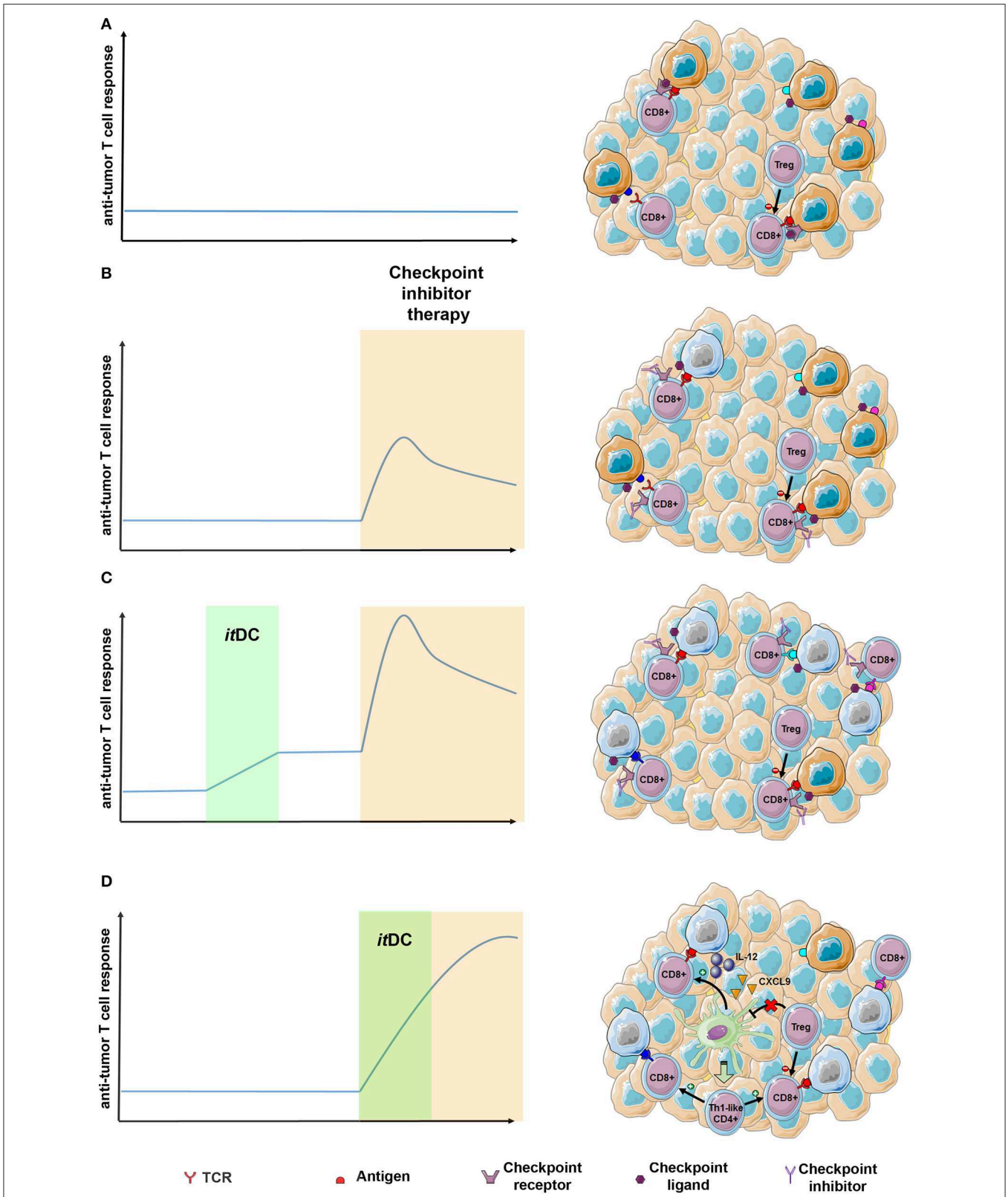

FIGURE 2 | Expected advantages of integrating the "*it*DC" approach in the context of the checkpoint blockade therapy. **(A)** In poorly immunogenic tumors, T cell response is usually low due to multiple checkpoint inhibitors expressed within the tumor microenvironment and immunosuppressive cells. **(B)** Checkpoint blockade

(Continued)

FIGURE 2 | therapy may result in increased antitumor T cell response of pre-existing antitumor clones. However, reduced richness and clonality, together with the presence of Treg cell limits checkpoint blockade efficacy, thus resulting in short-lived responses in the majority of patients. **(C)** *it*DC immunotherapy administered prior to checkpoint blockade therapy may lead to increased T cell clonality and richness. In this setting, subsequent checkpoint inhibitor administration is expected to lead to broader antitumor T cell activation. **(D)** *it*DC immunotherapy taking place during checkpoint blockade therapy may boost intratumor T cell activation by secreting IL-12, CXCL9. Additionally, it could overcome inhibitory signals from Treg cells, thus unleashing activation and infiltration of Th1-like $CD4^+$ T cells that can further potentiate antitumor T cell response (97–99). The figure was made using the Servier Medical ART set by Servier.

by intratumoral $CD103^+$ BATF3-dependent dendritic cells has also been correlated with response to checkpoint blockade (17, 96). Notably, *it*DC studies have already been shown to lead to increased tumor PDL1 expression and increased T cell responses in several tumor types (31, 35). In our recent study, combining NGS technology with *in silico* prediction, we analyzed T cell responses against patient specific mutations in follicular lymphoma patients before and after *it*DC and observed an increase in pre-existing T cell responses in some patients. This, thus, indicates increased T cell clonality and induction (within the limit of assay detection) of *de novo* T cell response, suggesting increased T cell richness of antigenic repertoire (30). Altogether, the evidences gained in clinical studies and animal models with *it*DC imply that checkpoint blockade therapy could be enhanced by prior itDC immunotherapy (**Figure 2**).

Interestingly, the role of intratumoral DC subsets in response to checkpoint blockade therapy has recently emerged, depicting two independent axis: an NK/cDC1/IL-12-CXCL9 axis needed for effective CD8 T cell response and a Treg/cDC2 axis for effective CD4 T cell response. In one study mainly focused on melanoma, Barry et al. have described the role of intratumoral NK cells in increasing cDC1 abundance within a tumor microenvironment by secreting FLT3LG, showing that the abundance of both populations positively correlates with the response to checkpoint blockade therapy (62). Further, recent literature has unraveled how intratumoral cDC1 "license" CD8 response during checkpoint blockade by secreting IL-12 and CXCL9, potentiating T cell activation (97, 98). On the other side, Binnewies et al. have discovered that levels of cDC2 populations relative to Treg abundance within tumor microenvironment are responsible of infiltration by CD4 T cells and correlate with the response to checkpoint blockade therapy (99). Whether *it*DC during checkpoint blockade therapy could potentiate T cell activity by secreting IL-12, CXCL9 or by overcoming inhibitory activity of Treg has not yet been analyzed. However, it is reasonable to expect that activated *it*DC will sum up with intratumoral DC in sustaining T cell responses during checkpoint blockade therapy (**Figure 2**).

CONCLUSIONS AND PERSPECTIVES

The intratumoral delivery of DC has been tested in several different clinical settings, where it has been proved to not only be feasible and safe, but also to be capable of enhancing and/or inducing a tumor-specific immune response.

The possibility to exploit, by an endogenous vaccination strategy, the broad tumor antigen repertoire promptly released by immunogenic tumor pre-conditioning, makes *it*DC a versatile cell therapy, potentially overcoming some of the limitations of therapies based on *ex-vivo* antigen loaded DC, such as the lack of dominant tumor antigens, the availability of tumor samples, and the possible emergence of neo-antigens.

Although the limited number of patients enrolled in phase I studies demands a prudent evaluation of the observed clinical results, data collected so far look promising, and encourages the application of *it*DC to hitherto unexplored clinical settings. More research efforts should yet be devoted to the identification of the optimal DC types to be used in *it*DC strategies, as well as of the most effective strategies for tumor microenvironment pre-conditioning tailored for specific clinical settings. Of note, the accumulating knowledge on their mechanism of action, by showing that *it*DC can affect tumor microenvironment at different levels (including cytokine release and NK cells stimulation), also provides the rationale for their use in combination with immunotherapy approaches currently used in oncology, such as immune checkpoint inhibitors. Based on the evidence available, summarized in this review, we envisage that *it*DC, administered prior or in concomitance with checkpoint inhibitors, by triggering a broader and more effective antitumor immune response, can not only prolong their efficacy, but also provide clinical benefit to patients showing limited responsiveness to checkpoint inhibitors *per se*.

AUTHOR CONTRIBUTIONS

LC, EA, and FB wrote the manuscript. LC and EA prepared the figures. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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CD137L-DCs, Potent Immune-Stimulators—History, Characteristics, and Perspectives

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Dendritic cell (DC)-based immunotherapies are being explored for over 20 years and found to be very safe. Most often, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4)-induced monocyte-derived DCs (moDCs) are being used, which have demonstrated some life-prolonging benefit to patients of multiple tumors. However, the limited clinical response and efficacy call for the development of more potent DCs. CD137L-DC may meet this demand. CD137L-DCs are a novel type of monocyte-derived inflammatory DCs that are induced by CD137 ligand (CD137L) agonists. CD137L is expressed on the surface of antigen-presenting cells, including monocytes, and signaling of CD137L into monocytes induces their differentiation to CD137L-DCs. CD137L-DCs preferentially induce type 1 T helper (Th1) cell polarization and strong type 1 $CD8^+$ T cell (Tc1) responses against tumor-associated viral antigens. The *in vitro* T cell-stimulatory capacity of CD137L-DCs is superior to that of conventional moDCs. The transcriptomic profile of CD137L-DC is highly similar to that of *in vivo* DCs at sites of inflammation. The strict activation dependence of CD137 expression and its restricted expression on activated T cells, NK cells, and vascular endothelial cells at inflammatory sites make CD137 an ideally suited signal for the induction of monocyte-derived inflammatory DCs *in vivo*. These findings and their potency encouraged a phase I clinical trial of CD137L-DCs against Epstein–Barr virus-associated nasopharyngeal carcinoma. In this review, we introduce and summarize the history, the characteristics, and the transcriptional profile of CD137L-DC, and discuss the potential development and applications of CD137L-DC.

Keywords: CD137L-DC, reverse CD137L siganling, moDC, Th1, Tc1, tumor immunotherapy

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INTRODUCTION

The past 10 years have witnessed a renewed enthusiasm for cancer immunotherapy, prompted by the success of chimeric antigen receptor-T cells (CAR-T) and immune checkpoint inhibitors (ICI). Despite impressive therapeutic responses in some patients of certain tumors, CAR-T and ICI failed to show efficacy in most patients of the majority of cancers, especially solid cancers (1, 2). Moreover, the high frequency of severe adverse effects and the risk of breaking immune tolerance (3) by CAR-T cells or ICI emphasize that these treatments need to be carried out with caution.

A much safer immunotherapeutic approach is the use of dendritic cells (DCs). Since DCs were first described (4), substantial knowledge about the ontogeny, functions, and therapeutic

applications of DCs has been accumulated, leading to the recognition that DCs exist in the form of several subsets, characterized by differences in ontogenies and functional properties (5–7). DC-based immunotherapies have been explored for two decades and are well-tolerated. Although DC-based cancer therapies has been proven to prolong the overall survival of patients, their efficacy and the clinical responses are far from satisfactory (8–10). Different strategies are being investigated to improve the efficacy, such as optimizing the tumor antigen source and loading, seeking optimal maturation methods, and the combination of DCs with ICI (11). However, first and foremost, a pivotal parameter is the source and type of DCs.

Due to the low frequency of natural DCs in blood, monocyte-derived DCs (moDCs), obtained by treating human peripheral monocytes with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4), are currently the most commonly used DC type in clinical trials. New methods to target DCs *in vivo* (12, 13), to enrich blood DCs in GMP facilities *ex vivo* (14, 15), or to differentiate myeloid DCs from stem cells (16, 17) have been explored. Yet the yield of DCs is limited. We have found a new type of human DC, CD137 ligand-induced DC (CD137L-DC), that is differentiated from peripheral monocytes by recombinant CD137-Fc protein or anti-CD137 ligand (CD137L) antibodies (18). Compared to the commonly used GM-CSF and IL-4-induced moDCs, CD137L-DCs have shown superior activities in inducing T cell responses (19, 20). In this review, we will give a systematic review on the development, the function, and the clinical application of this new type of DCs.

THE DISCOVERY OF CD137L-DC

CD137 (TNFRSF9, 4-1BB) is an important co-stimulatory molecule expressed strictly upon activation, predominantly on T cells, NK cells, and vascular endothelial cells (21–23). Engagement of CD137 potently costimulates T cells and induces effective anti-tumor immune responses (24–27). Two agonistic anti-CD137 antibodies (urelumab and utomilumab) have shown great potency in preclinical experiments, and are currently being tested in clinical trials (28). In CAR, the intracellular domain of CD137 delivers signals for CAR-T cell persistence and delays their exhaustion (29, 30). CD137 ligand (CD137L, TNFSF9, 4-1BBL) is expressed on all types of antigen-presenting cells (APCs), and expression levels of CD137L increase upon APC activation (31). In the 1990s, several tumor necrosis factor super family (TNFSF) members were reported to trigger reverse signals into APCs (32–34). Reverse signaling is possible when a ligand is not a soluble molecule but is expressed as a transmembrane protein on the cell surface and can transmit a signal into the cell it is expressed on. Thus, functionally, it is identical to a receptor but it is referred to as a ligand (1) due to historical reasons and/or (2) because its partner molecule is also a receptor. Hence, both interacting molecules send and receive signals, i.e., act at the same time as a receptor and ligand, thereby establishing bidirectional signaling (35).

Similarly, engagement of CD137L was found to cause T cell apoptosis (36) and to activate monocytes as evidenced by the induction of adherence and cytokine secretion (37). Further, immobilized CD137-Fc protein induced survival and even proliferation of monocytes, which are mainly mediated by CD137L-induced macrophage colony-stimulating factor (M-CSF) (38, 39). Reverse signaling of CD137L was further shown in monocytic cell lines (40), B cells (41), moDCs (42, 43), and myeloid DCs (44). Notably, cross-linking of CD137L matures moDCs and myeloid DCs *in vitro* as seen by the increased expression of costimulatory molecules and IL-12p40 (43, 44). Altogether, these findings demonstrate that CD137L, just like other TNFSF members, not only can deliver but also can receive a signal (**Figure 1**).

Human monocytes that were exposed to CD137L agonists adhered to cell culture dishes very rapidly and the resultant cells were morphologically different from resting or LPS-activated monocytes and from macrophages (37, 45). The cells exhibited extensions that were comparable with DCs but their morphology was different from DCs that were generated from monocytes by GM-CSF and IL-4 treatment. In 2009, it was found that an agonistic anti-CD137L antibody could replace GM-CSF in the differentiation of moDCs. The anti-CD137L antibody + IL-4-induced DCs stimulated stronger T cell proliferation and preferentially polarized naïve $CD4^+$ T cells toward type 1 T helper (Th1) cells compared to conventional moDCs (46). However, IL-4 is not required when employing CD137-Fc protein, which alone is sufficient to induce monocyte to DC differentiation. The resulting cells, later named CD137L-DC, had an enhanced expression of the DC maturation marker CD83 and enhanced endocytosis but reduced phagocytosis and oxidative burst (18). Despite lacking the conventional moDC markers CD1a and CD209, reverse CD137L signaling-induced cells could stimulate the proliferation of naïve T cells, which is the gold standard of defining a DC, justifying their naming as a type of DC (18). The T cell-activating capability of CD137-Fc-activated monocytes is gradually acquired because during the first 24 h of differentiation, the developing CD137L-DCs induce T cell apoptosis *via* elevated reactive oxygen species. This unexpected finding may have its physiological significance in a process called infection-induced T cell attrition, where old T cells are eliminated in order to create space for the new ones with specificity for the new challenge antigens (47). Altogether, these data made it clear that reverse CD137L signaling induced the differentiation of monocytes to a new type of DC, namely, CD137L-DC.

SPECIES DIFFERENCE IN CD137L-DC

The abovementioned reverse CD137L signaling has been reported in both human and mouse. However, murine reverse CD137L signaling is very different from human reverse CD137L signaling. In murine endothelial cells, reverse CD137L signaling leads to chemokine secretion (48). In murine macrophages, reverse CD137L signaling sustains TNF secretion induced by

Abbreviations: CD137L-DC, CD137 ligand-induced DC; DC, dendritic cell; ICI, immune checkpoint inhibitors; moDC, monocyte-derived DC.

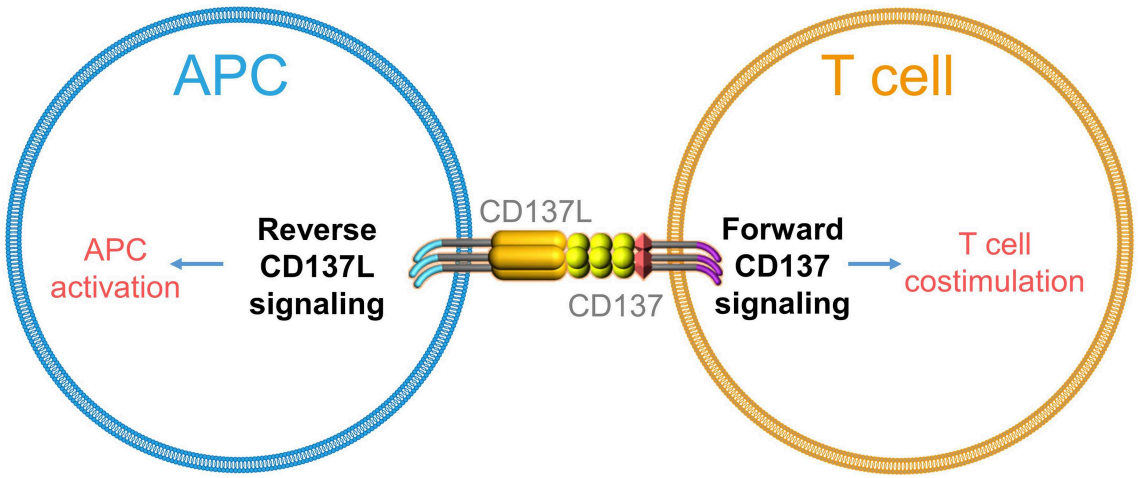

FIGURE 1 | Schematic depiction of bidirectional signaling by CD137–CD137L.

toll-like receptor 4 (TLR4) activation (49, 50). However, reverse CD137L signaling was reported by the same group to suppress the activation of macrophages and DCs *in vivo* in mice. Kang et al. (51) found that CD137$^{-/-}$ mice and agonistic anti-CD137 antibody-treated mice had better anti-tumor immune responses because of an increased differentiation of myeloid cells to CD103^{+} DCs and type 1 macrophages. They proposed that reverse CD137L signaling suppresses the generation of pro-inflammatory DCs and macrophages and thereby the induction of effective anti-tumor responses. This hypothesis is supported by immobilized CD137-Fc, which initiates reverse CD137L signaling, inhibiting the differentiation of CD103^{+} DCs and M1 macrophages *in vitro*. However, the more effective anti-tumor response was not seen in CD137L$^{-/-}$ or neutralizing anti-CD137L antibody-treated mice (51). Another concern is that CD137 is also expressed on and functional in macrophages (52, 53) and DCs (41, 54). It can therefore not be ruled out that CD137 deficiency may have disturbed the equilibrium and function of endogenous DCs and macrophages.

CD137L-DCs have only been differentiated from human but not from murine monocytes. Unlike other TNFSF members that generally share 60–80% homology between mouse and human, the amino acid sequence of human CD137L protein is only 36% identical to that of murine CD137L (55). While murine monocytes also proliferate and change their morphology in response to immobilized murine CD137-Fc protein, the resulting cells are not inflammatory DCs as evidenced by the absence of DC markers and their inability to induce allogenic T cell proliferation (56). Therefore, CD137L-DC specifically refers to *in vitro* generated human CD137L-DC in this review. At present, the functions and activities of CD137L-DC have just started to be evaluated *in vivo*.

THE SUPERIOR FUNCTION OF CD137L-DC

To achieve more effective anti-tumor immune responses, DCs must polarize naïve T cells preferentially to Th1 and type 1 CD8^{+} T cell (Tc1) responses. CD137L-DCs meet this requirement. CD137L-DCs enhance the subset of interferon (IFN)γ^{+} T cells, especially among the CD8^{+} cells, and no additional maturation of CD137L-DCs is required for this activity (18). CD8^{+} T cells activated by CD137L-DCs express more perforin and are more cytotoxic than T cells activated by conventional moDCs (18, 19). Most importantly, human TCR-redirected T cells are stronger activated and exert superior antigen-specific killing when activated by autologous peptide antigen-pulsed CD137L-DCs than by autologous moDCs or mature moDCs (19, 20). This superior function of CD137L-DCs has been observed with cytomegalovirus-, hepatitis B virus (HBV)-, and Epstein–Barr virus (EBV)-derived antigens. HBV and EBV are associated with various types of cancers (57, 58), implying that CD137L-DCs are a good candidate for virus-associated cancer immunotherapy. Classical DCs (cDCs) are also more potent than moDCs at activating Th1 and Tc1 responses. The direct comparison between CD137L-DCs and cDCs has not yet been done. What we know is that CD137L-DCs express low level of CD141 and no CD1c, markers for cDC1 and cDC2, respectively (59).

To achieve a successful DC-based immunotherapy, the maturation of DCs is of great importance. Although CD137L-DCs express the DC maturation marker CD83 and secrete pro-inflammatory cytokines, the cytokine levels are generally low. In order to increase the potency of CD137L-DCs, different combinations of cytokines and pattern recognition receptor agonists were compared regarding their ability to mature CD137L-DCs (19). Toll-like receptor 7/8 agonist R848 and IFNγ were found to generate the most potent mature CD137L-DCs. These mature CD137L-DCs significantly elevated the expression of CD40, CD70, CD80, CD86, CD137L, and HLA-DR (19, 60). Accordingly, mature CD137L-DCs more strongly enhance the proliferation and the percentage of IFNγ^{+} T cells (19).

The ability of DCs to migrate to the lymph nodes is another pivotal factor for a successful DC-based therapy (10, 11). To boost the expression of CCR7, and thus the migratory capacity of mature CD137L-DCs, prostaglandin E2 (PGE2) was included in the maturation cocktail (61). Even though PGE2 is known to

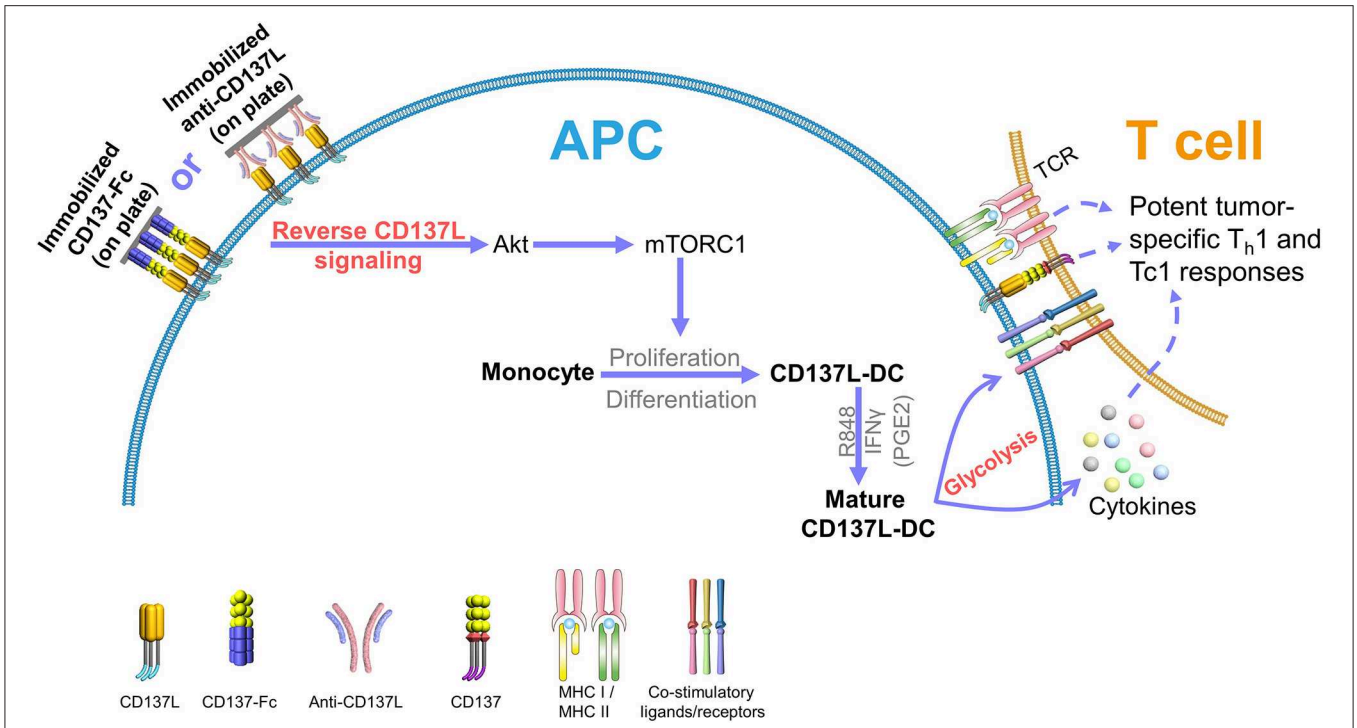

FIGURE 2 | CD137L-DCs induce potent Th1 and Tc1 responses against tumors. Reverse CD137L signaling activates monocytes and induces the proliferation and the differentiation of monocytes to CD137L-DCs. R848 and IFNγ (and PGE2) cause maturation of CD137L-DCs. Mature CD137L-DCs are characterized by a high Akt-mediated glycolysis rate, which is important for the inflammatory properties of CD137L-DCs. Mature CD137L-DCs preferentially induce Th1 and Tc1 polarization in T cells, leading to strong immune responses against virus-associated tumor cells, making them a promising candidate for virus-associated cancer immunotherapy.

decrease the secretion of IL-12 by DCs (62), the T cell-activating capability of mature CD137L-DCs is not significantly impaired by PGE2 (20). $CD8^+$ T cells activated by PGE2, R848, and IFNγ-matured CD137L-DCs are more cytotoxic, less exhausted, and metabolically more active than $CD8^+$ T cells activated by mature moDCs (20). A schematic summary of CD137L-DC functions is shown in **Figure 2**.

Based on these findings, we started a phase I clinical trial (NCT03282617) with CD137L-DCs to treat nasopharyngeal carcinoma (NPC). The CD137L-DCs are generated from patients' monocytes; matured with R848, IFNγ, and PGE2; and pulsed with peptide pools of EBV antigens. Currently, data are available on 10 patients and no immune-related adverse events have been observed, demonstrating an excellent safety profile of CD137L-DCs.

CHARACTERISTICS AND PHYSIOLOGICAL RELEVANCE OF CD137L-DC

The most commonly employed step between *in vitro* studies and clinical trials is the use of animal models, generally murine models. Demonstrating *in vivo* proof of principle of CD137L-DCs in murine tumor models was hindered by the above-described species difference in the molecular structure and in reverse CD137L signaling between mouse and human.

In order to investigate the *in vivo* relevance of CD137L-DC, Harfuddin et al. acquired the transcriptome of CD137L-DCs and compared it to the transcriptomes of different types of *in vitro*-generated human myeloid cells and *in vivo* inflammatory macrophages and DCs. By using hierarchical clustering and gene enrichment analysis, it was found that the gene signature of CD137L-DC is distinct from that of other myeloid cells, but is most similar to that of immature moDC and macrophages. Notably, the transcriptome of CD137L-DCs is enriched for the gene signatures of human inflammatory DCs and $BDCA1^+$ DCs as they occur *in vivo* at sites of inflammation (59), indicating CD137L-DCs may exist under inflammatory conditions *in vivo* in man.

Further support for this hypothesis comes from the presence of CD137 in blood vessels at sites of inflammation and the involvement of the CD137–CD137L system in monocyte recruitment into inflamed tissues. CD137 expression is induced on vascular endothelial cells by TNF, and CD137 on the vascular endothelial cells strengthens intercellular adhesion molecule 1 and lymphocyte function-associated antigen 1-mediated adhesion of monocytes. In *in vitro* systems, CD137 attracts monocytes to infiltrate into spheroids and matrigels (63, 64). Therefore, circulating monocytes that are recruited to sites of inflammation *via* CD137 on vascular endothelial cells would receive a CD137L signal during the recruitment that may initiate their CD137L-DC differentiation. Further CD137L

signaling would be induced by CD137-expressing leukocytes at the site of inflammation.

These data support the concept that reverse CD137L signaling in circulating monocytes may contribute to the physiological generation of inflammatory DCs in man. After all, CD137 expression is strictly activation-dependent and only found at sites of inflammation. Expression of CD137L is enhanced upon APC activation. This restricted expression of CD137 would confine the generation of CD137L-DCs to sites of inflammation.

The transcriptome profile of human CD137L-DC has generated additional interesting information. For example, CD137L-DCs strongly adhere to the plastic cell culture dishes. This feature finds its explanation in the gene ontology enrichment analysis. Compared to both immature and mature moDCs, CD137L-DCs express 22 genes involved in cell adhesion at more than 2-fold higher levels (59).

Another property of CD137L-DCs that was identified by the transcriptome analysis is their metabolism. Metabolic reprogramming is being increasingly appreciated as an important driving force of immune cell activation and effective immune responses (65). Our recent data demonstrate that Akt-driven glycolysis contributes to the superior function of CD137L-DCs. Compared to GM-CSF and IL-4-generated moDCs, CD137L-DCs show a significantly higher basal glycolysis rate and glycolytic capacity due to the elevated activation of the phosphoinositide 3-kinase (PI3K)–Akt–mechanistic target of rapamycin complex 1 (mTORC1) pathway. This higher rate of glycolysis is important not only for the maturation but also for the sustained activation of CD137L-DCs. The inhibition of the PI3K or Akt recapitulates the inhibition of CD137L-DC by suppressed glycolysis (**Figure 2**). In contrast to the flux of glycolysis intermediates into lipid synthesis in murine bone marrow-derived DCs (66), the higher glycolysis of mature CD137L-DCs leads to an increase in succinate and serine, which are known metabolites that regulate inflammation (60).

REVERSE CD137L SIGNALING MECHANISM

An interesting aspect is the signal transduction mechanism employed by CD137L. In human primary monocytes and THP-1 cells, protein tyrosine kinases, mitogen-activated protein kinase (MAPK) kinase, p38 MAPK, extracellular regulated protein kinases 1/2, PI3K-Akt-mTORC1, and protein kinase A are activated by recombinant CD137-Fc protein (60, 67). mTORC1 is critical for the differentiation of CD137L-DCs from monocytes (60). Interestingly, CD137L associates with several cell surface receptors with well-characterized signal transduction cascades. In human cells, CD137L associates with TNFRI, which is required for CD137L-induced cell adhesion, CD14 expression, and IL-8 production (68). In murine macrophages, the association of murine CD137L with TLR4 is required for TLR4-induced activation of the transcription factors cAMP response element binding protein (CREB) and CCAAT-enhancer-binding proteins (C/EBP), and for sustained TNF secretion by murine peritoneal macrophages (49). The murine TLR4–CD137L complex associates with toll–interleukin-1 receptor domain-containing adaptor protein (TIRAP), interleukin-1 receptor-associated kinase-like 2 (IRAK2), TNF receptor–associated factor 6 (TRAF6), transforming growth factor–b–activated kinase 1 (TAK1), and TAK-binding protein 1 (TAB1) to form a larger signaling complex (50). Further, CD137L associates with transmembrane protein 126A (TMEM126A), and knockdown of TMEM126A in murine macrophages prevents the induction of tyrosine phosphorylation and the secretion of M-CSF, IL-1β, and Tenascin C (69). It is currently not known whether these associations are species-specific or apply similarly to human and mouse. Nevertheless, the common theme from all these studies is that CD137L seems not to signal by itself, but to be part of a larger signaling complex.

On reverse CD137L signaling, there is an entirely different mechanism to consider. Upon cell–cell interaction of CD137- and CD137L-expressing cells, human CD137 gets transferred to the CD137L-expressing cells by trogocytosis and then forms a complex with CD137L, which gets internalized and degraded *via* the proteasome (70–72). Whether this process is involved in mediating reverse CD137L signaling has not yet been addressed.

FUTURE PERSPECTIVES

CD137L-DCs are an attractive candidate for cancer immunotherapy as CD137L-DCs are more potent at inducing and strengthening Th1 and Tc1 responses than the conventional GM-CSF and IL-4-induced moDCs. CD137L-DCs are showing an excellent safety profile in NPC patients. The evaluation of their immunological efficacy and clinical usefulness is pending. Apart from NPC, other virus-associated malignancies could also be targeted by CD137L-DCs (19, 20) such as the EBV-associated Hodgkin lymphoma and HBV-associated hepatocellular carcinoma (58). How to further enhance the efficacy of CD137L-DCs will be a topic of future research. Despite the addition of PGE2 to increase the mobility of DCs, Davignon et al. have shown that most DCs die at the site of injection (73). Improving the homing of functional DCs to the lymph nodes remains a challenge. Preconditioning the injection site may be one way to improve the efficacy of CD137L-DCs (10). Additionally, the combination of CD137L-DCs with ICI could be an approach to overcome the suppressive tumor microenvironment and boost the activation of T cells (74, 75).

An issue that a CD137L-DC therapy shares with most DC therapies and personalized therapies in general is the high cost of generating the cells. Monocytes need to be harvested and converted to CD137L-DC for each patient individually under good manufacturing practice conditions. It would be a huge advantage if an approach could be developed that enables the *in vivo* generation of CD137L-DC in patients, just like how inflammatory monocyte-derived DCs are generated at the site of inflammation, when and where they are needed. For instance, controlled and site-specific CD137L agonist delivery to monocytes may fulfill this requirement and mimic the *in vivo* generation of inflammatory monocyte-derived DCs. Practically, liposome- or exosome-mediated CD137 gene/protein delivery

could be suited for this task. Nevertheless, a comprehensive understanding of the mechanism of CD137L-DC differentiation is necessary for the generation of CD137L-DCs *in vivo*. CD137L likely forms a complex with other signaling mediators, and this complex formation may be necessary to induce the differentiation of CD137L-DC. A more detailed investigation of the molecules and signaling pathways leading to CD137L-DC differentiation will surely augment our understanding of the possible roles of CD137L-DCs under inflammatory conditions such as infection, autoimmune disease, and antibody treatment-induced inflammation.

CD137L-DCs have been studied for 10 years. Transcriptomic data indicate them to be close *in vitro* counterparts to *in vivo* monocyte-derived inflammatory DCs. Due to their potent immune-stimulatory activity, CD137L-DCs are being developed for cancer immunotherapy.

AUTHOR CONTRIBUTIONS

QZ, YZ, and HS organized, wrote, and edited the manuscript. Figures were drawn by QZ and edited by YZ and HS.

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Dendritic Cell Vaccination in Metastatic Melanoma Turns "Non-T Cell Inflamed" Into "T-Cell Inflamed" Tumors

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Dendritic cell (DC)-based vaccination effectively induces anti-tumor immunity, although in the majority of cases this does not translate into a durable clinical response. However, DC vaccination is characterized by a robust safety profile, making this treatment a potential candidate for effective combination cancer immunotherapy. To explore this possibility, understanding changes occurring in the tumor microenvironment (TME) upon DC vaccination is required. In this line, quantitative and qualitative changes in tumor-infiltrating T lymphocytes (TILs) induced by vaccination with autologous tumor lysate/homogenate loaded DCs were investigated in a series of 16 patients with metastatic melanoma. Immunohistochemistry for CD4, CD8, Foxp3, Granzyme B (GZMB), PDL1, and HLA class I was performed in tumor biopsies collected before and after DC vaccination. The density of each marker was quantified by automated digital pathology analysis on whole slide images. Co-expression of markers defining functional phenotypes, i.e., $Foxp3^+$ regulatory $CD4^+$ T cells (Treg) and $GZMB^+$ cytotoxic $CD8^+$ T cells, was assessed with sequential immunohistochemistry. A significant increase of $CD8^+$ TILs was found in post-vaccine biopsies of patients who were not previously treated with immune-modulating cytokines or Ipilimumab. Interestingly, along with a maintained tumoral HLA class I expression, after DC vaccination we observed a significant increase of $PDL1^+$ tumor cells, which significantly correlated with intratumoral $CD8^+$ T cell density. This observation might explain the lack of a significant concurrent cytotoxic reactivation of $CD8^+$ T cell, as measured by the numbers of $GZMB^+$ T cells. Altogether these findings indicate that DC vaccination exerts an important role in sustaining or *de novo* inducing a T cell inflamed TME. However, the strength of the

intratumoral T cell activation detected in post-DC therapy lesions is lessened by an occurring phenomenon of adaptive immune resistance, yet the concomitant PDL1 up-regulation. Overall, this study sheds light on DC immunotherapy-induced TME changes, lending the rationale for the design of smarter immune-combination therapies.

Keywords: melanoma, tumor microenvironment, T cell landscape, dendritic cell vaccine, immunomonitoring, immunotherapy, PDL1

INTRODUCTION

According to the cancer-immunity cycle, dendritic cells (DCs) play a fundamental role in setting off an anti-tumor specific immune response (1). Indeed, under ideal circumstances, DCs take up tumor antigens and promote the generation of anti-tumor specific T cells, which ultimately infiltrate the tumor bed and kill their target cells through cytolytic mechanisms (i.e., perforin and granzyme B). The translation of this concept into the clinic has led to the design of DC-based therapeutic vaccines (2). Since their first utilization, many clinical trials have been conducted in metastatic melanoma patients accounting for an objective response rate of 8.5%, as reported in a meta-analysis conducted in 1,205 advanced melanoma patients treated with DC vaccination monotherapy (3). Similar data were observed in our long-term follow-up series (4). Delayed-type hypersensitivity skin test (DTH) and quantification of peripheral antigen-specific anti-tumor T cell response with Enzyme-Linked immunoSPOT assay and/or tetramer analysis on peripheral blood samples, longitudinally collected during the treatment, are commonly used to evaluate the immunogenicity of DC vaccines. However, the reported induced tumor-specific immune responses measured in the blood seem to only partially correlate with efficacy (5). A higher enrichment of antigen specific T cells inside the tumor compared to the blood has been argued as a potential consequence of this phenomenon (6, 7). Intriguingly, it was also suggested that the presence of an anti-tumor specific blood response, even if weak, could serve to promote a local inflammation in the tumor microenvironment (TME), and intratumoral $CD8^+$ CTL infiltration has also been envisaged as the clinically relevant consequence of the eosinophilia found in DC vaccinated patients and associated with positive outcome (8). However, notwithstanding the recognized importance of the TME, no studies have been conducted to assess whether an *in situ* analysis could allow adding additional insight into the local immune modulation occurring in DC vaccinated patients. Besides systemic anti-tumor immunity, it becomes now clear that the immune contexture holds precious information endowed with clinical impact (9). In particular, the content of intratumoral immune cells, especially $CD8^+$ T cells, strictly correlates with patients' prognosis (10) across different tumor types, melanoma included (11). Accordingly, immunological characterization of the TME along treatment is increasingly utilized for identifying biomarkers of response and mechanisms of resistance to cancer immunotherapies (12, 13). Evidence available from the literature has been primarily obtained in patients treated with immune checkpoint inhibitors (14, 15), and aimed at identifying biomarkers predictive of response to therapy and finding potentially actionable synergistic targets for improving their clinical efficacy. In this line, clinical experience with combined anti-CTLA4 and anti-PD1 immunomodulating antibodies has shown better efficacy in melanoma patients, but at the expenses of more severe treatment-related toxicities. DC immunotherapy has a robust safety profile, which makes it an interesting good candidate for better-tolerated combination immunotherapies. Nonetheless, an extensive characterization of changes occurring in the TME upon DC vaccination is currently lacking. In order to fill this gap in the literature, we addressed by immunohistochemical (IHC) analysis the local modulation of the T cell landscape upon DC vaccination in a retrospective series of matched pre and post formalin-fixed paraffin embedded (FFPE) tumor lesions collected from a series of 16 metastatic melanoma patients treated with autologous DCs loaded with tumor lysate/homogenate. Our data show a DC vaccine-induced modulation of the TME, with the emergence of changes suggestive of a T cell inflamed TME, i.e., a robust $CD8^+$ T cell infiltration along with the up-regulation of PDL1. Altogether, our findings support the use of DC immunotherapy as a TME modulating therapeutic tool, which might broaden the effectiveness of anti-PD1/PDL1 therapies.

MATERIALS AND METHODS

Patients

In this study, we evaluated 16 patients with metastatic melanoma enrolled in different vaccination protocols from 2000 to 2015. All patients were given intradermally mature autologous dendritic cell pulsed with autologous tumor lysate (ATL) or autologous tumor homogenate (ATH) and keyhole limpet hemocyanin (KLH). DC vaccine was administered alone (mainly in a compassionate use program, CUP) or combined with different conditioning therapies, e.g., low doses of temozolomide prior to the vaccine (16) or INF alpha before leukapheresis (17) as described in **Table 1**. Pre-treatment tumor samples were obtained from tumor lesions surgically removed for the preparation of ATL or ATH. Post-vaccine biopsies were obtained for diagnostic and/or therapeutic purposes and were taken at least after the fourth induction dose of the vaccine. The median time from the pre-treatment biopsies to therapy was 3 months (0–29 months, average 6.25 months). All post-therapy lesions have been collected on-treatment besides Pt#2, Pt#6, and Pt#10 for whom the tumor was sampled after 18, 7, and 3 months from the last vaccine dose, respectively. The median time from start to biopsy was 5 months for the on-treatment samples (3–24 months, average 7 months) and 7 months for the post-treatment

TABLE 1 | Patients' characteristics ($n = 16$).

Pt#ID	BRAF status	Vax protocol	Tumor lesion, site		Months from surgery to first vax	Months from baseline vax to biopsy harvesting (on-treatment or post-treatment biopsy)	BOR (RECIST)/ duration	OS	Objective response of post-vax biopsy	DTH	Previous/ following treatments	Ratio post/ pre CD8 (cells/mm^2)	PDL1 in total cells (%)	
			Baseline	Post-vax									Pre	Post
1#	**V600E**	**CUP**	**Lymph node**	**Lymph node**	**1**	**17 (on-treatment)**	**CR/8**	**34**	**Progressing**	**+**	**No/CT, High dose IL-2**	**1,101**	**7,836**	**3,022**
2#	V600E	CUP	Lymph node	Subcutis	15	18 (post-treatment)	PR/68	87	Stable	+	BioCT/Surgery	0,913	0,726	0,402
3#	V600E	CUP	Omentum	Stomach	2	24 (on-treatment)	SD/50	108+	Stable	+	BioCT/Surgery,RT	0,823	16,750	6,185
4#	WT	Tem	Peritoneum	Subcutis	2	4 (on-treatment)	SD/4	16	Progressing	+	CT, Ipi/RT	0,173	4,744	4,793
5#	**na**	**Tem**	**Subcutis**	**Omentum**	**1**	**4 (on-treatment)**	**SD/10**	**45**	**Progressing**	**+**	**RT/Ipi**	**1,338**	**11,130**	**24,700**
6#	V600E	Tem	Lung	Skin	3	7 (on-treatment)	SD/9	62	Progressing	+	BioCT/low doses IL-2, Ipi	0,521	0.9748	9,203
7#	**V600E**	**CUP**	**Subcutis**	**Subcutis**	**10**	**5 (post-treatment)**	**SD/7**	**22**	**Progressing**	**+**	**CT*/Ipi**	**0,560**	**0,826**	**7,519**
8#	**WT**	**CUP**	**Jejunum**	**Adrenal gland**	**29**	**4 (on-treatment)**	**PR/57**	**87+**	**Stable**	**++**	**CT/Ipi, CT**	**3,010**	**0,000**	**33,639**
9#	**V600E**	**CUP**	**Lymph node**	**Subcutis**	**3**	**5 (on-treatment)**	**SD/6**	**23**	**Progressing**	**+**	**No/ Ipi,vemurafenib, Tem**	**4,039**	**0,602**	**6,530**
10#	V600E	CUP	Subcutis	Lymph node	12	3 (post-treatment)	SD/5	18	Progressing	+	Biot/RT	0,110	8,771	na
11#	**WT**	**CUP**	**Subcutis**	**Subcutis**	**3**	**9 (on-treatment)**	**SD/5**	**27**	**Progressing**	**+**	**No/CT, Ipi**	**1,051**	**1,255**	**13,999**
12#	WT	CUP	Subcutis	Subcutis	8	4 (on-treatment)	PD¥	13	Progressing	-	Biot/No	1,355	na	na
13#	**WT**	**Vax+INFα**	**Subcutis**	**Subcutis**	**5**	**3 (on-treatment)**	**PD**	**11**	**Progressing**	**+**	**No/Ipi**	**2,260**	**1,677**	**8,201**
14#	**WT**	**Vax+INFα**	**Lymph node**	**Subcutis**	**0**	**3 (on-treatment)**	**PD**	**7**	**Progressing**	**+**	**No/Ipi**	**5,013**	**2,465**	**11,765**
15#	WT	Vax only	Subcutis	Brain	4	6 (on-treatment)	SD/5	8	Progressing	+	CT, Ipi/No	1,080	2,379	0,893
16#	**V600E**	**CUP**	**Adrenal gland**	**Skin**	**2**	**5 (on-treatment)**	**PD**	**19**	**Progressing**	**+**	**No/Ipi, vemurafenib, BioCT, pembro**	**5,097**	**1,709**	**14,279**

Vax, dendritic cell vaccination; DTH, delayed-type hypersensitivity; WT, wild type; CUP, compassionate use program; CT, chemotherapy; CT, chemotherapy third line; RT, radiotherapy; Tem, temozolomide; IFNα, interferon alpha; IL-2, interleukine 2; Biot, low doses of cytokines (IL-2 and/or IFNα); BioCT, cytokine+chemotherapy; Ipi, ipilimumab; pembro, pembrolizumab. Naïve/CT/RT patients are highlighted in bold. ¥ Pt#12 was treated beyond progression after surgical removal of the progressing lesion.*

samples (3–18 months, average 9.33 months). Clinical response was defined according to RECIST 1.1 criteria (18) and surgically removed post-vaccine tumor lesions were classified as regressing if changes in their longest diameter were ≥−30% compared with the baseline, stable if changes were comprised between −30 and +20%, and progressing if ≥+20% (**Table 1**). All patients gave their informed consent to the study, which was conducted in accordance with the Declaration of Helsinki following a protocol approved by the local Institutional Review Board.

Generation and Administration of DCs

DCs were prepared following Good Manufacturing Practice (GMP) guidelines and according to Ridolfi et al. (16). Briefly, monocytes obtained by adherence of the leukapheresis product on culture flasks were cultured in CellGro DC medium supplemented with interleukin 4 (IL-4, Cell Genix) and granulocyte-macrophage colony stimulating factor (GM-CSF, Cell Genix) for 7 days. On day 6, 90% of the DC culture was pulsed with ATL or ATH (100 mg/ml), while the remaining 10% was pulsed with KLH (50 mg/ml). On day 7, the culture medium was discarded and immature DCs were cultured for further 2 days with a maturation cocktail comprising the following cytokines: TNFα, IL-1β, IL-6 (Cell Genix), and PGE2 (Pfizer). On day 9, mature dendritic cells (median 10^7, range 2.2–20.8 $\times$ 10^6) were recovered, washed, suspended in sterile saline solution and immediately injected. As part of our standard release criteria, before administration, DCs were checked for safety (sterility, mycoplasma, endotoxin), vitality, purity, and maturation phenotype. Purity was always reported to be >60% (average 63, 59%). The maturation phenotype of the infused DCs was confirmed by flow cytometry using the following markers: HLADR (accepted cut-off value ≥60%, average = 85, 8%), CD80 (accepted cut-off value ≥50%, average = 83, 4%), CD83 (accepted cut-off value ≥40%, average = 74,9%), and CD86 (accepted cut-off value ≥60%, average = 78,8%). Patients were given 10^7 DCs intradermally at the base of the thigh or groin every 2 weeks for 4 cycles, followed by monthly doses until progression, worsening of clinical conditions (ECOG performance status > 2), or autologous tumor lysate shortage. Patients who ran out of tumor lysate, but had additional surgically removable tumor lesions, were retreated utilizing tumor lysate obtained from newly removed lesions. Antitumor immune response to the DC vaccine was evaluated with Delayed-Type Hypersensitivity (DTH) test as follows: serial concentrations (100, 50, 20, 10, and 5 μg) of autologous tumor lysate and KLH were intradermally injected into the forearms of patients and erythema and induration were recorded after at least 24 h. DTH was considered as positive if the area of erythema and/or induration measured at least 5 mm at any antigen concentration.

Standard and Sequential Immunohistochemistry (IHC)

IHC stainings were performed on 4 μm FFPE tissue sections. Briefly, after deparaffinization in xylene and rehydration in graded ethanol, sections were washed in phosphate-buffered saline (PBS). After an antigen retrieval step, sections were incubated with the primary antibody (see **Table 2** for details). Reactions carried out on Ventana BenchMark automated slide stainer were performed according to manufacturer's instructions. The sections were then kept for 15 min at room temperature (RT) before further PBS washing and immunostained with a standard streptavidin-biotin-peroxidase procedure, followed by a 3,3-diaminobenzidine (DAB) color reaction and counterstaining with hematoxylin. Experimental conditions and list of primary antibodies clones are reported in **Table 2**. All the antibody conditions were validated on tissue microarrays (TMAs) containing different positive control tissues. For sequential IHC, a non-biotin Poly HRP conjugate system followed by aminoethyl carbazole (AEC) substrate reaction was used instead of DAB. For consecutive cycles of staining, a chromogen destaining step (in alcohol) and a stripping step (in citrate buffer) were applied according to a previously published protocol (19). Reproducibility of the staining along increasing cycles of staining/destaining was checked for each marker utilized in sequential staining (**Supplementary Figures 1**, **2**).

Image Acquisition

High-resolution whole slide images (40x and 20x magnifications) (WSI) of IHC stained slides were acquired using the Aperio CS2 slide scanner (Leica Biosystems Nussloch GmbH) or the MicroVisioneer Manual WSI system (MicroVisioneer, 20x magnification).

Software Assisted Quantification of Single IHC Stains

Digital pathology analysis was performed on WSIs with *QuPath*, an open source image analysis software (20). Quantification of IHC stains was supervised by an expert Pathologist (MG): tumor areas, non-tumoral stroma, and necrotic areas were separately annotated, and artifacts (e.g., tissue folding) deselected and excluded from the analysis. Quantification of marker positive cells was performed in tumor areas, excluding non-tumoral stroma and necrosis. In the analysis of the lymph node metastasis ($n = 5$) attention was paid to only count for tumor-infiltrating T lymphocytes, excluding those associated to the lymph node tissues. The density of positive cells (i.e., the number of positive cells per mm^2) was calculated for CD4, CD8, FoxP3 and Granzyme B; the percentage of positive cells on the total was instead calculated for PDL1. Detection of positive cells was performed using *QuPath's Simple Tissue Detection* and *Positive Cell Detection* methods. Briefly, bright-field images were analyzed using the setup parameter *optical density sum* to avoid nuclei detection loss in samples showing weak haematoxylin counterstain. Alternatively, the *Hematoxylin OD* setup parameter was preferentially used to avoid overestimating the total cell number (due to background artifacts). For nuclear markers detection (e.g., Foxp3) the AEC or DAB signal was assessed using the command *Nucleus DAB OD mean* or *max*, whereas *Cell DAB OD mean* or *Cytoplasm DAB OD mean* commands were applied for surface or cytoplasmatic markers detection, respectively. Otherwise, when active the *Optical density sum* parameter, the intensity of the cell and cytoplasmatic signal was assessed in the cell nuclei, since this parameter tends to include in the nucleus the AEC or DAB signal that comes from the membrane

TABLE 2 | Details of IHC antibodies.

Antibody	Clone	Isotype /host	Supplier cat#	Dilution/Ab reaction	Antigen retrieval	Ab diluent	Position in sequential IHC/AEC reaction time
CD45	2B11+ PD7/26	IgG1/Mouse monoclonal	Dako Cat#M0701	1:50/1 h RT	EDTA (Ph8) water bath 100°C, 40 min	PBS + 1%BSA + 0,02% sodium azide	2°/30 min
CD4	**EPR6855**	**IgG/rabbit monoclonal**	**Abcam Cat#ab133616**	**1:100/1 h RT**	**EDTA (Ph8) water bath 100°C, 40 min**	**PBS + 1%BSA + 0,02% sodium azide**	**4°/30 min**
	4B12	IgG1/mouse monoclonal	Dako Cat#M7310	1:100/1 h RT	TRIS EDTA (Ph9) water bath 98.5°C, 20 min	Ventana Antibody Diluent Cat#251-018	
CD8	**4B11**	**IgG2b/mouse monoclonal**	**Thermo scientific Cat#MA1-80231**	**1:40/1 h RT**	**EDTA (Ph8) water bath 100°C, 40 min**	**PBS+1%BSA+ 0,02% sodium azide**	**4°/30 min**
	4B11	IgG2b/mouse monoclonal	Novocastra Cat#NCL-L-CD84B11	1:100/1 h RT	TRIS EDTA (Ph9) water bath 98.5°C, 20 min	Ventana Antibody Diluent Cat#251-018	
Foxp3	**SP97**	**IgG/rabbit monoclonal**	**Thermo Scientific Cat#MA5-16365**	**1:100/1 h RT**	**EDTA (Ph8) water bath 100°C, 40 min**	**PBS + 1%BSA + 0,02% sodium azide**	**2°/30 min**
	236A/E7	IgG1/mouse monoclonal	Abcam Cat#ab20034	1:100/1 h RT	Citrate buffer (Ph6) water bath 98.5°C, 20 min	Ventana antibody diluent Cat#251-018	
Granzyme B	GrB-7	IgG2a/mouse monoclonal	Merk/Millipore Cat#MAB3070	**1:20/1 h RT**	**EDTA (Ph8) water bath 100°C, 40 min**	**Ventana Antibody Diluent Cat#251-018**	**1°/30 min**
				1:20/1 h RT	Citrate buffer (Ph6) water bath 98.5°C, 20 min	Ventana Antibody Diluent Cat#251-018	
PDL1	SP142	IgG/rabbit monoclonal	Spring Bioscience Cat#M4424	**1:100/1 h RT**	**EDTA (Ph8) water bath 100°C, 40 min**	**PBS + 1%BSA + 0,02% sodium azide**	**1°/30 min**
				1:40/1 h RT	Cell conditioning solution (CC1) ventana BenchMark Cat#950-124	Ventana antibody diluent Cat#251-018	

Antibodies and conditions applied in the optimized sequential IHC protocol are indicated in bold.

or cytoplasm. Parameters were set-up for each slide on at least three fields selected for optimal or suboptimal staining (e.g., high melanin content) to obtain the better parameters combination for total cells and positive cells enumeration. Then, the number of positive cells detected per area was used to calculate the average number of positive cells per mm^2, and these results exported along with mark-up images showing the detected cells for visual verification.

Processing of Multiplex/Sequential IHC Images

To perform co-localization analyses, we designed *Data Science for Health (DS4H) Image Alignment*, a user-friendly tool freely provided as an *ImageJ/Fiji* plugin. With *DS4H Image Alignment*, multiplex/sequential IHC images can be easily co-registered by defining with a few clicks some well-visible reference marks. The implemented least-squares method automatically approximates the solution of the mathematical over-determined system, so to define the registration matrix then used to align the different images (21, 22). It also considers rotations and scale changes in case the staining/destaining/stripping steps generated a tissue dilation/shrink (23). Finally, it provides an iterative subroutine for a fine alignment, to easily reach a very good image co-registration quality. Practically speaking, the sequential IHC images considered in this work have been: (a) imported into *Fiji*; (b) cropped to extract corresponding, significant Regions Of Interest (ROI); (c) aligned with *DS4H Image Alignment*; (d) separated into single channels using the *H AEC* option of the *ImageJ/Fiji Color Deconvolution* tool (24); then, (e) the AEC channels have been re-aligned into a *z*-stack for final comparisons. *DS4H Image Alignment* has been implemented in *Java* as a plugin for *ImageJ/Fiji*. It works with "*.svs*" files, but also all the medical imaging formats included in *Bio-Formats* (25, 26). *DS4H Image Alignment* version 1.0 is freely available at:

www.filippopiccinini.it/DS4H-IA.html, together with a sample dataset and a video tutorial.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (version 6, Jolla, CA, USA). A non-parametric two-tailed Wilcoxon signed-rank test was used to evaluate differences in the distribution of the number of cells positive for a given marker per square millimeter between pre- and post-vaccine biopsies. Correlations of PDL1 expression with the immune infiltrate were analyzed by Spearman's rank correlation coefficient. A p-value of <0.05 was considered as statistically significant.

RESULTS

Patients and Clinical Outcomes

Based on the availability of both pre- and post-vaccine tumor biopsies, 21 patients treated with DC vaccination at Morgagni Hospital (Forlì, FC, Italy) and IRCCS-IRST (Meldola, FC, Italy) between 2000 and December 2015 were initially selected: in five cases pre-treatment or post-treatment biopsies were not evaluable (mainly for insufficient tumor tissue left). Detailed information on patient characteristics and vaccine administration is provided in **Table 1**. All patients, except one (Pt#12), were immunoresponsive to the vaccine, as shown by positivity to DTH tests performed after at least four induction immunizations. Patients' median age at study entry was 51 years (range 31–73) and both genders were equally represented (nine males and seven females). In 11 out of the 16 cases indagated the sites of tumor biopsies taken before and after vaccination were of the same type, i.e., soft tissue/nodal or visceral, according to the classification provided in Bartlett et al. (27), thus avoiding any statistically significant imbalance in the level of CD8 expression in the selected pre-treatment sample cohorts. Best overall response (BOR) to the treatment per RECIST 1.1 criteria was complete response (CR) in one patient, partial response (PR) in two patients, stable disease (SD) in nine patients, while the remaining four patients showed progressive disease (PD) at the first radiological tumor assessment. Retrospective evaluation confirmed that Pt#12, Pt#13, Pt#14, and Pt#16 were all confirmed PD even when immune-related response criteria were applied. Median duration of response was 7.5 months (range 4–68 months). Median overall survival was 22 months (range 7–108 months). Six patients were given DC vaccine as a first line therapy, whereas the remaining had received at least one therapy line before (chemotherapy, radiotherapy, biochemotherapy or immunotherapy).

Increase of CD8$^+$ T Cell Characterizes Post-DC Vaccine Tumor Lesions of Naïve and Chemo/Radiotherapy Treated Melanoma Patients

To gain insight into the intratumoral T cell landscape of DC vaccinated patients, quantification of CD8 and CD4 positive cells was performed on matched pre and post FFPE tumor biopsies. Globally, the amount of intratumoral CD8$^+$ T cells increased in post-treatment tumor biopsies compared with pre-treatment ones, although this change did not reach statistical significance (mean $\pm$ SEM 597.9 ± 132.7 vs. 731.5 ± 159.0 CD8$^+$ cells/mm^2 in pre-treatment and post-treatment biopsies, respectively; $p = 0.2114$; **Figure 1A**). In six cases the content of intratumoral CD8$^+$ T cells decreased after treatment: interestingly, one of these patients (Pt#4) started DC vaccination after failure on Ipilimumab and showed a very high pre-treatment level of intratumoral CD8$^+$ T cells (1,068.927 cells/mm^2 vs. 185.006 cells/mm^2, in pre- vs. post-vaccine lesion, respectively; **Table 1**, **Supplementary Table 1**). Moreover, four out of the five remaining patients had previously received cytokines either in combination with chemotherapy (BiotCT) or as low doses IFNalpha and IL-2 (Biot), and showed higher levels of pre-treatment levels CD8$^+$ T cells as well (**Table 1**, **Supplementary Table 1**). Along this line, patients were stratified into two separate groups: 1) *naïve/CT/RT*, comprising patients who received the vaccine as a first line therapy ($n = 6$), after chemo- ($n = 2$), or radiotherapy ($n = 1$); 2) *immuno_treated*, accounting for all patients ($n = 7$) previously treated with immunomodulating cytokines or anti-CTLA4 (Ipilimumab, Ipi). Intriguingly, a selective significant increase of tumor-associated CD8$^+$ T cells in post- vs. pre-vaccine samples was observed in the *naïve/CT/RT group* (mean $\pm$ SEM 533.2 ± 201.7 vs. 878.6 ± 220.3 CD8$^+$ cells/mm^2 in pre-treatment and post-treatment biopsies, respectively; $p = 0.0195$, **Figure 1B**, **Supplementary Table 1**) compared to the *immuno_treated* one (mean $\pm$ SEM $681.0 \pm 1{,}649.7$ vs. 542.4 ± 224.9 CD8$^+$ cells/mm^2 in pre-treatment and post-treatment biopsies, respectively; $p = 0.2969$; **Figure 1B**, **Supplementary Table 1**). **Figure 1C** shows the *QuPath*-generated mark-up WSI of CD8 stain in the pre and post-vaccine lesions of Pt#16 displaying a remarkable increase in tumor-associated CD8$^+$ T cells (269,037 vs. 1,371,354 cells/mm^2, in pre- and post-vaccine, respectively). Of note, while lesions analyzed upon DC vaccination were mainly resected during treatment (on-treatment samples), as for all those of the *naïve/CT/RT group*, three out of seven samples belonging to the *immuno_treated group* (identified with square within the graphs) represent lesions sampled after the last vaccine dose (post-treatment samples). To reinforce our results and data interpretation we confirmed the absence of statistically significant difference in the post/pre CD8 ratio between these post-treatment samples ($n = 3$) and those harvested on-treatment ($n = 4$) (Mean $\pm$ SEM; 0.5147 ± 0.2318 and 0.8578 ± 0.2528, respectively).

No significant change in the number of intratumoral CD4$^+$ T cells upon DC vaccination was observed in any of the patient groups (**Figures 1D,E**), speaking in favor of a specific DC vaccine-mediated modulation of the CD8$^+$ Cytotoxic T Cell arm of the adaptive immune system.

CD8$^+$ T Cell Infiltration Is Paralleled by a Concurrent Increase of PDL1 Expression in Tumor Cells

Anti-tumor T cell activity requires antigenic presentation in the context of human leukocyte antigen (HLA) molecule and

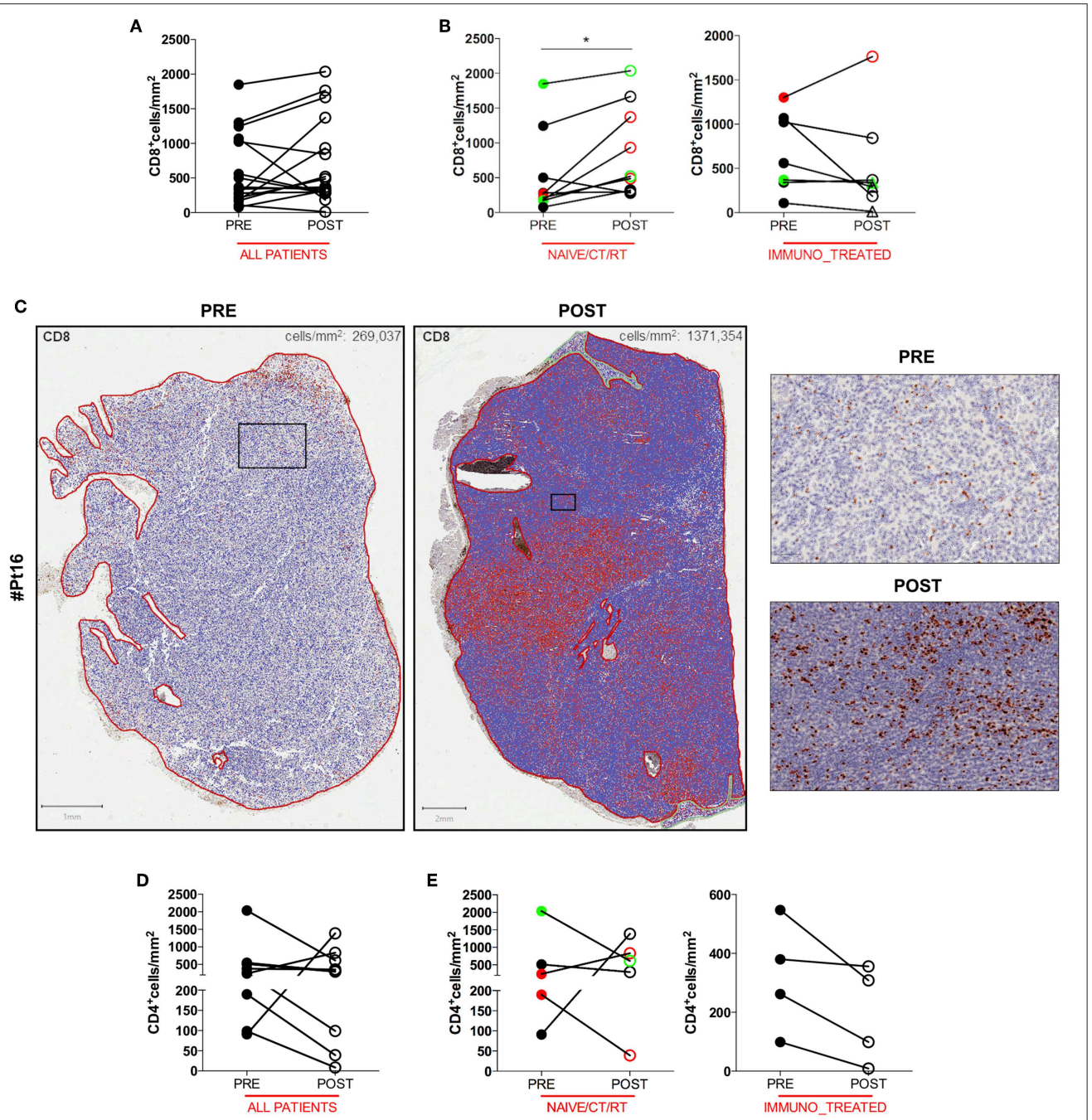

FIGURE 1 | Quantitative analysis of intratumoral CD8$^+$ and CD4$^+$ T cells of serial tumor biopsies from DC vaccine treated patients. The number of intratumoral CD8$^+$ T cells per mm^2 in matched pre- and post- treatment samples is plotted in graphs. Graph showing *all patients* ($n = 16$, $p = 0.2144$) **(A)**. Right and left graphs showing *naïve/CT/RT* ($n = 9$, $^*p = 0.0195$), and *immuno_treated* ($n = 7$, $p = 0.2969$), respectively **(B)**. Whole slide images (WSI) of CD8 staining in the pre- and post-vaccine lesions of one representative patient (Pt#16). Scale bars 1 and 2 mm for the left and the right WSI panel, respectively. Higher magnification images for the mark-up CD8 stain in pre- and post- treatment samples are shown. Scale bars, 100 μm **(C)**. The number of intratumoral CD4$^+$ T cells per mm^2 in matched pre- and post-treatment samples is plotted in graphs. Graph showing *all patients* ($n = 9$, $p = 0.4258$) **(D)**. Right and Left graphs showing *naïve/CT/RT* ($n = 5$, $p > 0.9999$) and *immune_treated* ($n = 4$, $p = 0.1250$), respectively **(E)**. CR and PR Patients are displayed in green, SD in black, and PD in red. Open circles denote on-treatment samples, open triangles post-treatment ones. Statistical comparisons are based on the non-parametric two-tailed Wilcoxon signed-rank test. Only values statistically significant are reported: $^*p < 0.05$.

loss or inability to up-regulate HLA class I expression is a common mechanism of tumor immune escape. Another crucial way tumor cells avoid immune-mediated killing is the up-regulation of the immune checkpoint molecule PDL1. Therefore, we assessed whether the increase in intratumoral CD8$^+$ T cells after DC vaccination was associated with relevant

changes in the expression of HLA class I by melanoma cells or in the pattern of expression of PDL1. No difference was found in HLA class I expression between pre and post-DC therapy lesions (**Figure 2A**), indicating that loss/downregulation of HLA class I molecules is unlikely involved in immune escape after DC vaccine in our series. Intriguingly, when PDL1 expression was evaluated, a significant increase in the number of $PDL1^+$ tumor cells was detected in post-vaccination tumor biopsies (11 out of 14 assessable paired biopsies, mean ± SEM 3.721 ± 1.316 vs. 10.37 ± 2.456 PDL1% in pre-treatment and post-treatment biopsies, respectively; $p = 0.0353$) (**Figure 2B**). A representative case is shown in **Figure 2C**. Of note, we found that this up-regulation was stronger in the *naïve/CT/RT cohort* ($n = 9$, mean ± SEM 3.056 ± 1.268 vs. 13.74 ± 3.242 PDL1% in pre-treatment and post-treatment biopsies, respectively; $p = 0.0078$), while it did not reach statistically significance in the *immune_treated one* ($n = 5$, mean ± SEM $4{,}920 \pm 3{,}068$ vs. $4{,}295 \pm 1{,}653$ PDL1% in pre-treatment and post-treatment biopsies, respectively; $p = 0.6250$), suggesting a positive correlation with the observed higher density of intratumoral $CD8^+$ T cell infiltrate (**Figure 2D**). Indeed, Spearman's correlation analysis showed a significant positive correlation between the density of $CD8^+$ T cells and the percentage of $PDL1^+$ cells (Spearman $r = 0.4948$, $p = 0.0074$; **Figure 2E**). Mark-up WSI overview of PDL1 stain for Pt#16 (**Supplementary Figure 3A**) shows the spatial distribution of PDL1 expression in post-therapy biopsy and clearly highlights that the enriched expression of PDL1 was topographically associated with the $CD8^+$ T cell infiltrated areas (**Figure 1B**). Sequential staining on the same tissue section of CD45 and PDL1 allowed discerning its relative expression on tumor cells and immune cells. Pseudo-fluorescence double images (**Supplementary Figure 3B**) confirmed that PDL1 was expressed almost exclusively in tumor cells and underscore the proximity of $CD45^+$ immune cells to that of $PDL1^+$ tumoral cells, stressing the inducible nature of PDL1 expression.

Intratumoral PDL1 Counteracts Cytotoxic Activation of Intratumoral $CD8^+$ T Cells

To further understand the activation extent of tumor-infiltrating $CD8^+$ T cells we examined paired pre and post DC vaccine tumor samples for the presence of Granzyme B (GZMB), a key functional marker of effector cytotoxic $CD8^+$ lymphocytes. Thirteen cases were assessable for the GZMB staining. Co-expression of GZMB and CD8 was assessed by sequential IHC, and confirmed that a considerable fraction of $CD8^+$ T cells were activated cytotoxic $CD8^+$ lymphocytes, rather than potentially activated $GZMB^+CD8^-$ natural killer cells. A representative image of the reconstructed double pseudo-fluorescence image is shown in **Figure 3A** (Pt#16). Moreover, the tight association of apoptotic or necrotic tumor cells with $CD8^+$ TILs together with the polarization of the cytotoxic granules toward melanoma cells (data not shown), strongly support the functional relevance of the defined phenotype. The GZMB:CD8 ratio was used to define the effective fraction of intratumoral cytotoxic T cells. Unexpectedly, no significant change in the amount of $GZMB^+$ cells was found between pre- and post-treatment tumor biopsies (mean ± SEM 118.3 ± 47.68 vs. 137.2 ± 51.98 $GZMB^+$ cells/mm^2 in pre-treatment and post-treatment biopsies, respectively; $p = 0.5830$; **Figure 3B**). In addition, the observed increase in the number of $CD8^+$ cells in the *naïve/CT/RT group* was not paralleled by a concurrent increase in GZMB-expressing cells, suggesting that cytotoxic activation of vaccine-induced intratumoral $CD8^+$ T cells may have been hampered by the up-regulation of PDL1 on tumor cells. Supporting this hypothesis, we found a significant inverse correlation (Spearman $r = -0.8667$, $p = 0.0045$) between the GZMB:CD8 ratio and the percentage of $PDL1^+$ cells (**Figure 3C**). An additional crucial mechanism involved in impairing the CD8 effector program is the presence in the TME of immune suppressive cells, like $Foxp3^+$ regulatory T cells (Tregs). In this respect, we evaluated the density of intratumoral $Foxp3^+$ cells in our series. Sequential IHC stainings (**Figure 3D**) showed that Foxp3 was expressed exclusively in the $CD4^+$ compartment, thus excluding any association with CD8 potentially accounting for early effector $CD8^+$ T lymphocytes (28) and confirming that in our samples $Foxp3^+$ cells were for the large majority Tregs. Due to the shortage of tumor material, four samples could not be assessed (**Supplementary Table 1**). In the remaining 12 patients we observed a trend (7 out of 12 cases) toward a decrease in the number of $Foxp3^+$ cells (mean ± SEM 163.7 ± 101.7 vs. 110.5 ± 44.56 $Foxp3^+$ cells/mm^2 in pre-treatment and post-treatment biopsies, respectively; $p = 0.7344$, **Figure 3E**). Similarly, no significant increase of this immunosuppressive population was observed in any of the analyzed groups (**Figure 3E**), indicating that this mechanism is unlikely involved in decreasing cytotoxic activation of $CD8^+$ effector cells.

DISCUSSION

It is widely recognized that DC vaccination as monotherapy has a limited clinical efficacy, particularly in heavily pretreated Patients. However, DC vaccines are very well-tolerated, and in the actual clinical scenario they could have a role in combination immunotherapy with immune checkpoint inhibitors. Nowadays, their interaction is still poorly understood and clinical trials are needed to identify the best sequential or combination regimen. In this respect, immune monitoring within the TME has been fundamental for the discovery of mechanisms of response and resistance to treatment, as well as instrumental for the design of combination regimens to enhance anti-tumor immunity and clinical responses. Accordingly, this study describes the qualitative and quantitative changes of immune cell subpopulations occurring in the TME in a cohort of metastatic melanoma patients treated with autologous tumor lysate loaded mature DCs for whom matched pre and post-therapy material was available from our Institutional repository. Likewise, Gross et al. attempted to score $CD3^+$ lymphocytic infiltration upon DC vaccination, but the analysis was conducted on pre-vaccination metastases in 17 patients and post-vaccination metastases in 17

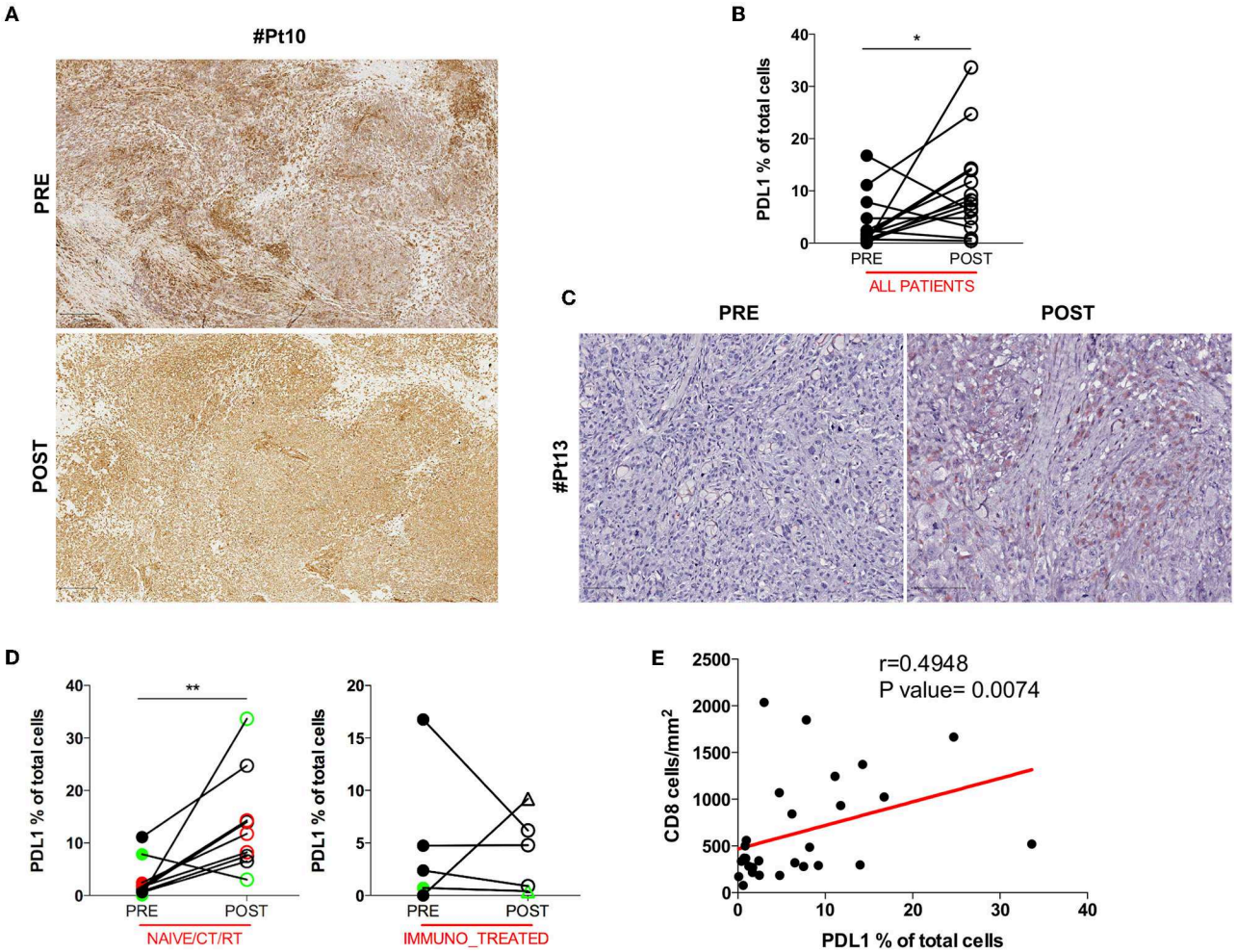

FIGURE 2 | HLA class I and PDL1 expression in serial tumor biopsies from DC vaccine treated patients. The pattern of HLA class I expression by melanoma cells is shown in matched pre- and post-therapy lesions for one representative patient (Pt#10). Scale bars, 250 μm **(A)**. Differences in intratumoral PDL1 expression are illustrated in the graph as the percentage of PDL1 expressing cells on the total cell number ($n = 14$, $^{*}p = 0.0353$) **(B)**. A representative example of the staining is shown (Pt#13). Scale bars, 100 μm **(C)**. PDL1 expression distribution in matched samples within the *naïve/CT/RT* cohort and the *immuno_treated* cohort is reported in right and left graphs ($n = 9$, $^{**}p = 0.0078$ and $n = 5$, $p = 0.6250$), respectively. CR and PR Patients are displayed in green, SD in black, and PD in red. Open circles denote on-treatment samples, open triangles post-treatment ones **(D)**. A positive correlation was found between CD8 and PDL1 expression (Spearman $r = 0.4948$, $p = 0.0074$) **(E)**. Statistical comparisons are based on the non-parametric two-tailed Wilcoxon signed-rank test. Only values statistically significant are reported: $^{*}p < 0.05$ and $^{**}p < 0.01$.

patients, with paired samples available in only seven patients (5). However, albeit limited by the number of samples, a higher lymphocytes score was recorded in post-vaccine tumor samples compared to pre-vaccine ones. Additionally, a higher immune infiltration following DC vaccination was also highlighted in glioblastoma multiforme, particularly including a $CD8^{+}$ T cell population (29). To the best of our knowledge, our study represents the largest retrospective analysis of a unique cohort of matched pre and post samples from metastatic melanoma patients treated with an autologous DC vaccine. Remarkably, our quantitative analysis was conducted on WSI, rather than selected areas, and using resected surgical specimens, which are more representative of the entire TME compared to core needle biopsies or frequently used tissue microarrays. Our data showed that DC vaccination increases the number of intratumoral $CD8^{+}$ T cells, although the differences observed between pre-treatment and post-treatment biopsies were not statistically significant in the whole series. However, when patients were analyzed according to the type of treatment they received before DC vaccination some differences emerged. In particular, patients who previously failed immunological treatments did not show significant changes in the density of intratumoral $CD8^{+}$ T cells, suggesting that the DC vaccine-driven effects on the TME might have been hampered by different mechanisms of immune escape that have led to the failure of previous immunotherapy. Indeed, it has been extensively reported that in the metastatic disease setting, one of the major obstacles to DC vaccine efficacy is represented by the occurrence of multiple mechanisms of tumor-induced immunosuppression (30). Accordingly, it can be assumed that,

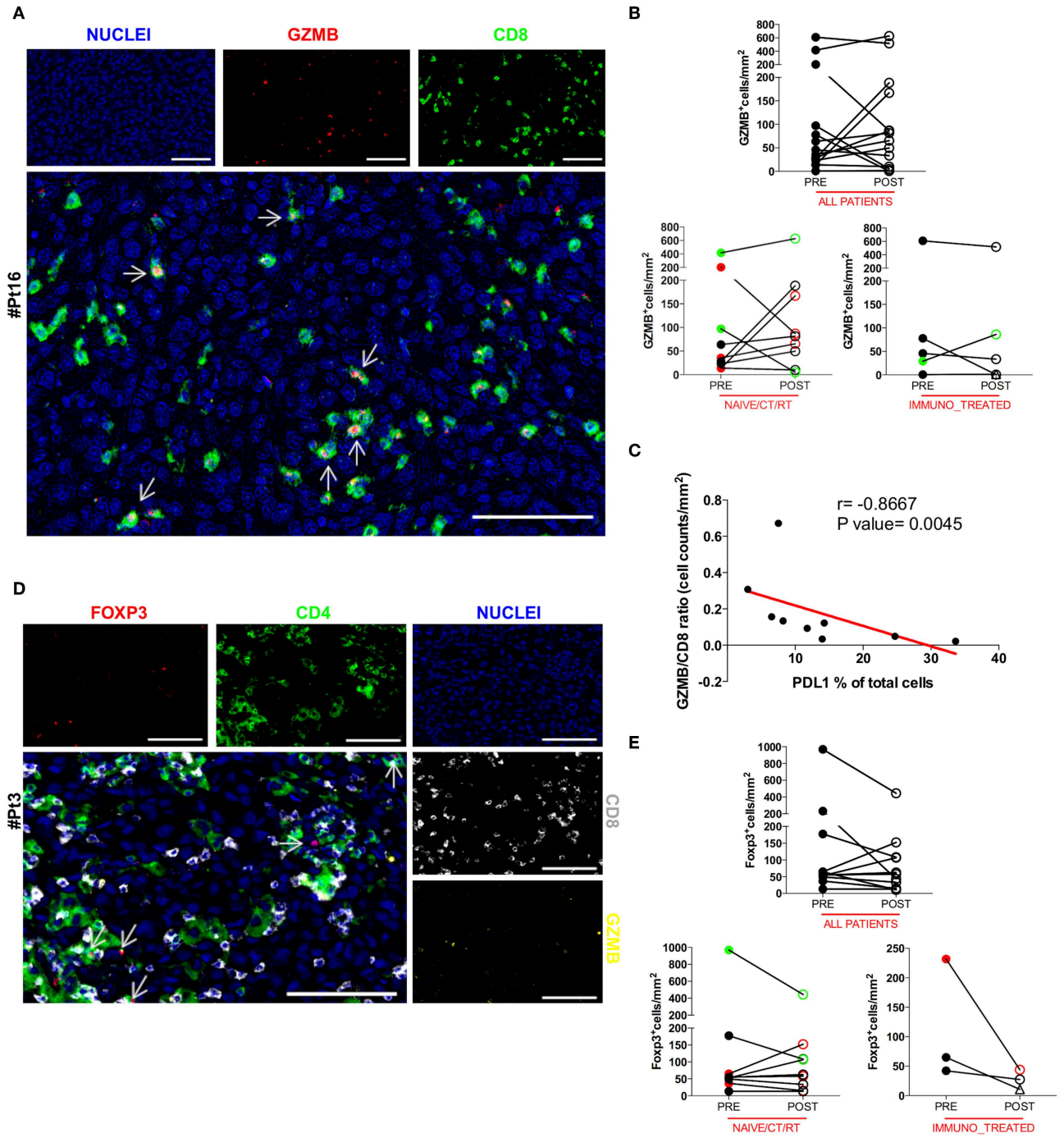

FIGURE 3 | Analysis of the functional phenotype of intratumoral $CD8^+$ and $CD4^+$ T cells. The co-expression of GZMB and CD8 was evaluated by sequential IHC. AEC color signals were extracted from each digitized single-marker image by color deconvolution, followed by pseudo-coloring. A representative image is shown (Pt#16). Nuclei (blue), GZMB (red), CD8 (green). Scale bars, 100 μm **(A)**. The GZMB expression between matched pre- and post-therapy samples, either in the total patient cohort or in any of the defined patient groups is illustrated in graphs (*all patients* $n = 14$ $p = 0.5830$, *naïve/CT/RT* $n = 9$ $p = 0.2500$, *immuno_treated* $n = 5$ $p = 0.4375$) **(B)**. Correlation between the GZMB ÷ CD8 ratio and the percentage of PDL1 over total cells in the *naïve/CT/RT* patient cohort (Spearman $r = -0.8667$, $^{**}p = 0.0045$) **(C)**. FFPE pre-therapy sections were analyzed by multiplex IHC. Results from a representative patient (Pt#3) are shown. Nuclei (blue), GZMB (yellow), CD8 (gray), CD4 (green), and Foxp3 (red). Scale bars, 100 μm **(D)**. Changes in the expression of Foxp3 marker in matched pre- and post-therapy lesions are shown in graphs for *all patients* ($n = 12$, $p = 0.2334$) as well as for the two defined patient sub-groups (*naïve/CT/RT* $n = 9$, $p = 0.7334$, *immuno_treated* $n = 3$, $p = 0.2500$) **(E)**. CR and PR Patients are displayed in green, SD in black, and PD in red. Open circles denote on-treatment samples, open triangles post-treatment ones. Statistical comparisons are based on the non-parametric two-tailed Wilcoxon signed-rank test. Only values statistically significant are reported.

upon failure to the previous immune-based treatments, patients belonging to the *immuno_treated group* could have developed strong immunosuppression, which negatively affects the ability of DC vaccination to increase intratumoral $CD8^+$ T cells, as instead observed in the *naïve/CT/RT group*. Besides not being forms of immunotherapy, we know that chemotherapy (CT) and radiotherapy (RT) might exert immune-modulating effects (31, 32). In light of this, while in all the naïve patients ($n = 6$) we observed a marked $CD8^+$ T cell up-regulation compared to baseline treatment (**Supplementary Table 1**), one CT-treated patient (Pt#7) did not actually display a $CD8^+$ T cell increase, thus behaving more similar to the *immune_treated group*. The second striking effect we observed after DC vaccination was the marked up-regulation of PDL1 expression. Similar to what previously described (33) and in accordance to its inducible profile, we found that the intratumoral PDL1 expression was a reflection of the endogenous $CD8^+$ T cell abundance, as shown by the positive correlation between the percentages of $PDL1^+$ cells and $CD8^+$ T cell density. Again, WSI distribution analyses showed a strictly related spatial distribution of $PDL1^+$ cells and $CD8^+$ TILs. Multiplex IHC for CD45 and PDL1 further confirmed that PDL1 was largely expressed in $CD45^-$ tumor cells in close proximity to $CD45^+$ immune cells. Our data are also consistent with the hypothesis that cytotoxic activation of $CD8^+$ T cells recruited in the TME after DC vaccination can be strongly inhibited by PDL1 concurrently induced in tumor cells, as suggested by the significant negative correlation between $PDL1^+$ cells and the GZMB:CD8 ratio. A comprehensive evaluation of the tolerability and clinical efficacy of our DC vaccination protocols has been already provided (4), and an association of the immune contexture with the clinical activity was out of the scope of the current study. Interestingly, in three out of the four truly progressing patients (Pt#13, Pt#14, and Pt#16), the strong rise of $CD8^+$ T cells in the tumor microenvironment was matched by an increased expression of the PDL1 inhibitory molecule, thus suggesting an immune escape–associated progression. Although the underlined PDL1 pattern partially explains the limited functionality of intratumoral T cells, we recognize that changes in the immune signature observed did not fully correlate with the clinical outcome of the vaccination. In addition, the lack of correlation between $Foxp3^+$ cell densities and cytotoxic activation of $CD8^+$ T cells further reinforces the role of PD1/PDL1 axis activation in suppressing DC vaccine-induced cytotoxic immune response. Additional studies, albeit hampered by the limited accessibility to this type of samples, will be needed to confirm our findings and potentially also shed light on other markers/immune populations. Indeed, we do not exclude that other phenomena, such as the variation in the number of intratumoral $Foxp3^+$ cells, be involved in the limited clinical efficacy of DC vaccination, and could have been detected if the analysis was conducted on a greater number of cases. DCs by nature are crucial for immunosurveillance and thus more likely for the *de novo* induction of anti-tumor immunity, although DC vaccination could hardly overcome profound tumor-induced immunosuppression. Accordingly, DC vaccination monotherapy is increasingly utilized in the adjuvant setting (34, 35). Conversely, it has been shown that pre-existing spontaneous immune response largely directed against neoantigens are frequently associated with a "hot" (i.e., T cell inflamed) TME, characterized by high levels of $CD8^+$ T cells together with immune-mediated adaptive PDL1 up-regulation. Of note, T cell inflamed tumors more likely respond to therapeutic PD-1 blockade (36). Interestingly, a recent phase 3 clinical trial with the combination of the anti-CTLA4 monoclonal antibody ipilimumab and the anti-PD1 nivolumab in metastatic melanoma showed an advantage, both in terms of PFS and OS, of the combination over nivolumab alone in patients with PDL1 negative tumors (37), but at the expenses of much higher toxicity. In this line, synergism might be observed between PD1/PDL1 blockade and treatments up-regulating PDL1 expression in the TME in patients carrying PDL1 negative melanomas. On these grounds, the very favorable toxicity profile, together with its ability to turn "cold" into "hot" tumors, define DC vaccination as a promising candidate for combination with inhibitors of the PD1/PDL1 immune checkpoint.

DATA AVAILABILITY STATEMENT

All raw data to the main and supplementary data figures are provided in an anonymous format as a FigShare Collection named "2019_BulgarelliEtAl_Patients_Figures", available at https://doi.org/10.6084/m9.figshare.c.4584041.v3.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico della Romagna (CEROM). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JB and MT conceived the study, performed the experiments, analyzed and interpreted the data, and wrote the manuscript. AG helped performing some of the experiments. LR provided support with the clinical data and with critical feedback. SM helped performing WSI quantification with *QuPath* software and sequential IHC. FP and AC developed the corner-based 2D image alignment tool, designed the image processing pipeline, and provided support in image analysis. FR and GG provided support with the clinical data. BV and BL provided the Tissue Microarray and technical feedbacks. MP and EP were responsible of the DC vaccine preparation. VA and GF helped collecting and sectioning the FFPE tumor samples. MF arranged the collection of patient samples. MG conceived the study, interpreted the data, and critically reviewed the manuscript. All 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.2019.02353/full#supplementary-material

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Conflict of Interest: The 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.

frontiers in Immunology

REVIEW
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Induction of Tolerance and Immunity by Dendritic Cells: Mechanisms and Clinical Applications

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Dendritic cells (DCs) are key regulators of immune responses that operate at the interface between innate and adaptive immunity, and defects in DC functions contribute to the pathogenesis of a variety of disorders. For instance, cancer evolves in the context of limited DC activity, and some autoimmune diseases are initiated by DC-dependent antigen presentation. Thus, correcting aberrant DC functions stands out as a promising therapeutic paradigm for a variety of diseases, as demonstrated by an abundant preclinical and clinical literature accumulating over the past two decades. However, the therapeutic potential of DC-targeting approaches remains to be fully exploited in the clinic. Here, we discuss the unique features of DCs that underlie the high therapeutic potential of DC-targeting strategies and critically analyze the obstacles that have prevented the full realization of this promising paradigm.

Keywords: dendritic cells, immunotherapy, cancer, autoimmune disorders, vaccine preparation

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INTRODUCTION

Immune responses result from a complex interplay between the innate and adaptive immune system. Dendritic cells (DCs) are an important subset of antigen-presenting cells (APCs) that specialize in priming different types of effector T cells and, thus, tailor the outcome of an immune response and having a central role in the immune system with a unique ability to control both immunity and tolerance. Compared to other APCs such as macrophages and B cells, DCs are considered the most efficient APCs capable of efficiently processing and presenting exogenous antigens on both MHCII and MHC I molecules to naïve $CD4^+$ and $CD8^+$ T cells, respectively, thus initiating the adaptive immune response. DCs were first discovered in 1973 by Ralph Steinman, who was awarded a Nobel Prize in 2011 for that discovery. DCs comprise a heterogeneous population of bone-marrow-derived cells that are seeded in all tissues. Five major types of DCs can be distinguished: plasmacytoid DC (pDCs), type 1 conventional DCs (cDC1), type 2 cDCs (cDC2) also referred to as myeloid DCs (mDCs), Langerhans cells and monocyte-derived DCs (MoDCs) (1–4), which differ in their phenotype, localization, and function as summarized in **Table 1** (2, 5–7). In peripheral tissues, DCs capture antigens using different mechanisms. DCs loaded with antigens subsequently migrate into the draining lymph nodes via afferent lymphatics, where peptides loaded on DCs histocompatibility complex (MHC) class I and II molecules to be recognized by T-cell receptor (TCR) on T lymphocytes (8). Immature DCs (iDCs) can present self-antigens to T cells to maintain immunological tolerance either through T cell deletion, induction of T cell anergy or the differentiation of regulatory $CD4^+CD25^+FoxP3^+$ T cells (Tregs) (9). After encountering appropriate stimuli, DCs differentiate into mature DCs, which are characterized by a decrease in

TABLE 1 | Human DC subsets.

DC subtype	Main surface markers	Main PRRs	Presence *in vivo*	Main functions	Specific mediators produced upon activation	T cell priming ability	The role in cancer immunotherapy	The role in autoimmune diseases
pDC	$CD11c^{-}$; $CD123^{+}$; $CD303^{+}$; $CD304^{+}$; $CCR2^{+}$; $CXCR3^{+}$; $HLA\text{-}DR^{low}$	STING; TLR7; TLR9; CLEC12A	Resident in lymphoid tissues; also present in tonsils	Antiviral immunity	Type I and III IFN secretion	Poor priming of naive T cells; present and cross-present peptides only after activation	Negatively correlate with prognosis in cancer	Implicated in progression of autoimmune diseases by increased IFNα production and decreased ability to prime Treg cells
cDC1	$CD11c^{low}$; $HLA\text{-}DR^{+}$; $DEC205^{+}$; $XCR1^{+}$	STING; TLR1; TRL3; TLR6; TRL8; TLR10; CLEC12A	Resident in lymphoid tissues and also present in blood, peripheral tissues, and lymph nodes	$CD8^{+}$ T cell and T_H1 priming Cross presentation	Not well-defined	Efficient processing and cross-presentation of exogenous antigens on MHC class I molecules to activate $CD8^{+}$ T cells and prime T_H1 response	*Cellular immunity against tumor cells and correlates with beneficial prognosis in cancer *Produce CXCL9 and CXCL10 in the TME to promote the recruitment of $CD8^{+}$ T cells into the TME	Implicated in progression of autoimmune diseases by increased production of pro-inflammatory cytokines and T cell activation
cDC2	$CD11c^{+}$; $HLA\text{-}DR^{+}$; $C11b^{+}$; $CD172a^{+}$	STING; TLR1-9; CLEC4A; CLEC6A; CLEC7A; CLEC10A; CLEC12A	Resident in lymphoid tissues and also present in blood, peripheral tissues, and lymph nodes	$CD4^{+}$ T cell priming; T_H17 activation; T_H1, T_H2 response induction; Tregs activation	IL-6 and IL-23	Present peptides on MHC class II molecules to $CD4^{+}$ T cells	Inducing $CD4^{+}$ T cell-mediated immunity in cancer	
Langerhans cells	Langerin; Epcam; $BDCA1^{+}$; $CD1a^{+}$; $CD11c^{High}$		Resident in epidermis	Tolerance and priming of immune response	Not well-defined	Not well-defined	Not well-defined	Not well-defined
MoDCs	$CD11c^{+}$; $CD11b^{+}$; $HLA\text{-}DR^{+}$; $CD1c^{+}$; $CD206^{+}$; $CD209^{+}$; $CD1a^{+}$; $CD172a^{+}$; $CCR2^{+}$		Differentiate from monocytes in peripheral tissues on inflammation	Inflammation	TNF and iNOS	Induce context dependent differentiation of $CD4^{+}$ T cells into T_H1, T_H2 or T_H17 cells	Mostly studied and used in *ex vivo* generated immunotherapy protocols	Mostly studied and used in *ex vivo* generated immunotherapy protocols

Overview of key characteristics of the predominant human dendritic cells (DC) subsets: pDCs, plasmacytoid DCs; cDC1s, conventional type I DCs; cDC2s, conventional type 2 DCs; MoDCs, Langerhans cells and monocyte-derived DCs. CCR, chemokine receptor; CXCR3, CXC-chemokine receptor 3; PRR, pattern recognition receptor; T_H1 cell, type 1 $CD4^{+}$ T helper cell; T_H2 cell, type 2 $CD4^{+}$ T helper cells; T_H17 cell, IL-17-producing $CD4^{+}$ T helper cell; TLR, Toll-like receptor; Treg cell, regulatory $CD4^{+}$ T cells. References are provided throughout the main text.

endocytic activity, upregulation of MHC class I and II molecules and costimulatory molecules and responsiveness to inflammatory chemokines (10). Mature, antigen-loaded DCs promote the differentiation and activation of T cells into effector T cells with unique functions and cytokines profiles by providing immunomodulatory signals through cell-cell contacts and cytokines (8, 11). As a result of the progress made by research studies worldwide, there is now evidence of a central role for DCs in initiating antigen-specific immunity and tolerance, which has been widely translated into different approaches for vaccine design in preclinical and clinical programs (12–15).

DCs SUBSETS

DCs comprise two major classes: plasmacytoid DCs (pDCs) and conventional or classical DCs (cDC) (**Table 1**) (11, 16). pDCs represent a small subset of DCs which accumulate mainly in the blood and lymphoid tissues and enter the lymph nodes through the blood circulation. For maturation, pDCs selectively express activating FcR as well as Toll-like receptor 7 and 9 (TLR7 and TLR9). On the contrary, they express low levels of MHC class II and costimulatory molecules in the steady state. Upon recognition of foreign nucleic acids, they start to produce type I interferon (1, 11). pDC-derived IFNα can also induce the activation of other DC subsets or B cells into plasma cells via cytokines and surface signaling (17). cDCs form a small subset of tissue hematopoietic cells present in most of lymphoid and non-lymphoid tissues, where they constantly acquire tissue and blood antigens. cDCs excel in priming naïve T cells due to their superior ability to migrate loaded with antigens to T cell one of lymph node and to process and present antigens. Moreover, cDC1 have a unique potential to induce cellular immunity against intracellular pathogens and malignant cells due to the processing and cross-presentation of exogenous antigens on MHC class I molecules to activate $CD8^+$ T cells and T_H1 cells. On contrary, cDC2 are known potent inducers of $CD4^+$ T cell response (1, 11). MoDCs mainly differentiate from monocytes in peripheral tissues during inflammation and induce context dependent differentiation of $CD4^+$ T cells into T helper 1 (T_H1), T helper 2 (T_H2) or T helper 17 (T_H17) cells (7).

DC ACTIVATION

DCs in the resting state are considered to be immature but primed to acquire pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) *in situ* through a variety of surface and intracellular receptors, namely (1) cell surface C-type lectins, (2) surface and intracellular TLRs, and (3) intracellular helicases that recognize nucleic acids, such as retinoic acid-inducible gene I (RIGI) (18) (**Table 1**). iDCs are potentially tolerogenic due to their capacity to facilitate the suppression of autoreactive T cells and the clonal expansion of Tregs, which might be addressed in the manufacturing of DC-based vaccines for autoimmune disease treatment (19) (**Figure 1**). DCs undergo a series of phenotypic and functional changes upon exposure to activation signals, leading to their maturation (10). This process is associated with the following events: (1) downregulated antigen-capture activity, (2) increased expression of surface MHC class II molecules and enhanced antigen processing and presentation, (3) increased levels of chemokine receptors, e.g., CCR7, which allows migration of the DC to lymphoid tissues; (4) increased expression of costimulatory molecules associated with the capacity to stimulate or suppress T cells through different signaling axes: CD80/CD86-CD28, CD40-CD40L, OX40L-OX40, ICOSL-ICOS and galectin (GAL)9-TIM3, CD80-CTLA4, PDL1-PD1, PDL2-PD1, respectively (**Figure 2**); and (5) enhanced secretion of cytokines and chemokines, leading to the development of an immune response T cell subtypes, e.g., $CD4^+$ T cells such as T_H1, T_H2 and Tregs (8, 20) (**Figure 1**).

INDUCTION OF T CELL TOLERANCE vs. ACTIVATION BY DCs

Different DCs subsets are specialized to capture and process antigens that are presented on MHC molecules and recognized by T cells, resulting in final clonal T cell selection leading to a wide T cell repertoire as summarized in **Table 1** (21). Among DC subsets, pDCs show relatively limited priming of naïve T cells, unless stimulated to induce $CD8^+$ T cells (22). Conversely, cDC1 provide efficient processing and cross-presentation of exogenous antigens on MHC I molecules to activate $CD8^+$ T cells and T_H1 cell responses as a response to tumor cells or intracellular pathogens (23, 24) and cDC2 are known to be inducers of $CD4^+$ T cell responses (25, 26). Importantly, MoDCs can be generated to promote context-dependent differentiation of $CD4^+$ T cells toward a T_H1, T_H2, or T_H17 phenotype (27). This variety of T cells represents an infinite tool for specific therapies that increase or decrease T-cell function. The efficient activation of naïve T cells requires the following: (1) binding of the TCR to the peptide-MHC complex on DCs, (2) the interaction of costimulatory molecules at the interface between DCs and T cells, and (3) additional signals from the local environment (28). The presence of these three signals is crucial for full T cell activation (**Figure 2**). Under inflammatory conditions, large numbers of mature DCs accumulate in T cell areas of the draining lymph nodes for a sustained period of time (29). Mature DCs presenting high levels of antigen/MHC complexes allow strong and sustained TCR occupancy, delivering T cells the main stimulatory signal (30). Simultaneously, high levels of costimulatory and adhesion molecules expressed on mature DCs are required for amplification of the signal initiated by the TCR and for increased adhesion between the DC and the T cell, thus increasing the strength and duration of the interaction, respectively (10). Subsequent strong activation of signaling pathways downstream of the TCR and the costimulatory receptors in the presence of cytokines or factors eliciting immunostimulation and the effector T cell phenotype results in full T cell activation, proliferation, and differentiation into effector and memory cells (**Figures 1**, **2**) (31).

In contrast, DCs that engulf the antigen in the absence of a local inflammatory signal remain in the immature, tolerogenic

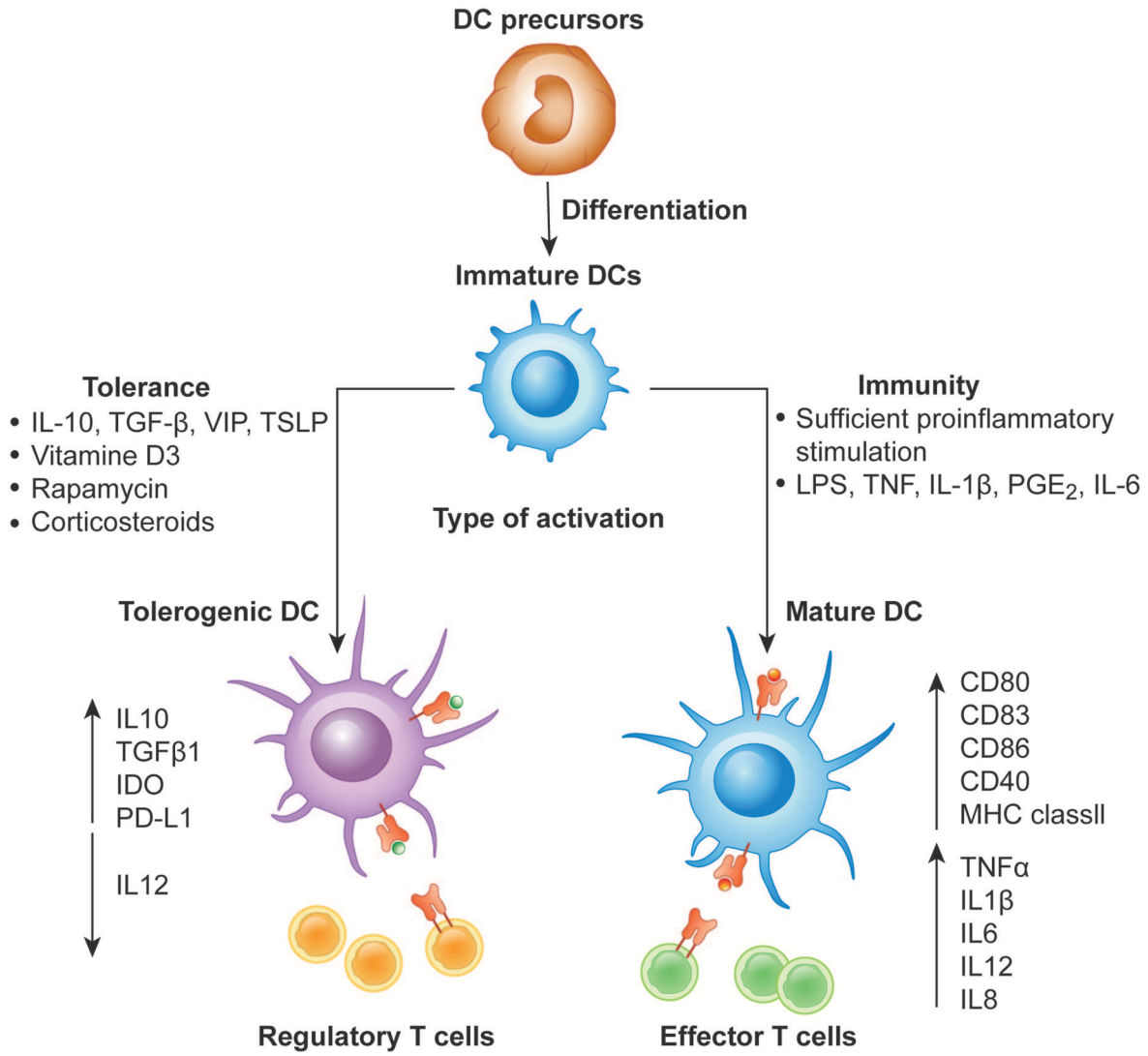

FIGURE 1 | Differentiation of monocyte-derived activated vs. tolerogenic dendritic cells. Dendritic cells (DC) differentiate from DC precursors into immature DCs (iDCs) in the presence of IL-4 and GM-CSF. In the presence of a maturation signal (proinflammatory cytokines and Toll-like receptor ligands), DCs become activated and transition to a stimulatory phenotype, which subsequently leads to the induction of effector/cytotoxic T cell responses. In contrast, incubation of iDCs with different mediators or genetic modification of DCs in the absence of maturation factors can lead to the generation of tolerogenic DCs, which induce anergy, apoptosis or activation of Tregs.

state with low expression of MHC molecules and costimulatory molecules, such as CD80 and CD86 (9, 32, 33). Presentation of antigen to T cells in the absence of sufficient CD80/CD86 stimulation of CD28 molecules on T cells leads to the activation of anergy-associated genes under the control of nuclear factor of activated T cells (NFAT) and induction of T cell anergy (34, 35) (**Figures 1**, **2**). Moreover, low or no signal through the CD28 receptor is a prerequisite for the induction of Treg differentiation (36). Thus, tolerogenic DCs (tolDCs), DCs with regulatory properties, play a pivotal role in immune tolerance (37).

The tolDC population consists of naïve iDCs or alternatively activated semimature DCs induced by apoptotic cells or regulatory cytokine milieu, such as IL-10 and transforming growth factor β (TGF-β) (20). Immunosuppressive DCs can also be generated under tumor microenvironment-derived factors, such as β-catenin, indoleamine 2,3-dioxygenase (IDO), endoplasmic reticulum (ER) stress, lactate, vascular endothelial growth factor (VEGF), IL-10, TGF-β, prostaglandins, accumulation of adenosine, increased levels of lactate and hypoxia (38–42). TolDCs contribute significantly to the induction and maintenance of immune tolerance through various mechanisms. They promote effector T cell anergy and elimination of autoreactive T cells, participate in the generation and maintenance of a population of naturally occurring Tregs, allow the generation of IL-10-producing T_H1 and T_H3 regulatory cells, and allow the conversion of differentiated T_H1 cells into

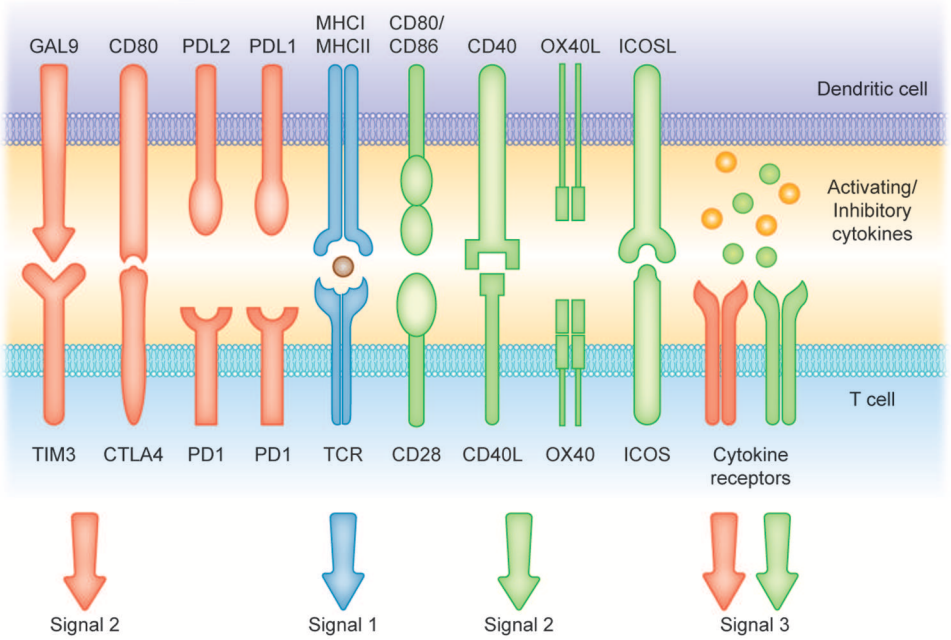

FIGURE 2 | Induction of T cell-mediated immunity or tolerance by DCs. Signal (1) Antigen presentation. Dendritic cells (DCs) can present antigens on MHC I and MHC II molecules to mediate T cell activity. Signals (2) and (3) Costimulatory molecules [belonging to the B7 and tumor necrosis factor (TNF) protein families] and soluble cytokines can provide positive signaling (green arrows and receptors) to prime T cell response. Conversely, CTLA4, cytotoxic T lymphocyte antigen 4; PD1, programmed cell death protein 1; PD-L1, programmed cell death 1 ligand 1 and TIM-3, T cell immunoglobulin and mucin-domain containing-3 and soluble factors such as IL-10 can represent suppressors of T cell activation (red arrows and receptors).

T_H2 cells (43, 44). These processes are mainly due to the high production of the regulatory cytokine IL-10, which promotes the generation of Tregs and T_H2 cells and inhibits DC maturation in a paracrine manner (45). Furthermore, regulatory DCs express various immunomodulatory molecules and immunosuppressive molecules that inhibit proinflammatory immune responses and induce immune tolerance. Indeed, the expression of PD-L, ICOS-L, thrombospondin, prostaglandins, and adenosine was documented to participate in the induction of T cell anergy. A number of mechanisms contribute to the clonal deletion of T cells including the interaction between FasL on DCs and Fas molecules on T cells, the expression of GAL-3 that binds to TIM3 on T cells or the production of IDO that leads to subsequent tryptophan depletion. TolDCs were also reported to induce Tregs or B regulatory cells (Bregs) by the expression of PD-L molecules, Ig-like inhibitory receptors IL-T3 and IL-T4, human leukocyte antigen G (HLA-G), anti-inflammatory cytokines IL-10, TGF-β, IL-27 and IL-35, retinoic acid, heme-oxygenase and IDO (9, 46). Finally, the functionality of tolDCs is connected with their metabolic activity, such as lipid accumulation, enhanced oxidative phosphorylation, fatty acid oxidation, and modulation of glycolysis (39, 47).

THE ROLE OF DCs IN CANCER

The immune system plays a critical role in the control of tumorigenesis based on experimental and clinical observations in both mice and humans, as formulated by the cancer immunosurveillance and immunoediting hypothesis (48). The plasticity of malignant cells resulting from their genetic instability may eventually give rise to new phenotypes with reduced immunogenicity and various mechanisms for the evasion of tumor cells from immunosurveillance, leading to malignant proliferation (49). Malignant cells escape immunosurveillance by different mechanisms, some of which are: (1) reduced immune recognition (including loss of tumor antigen expression and MHC class I and costimulatory molecule expression), (2) increased resistance to apoptosis (through STAT3 signaling), or (3) development of an immunosuppressive tumor microenvironment (including the production of cytokines, e.g., VEGF, TGF-β, IL-10 and increased expression of immunoregulatory molecules, e.g., PD-1/PD-L1, TIM-3, LAG-3), which lead to the development of malignant diseases (48, 50). Different DC subsets can be found in the majority of human tumors and play a crucial role in cancer immunosurveillance, as tumor-infiltrating DCs can migrate to regional lymph nodes to present tumor antigens to naïve tumor-specific T cells (51). However, naïve antigen-specific $CD8^+$ T cells cannot directly eliminate malignant cells. Thus, to become effector cytotoxic T cells, they need to be activated by professional APCs. Cross-presentation is an essential mechanism that allows DCs to present exogenous antigens on MHC I molecules to $CD8^+$ T cells, which become the main mediators of anti-tumor immunity (52). Importantly, the contribution of the different DCs subtypes to cross-presentation a cross-priming (in induction of effector $CD8^+$ T cells *in vivo*) varies depending

on the experimental setting. cDC1 are mainly associated with superior cross-presentation of tumor antigens to $CD8^+$ T cells and polarization of $CD4^+$ T cells into T_H1 phenotype resulting in induction of anti-tumor immunity (53–55). cDC2 and MoDCs may also cross-present tumor antigens and cDC2 are known to be essential for priming of anti-tumor $CD4^+$ T cell response (56). Moreover, the effector activity of T cells depends on DC-derived cytokines, including IL-12 and type-I IFN. Both cDC1s and cDC2s produce IL-12 following TLR stimulation. Tumor infiltrating cDC1s are also the main producers of different chemokines, including CXCL9 and CXCL10, which help to promote the recruitment of $CD8^+$ T cells into the tumor microenvironment (TME) (57). Therefore, elevated levels of tumor-infiltrating DCs inversely correlate with tumor grade and stage and have a robust prognostic value in multiple cancers, including non-small-cell lung carcinoma (NSCLC), melanoma, renal cell carcinoma, breast cancer, ovarian, and colorectal carcinoma (58–63).

However, the tumor microenvironment employs various mechanisms that lead to the functional impairment of DCs (7). First, in the TME, iDCs differentiate from hematopoietic progenitors following an encounter with an antigen/danger signal (64). However, the differentiation of DCs in the TME is often mediated by the interplay between IL-6 and macrophage colony-stimulating factor (M-CSF), resulting in the recruitment and accumulation of functionally deficient and frequently iDCs unable to induce the proliferation of tumor-specific $CD4^+$ and $CD8^+$ T cells (65, 66). Second, DCs in their function as APCs are sampling tumor antigens through the capture of dying tumor cells and initiating the anti-tumor immune response. Dying tumor cells provide three different signals to DCs and other phagocytes: "find-me," "eat-me "and "do not eat me" (67). A number of find-me signals have been characterized that act in a context-dependent manner, including lipid lysophosphatidylcholine (LPC), sphingosine 1-phosphate (S1P), CX3CL1 and the nucleotides adenosine triphosphate (ATP) and uridine triphosphate (UTP) (67). Immunogenic phagocytosis is mediated by eat-me signals, namely, ecto-calreticulin (CALR), surface-heat shock protein (HSP) 90, and phosphatidylserine (68, 69). The "do not eat me" signals serve as negative regulators of phagocytosis, mainly including CD47 and lactoferrin (70). Therefore, homeostatic clearance of dying cancer cells could be accelerated or impaired by the different molecules provided by tumor cells, which results in enhanced or impaired phagocytosis of malignant cells (71). Third, the functional capacity of DCs in the TME is negatively impacted through different mechanisms, including the activation of STAT3 signaling in DCs via different cytokines frequently expressed in tumors (IL-6, VEGF and IL-10) (72). Moreover, tumors may condition local DCs to form suppressive T cells, such as Tregs, IL-13-producing $CD4^+$ T cells and natural killer T cells (NKT cells), leading to a tumor-induced functional deficiency of DCs that results in decreased expression of costimulatory molecules, decreased production of IL-12, suppressed endocytic activity, inhibited antigen-processing machinery, and poor viability (73–77). Altogether, these and other findings suggest that malignant cells can exploit DCs to evade immunity. However, the majority of clinical protocols harnessing patient DCs do not consider the fact that DCs once administered back to patients might quickly lose their activity.

THE ROLE OF DCs IN AUTOIMMUNITY

Previous studies have described the link between peptide presentation by HLA class II molecules expressed on APCs and autoimmune diseases. In different autoimmune diseases, DCs are bearing certain autoimmune risk-conferring HLA class II molecules with the distinct hotspots in the peptide-binding groove that favor the presentation of particular self-antigens that will ultimately be recognized by self-reactive TCR. In the case of type 1 diabetes (DM1), the presence of specific amino acid in the binding groove of HLA-DQ8 alleles favors the binding of insulin-derived peptides. Similarly, in the case of rheumatoid arthritis (RA), HLA-DR4 molecules bearing the conserved amino acid motif (shared epitope) favor the presentation of citrullinated self-peptides leading to activation of citrulline-specific $CD4^+$ T cells and subsequent production of anti-citrulline antibodies that foster RA but prevent natural ligands bearing arginine instead of citrulline (78).

Aberrant cDC and pDC phenotypes and functions due to underlying genetic defects or a chronic inflammatory environment were shown to be associated with the development of various autoimmune diseases, such as RA, systemic lupus erythematosus (SLE), multiple sclerosis (MS) or DM1 (45, 79–81). DCs can either induce or suppress the autoreactive T cell response, and their effect depends on the DC subset, the degree of maturity, signals obtained from the local microenvironment and crosstalk with other immune and stroma cells. Under non-inflammatory conditions, lymphoid-resident immature cDCs or specialized types of tolDCs bearing self-antigens suboptimally activate naïve $CD4^+$ and $CD8^+$ T cells, thus maintaining immune tolerance and affecting the regulation of autoimmune diseases. Aberrant intrinsic tolDC function, such as impaired IL-10 secretion, defective ability to remove apoptotic cells, defective antigen processing machinery or absent negative regulators of inflammation, can contribute to DC hyperactivation and trigger autoimmunity (82–85). DC hyperactivation might also result from environmental triggers such as an inflammatory cytokine milieu induced by bacteria (86, 87), excessive IFN production in response to viral infection as observed in DM1 (88), oxidative stress induced by noxious agents as observed in RA (89) or danger signals released under cell stress or from necrotic and late apoptotic cells as documented in SLE and DM1 (90, 91). Activated cDCs accumulate in lymphoid and non-lymphoid tissues during autoimmune disease progression. Hyperactivated cDCs present self-antigens, prime naïve autoreactive $CD4^+$ T cells including follicular helper T cells, promote cross-priming of $CD8^+$ T cells and orchestrate the maturation of B cells leading to the subsequent expansion of autoantibodies and immune complex formation (81). Furthermore, mature cDCs generate an inflammatory environment by producing high levels of pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, and IL-23 that induce a deleterious imbalance between T_H1, T_H2, and T_H17

cells and contribute to local inflammation and tissue destruction. Although partially regulated, the autoimmune response persists due to ongoing stimulation of autoreactive T cell clones and B cell clones. pDCs play a central role in the pathogenesis of IFN-driven autoimmune diseases such as SLE and psoriasis. In SLE, pDCs are activated by immune complexes formed by the aggregation of autoantibodies, stress proteins, such as high mobility group box 1 (HMGB1), and self-DNA released from apoptotic cells that have not been cleared or by nucleic acid-containing nets released from activated neutrophils. These complexes are delivered to endolysosomes to activate TLR7 or intracellular DNA sensors, such as cGAS-STING, to further activate pDCs and IFN-α secretion (92–94). On the other hand, pDCs can also reduce autoimmune responses by secreting IDO and inducing Tregs depending on the disease stage and signals from local tissues (95, 96).

DC-BASED CANCER IMMUNOTHERAPY

Immunotherapy strategies harnessing DCs have been developed based on their unique capacity to coordinate innate and adaptive immune responses (10). The main aim of DC-based cancer vaccination is to induce tumor-specific cellular and humoral immunity resulting in the reduction of tumor mass and induction of immunological memory, which will control cancer relapse. Therefore, a critical step in cancer vaccine preparation is to provide mature DCs with specific tumor antigens. This can be achieved by the following: (1) culturing *ex vivo* DCs derived from patients with tumor antigens and activation stimuli and subsequently transferring the activated DCs back into patients or (2) inducing tumor antigen uptake by DCs directly *in vivo* (7, 97) The first proof-of-principle studies exploring DC immunotherapy were performed in the early 1990s based on the discovery that DCs can be obtained from $CD14^+$ monocytes or $CD34^+$ progenitors from leukapheresis products by culturing the cells *in vitro* in the presence of IL-4 and GM-CSF for 5–6 days (98). The first clinical study of a DC anti-cancer vaccine in B-cell lymphoma patients was reported by Hsu and colleagues in Nature Medicine in 1996 (99). Since then, ∼200 clinical studies have been performed of single treatments using mostly monocyte-derived DCs and measuring the immune response, which have been comprehensively reviewed elsewhere (12, 13, 97, 100). These studies concluded that DC-based vaccines are safe and potent for inducing the expansion of circulating tumor-specific $CD4^+$ and $CD8^+$ T cells (101–103). Although an anti-tumor immune response is frequently observed, objective clinical responses remain low, with a classic objective tumor response rate rarely exceeding 15%, as currently concluded in the meta-analysis provided by Anguille and colleagues (13, 14, 21). Although considerable progress has been made over the years, most of the studies have, unfortunately, been performed in late-stage patients with strong immunosuppression mechanisms already in place (104–106). To date, limited phase II and III trials (**Table 2**) have been performed with DC-based immunotherapy and, therefore, more clinical studies evaluating early stage patients or patients with preneoplasia are strongly needed.

EX-VIVO DC-BASED VACCINES

Different *ex vivo* DC-based immunotherapy clinical trials have recently been concluded with encouraging clinical outcomes (100). Completed clinical studies have analyzed the following: (1) different protocols for DC preparation, (2) different DC activation stimuli, (3) different forms of antigen preparations from short peptides to complex whole-tumor-cell hybrids, and (4) different types of DC vaccine applications. First, the FDA-approved cell-based therapy for the treatment of hormone-refractory prostate cancer Provenge (Sipuleucel-T) is a vaccine consisting of autologous peripheral blood mononuclear cells (PBMCs) obtained by leukapheresis, including DCs activated with a fusion protein of a prostate antigen (prostatic acid phosphatase; PAP) and GM-CSF. Treatment with Sipuleucel-T resulted in a 4.1-month-prolonged median survival compared with placebo (25.8 vs. 21.7 months). The impact of this first FDA-approved cancer vaccine has been significant, however this product is not readily available for different reasons, including logistic and financial problems (107). More phase II and III clinical trials using autologous MoDCs obtained from patient-derived $CD14^+$ blood monocytes or from the $CD34^+$ progenitors are shown to be effective against different cancer types and are summarized in **Table 2**. Phase III clinical trials using Mo-DC-based cancer vaccination are ongoing in metastatic colorectal cancer (NCT02503150, autologous tumor lysate), castration-resistant prostate cancer, which is combined with first-line chemotherapy (NCT02111577; VIABLE, MoDC vaccine loaded with antigens from an allogeneic apoptotic tumor cell line) and melanoma (NCT01983748, autologous tumor RNA antigen). In addition to colorectal, prostate cancer and melanoma cancer, DCs are intensively studied in glioma and renal and ovarian carcinoma (**Table 2**) (108, 109).

IN VIVO DC TARGETING

Another approach to recruit natural DCs for cancer immunotherapy is to target DC subsets *in vivo* via specific receptors, e.g., DEC205, CLEC9A, and langerin to target cDC1s; CLEC4A4 to target cDC2; CLEC7A (dectin 1) to target cDC2 and MoDCs; CD209 (DC-SIGN), mannose receptor and macrophage galactose-type lectin to target macrophages, using antibodies to deliver antigens and activating agents (110–112). Compared to *ex vivo* DC generation protocols, *in vivo* targeting allows vaccines to be produced on a larger scale and, most importantly, allows direct activation of natural DC subsets in the patient's body. Importantly, in the absence of adjuvants, targeting antigens to DCs might induce tolerance rather than anti-tumor immunity, which would have substantial value in the context of autoimmunity. Currently, numerous *in vitro* and *in vivo* studies in humans are focused on DC-targeting vaccine development. In a phase I trial, a DC-based vaccine consisting of a fully human anti-DEC205 monoclonal antibody fused to the tumor antigen NY-ESO-1 and accompanied by a topical or subcutaneous application of TLR agonists (resiquimod) showed the efficient generation of NY-ESO-1-specific cellular and humoral responses and led to partial clinical responses without toxicity (113).

TABLE 2 | Phase II and III clinical trials currently testing the therapeutic efficacy of dendritic cell-based anticancer immunotherapy.

Cancer type	Trial phase	Type of vaccine	Status	Intervention	ClinicalTrial.gov identifier
Breast cancer	II	Autologous DC-CIK combinations	Active, not yet recruiting	CIKs, Capecitabine	NCT02491697
CRC	III	Autologous DCs loaded with tumor cell lysate	Active, not yet recruiting	DCs+FOLFOX6 (Oxaliplatin, 5-Fluorouracil)	NCT02503150
Follicular lymphoma	II	Autologous DCs	Active, recruiting	Intranodal DCs+pembrolizumab	NCT02677155
GBM	II	Autologous DCs loaded with tumor cell lysate	Active, not yet recruiting	Tetanus Diphteria toxoid, Basiliximab	NCT02366728
	II	Autologous DCs loaded with tumor cell lysate	Active, not yet recruiting	Nivolumab	NCT03014804
	II	Autologous DCs	Active, recruiting	Tetanus Diphteria toxoid, GM-CSF	NCT02465268
Melanoma	II	Autologous DCs loaded with tumor cell lysate	Active, recruiting	DCs+IL-2	NCT02718391
	II	Autologous DCs loaded with tumor cell lysate	Active, recruiting		NCT02301611
	II	Autologous DCs loaded with TAAs	Active, not yet recruiting	Hiltonol	NCT02334735
	III	Autologous DCs loaded with TAAs	Active, recruiting		NCT02993315
	III	Autologous DCs + irradiated autologous tumor cells	Terminated		NCT01875653
Multiple myeloma	II	Dendritomas	Active, not yet recruiting	Autologous stem cell transplant with Melphalan, lenalidomide	NCT02728102
NSCLC	II	Autologous DCs + HHP-treated tumor cells	Completed	DCs+carboplatin, paclitaxel	NCT02470468
Ovarian cancer	II	Autologous DCs + HHP-treated tumor cells	Active, recruiting	DCs+carboplatin, paclitaxel	NCT02107937
	II	Autologous DCs + HHP-treated tumor cells	Completed	DCs+carboplatin, paclitaxel	NCT02107950
Prostate	II	Autologous DCs loaded with TAAs	Active, recruiting		NCT02362451
	II	Autologous DCs loaded with TAAs	active, not yet recruiting		NCT02692976
	II	Autologous DCs + HHP-treated tumor cells	Completed	DCs+docetaxel	NCT02105675
	II	Autologous DCs + HHP-treated tumor cells	Completed	DCs+standart of care hormone therapy (Leuprolid, Goserelin)	NCT02107391
	II	Autologous DCs + HHP-treated tumor cells	Completed		NCT02107404
	II	Autologous DCs + HHP-treated tumor cells	Completed	DCs+standart radiotherapy	NCT02107430
	III	Autologous DCs + HHP-treated tumor cells	Active, recruiting	DCs+docetaxel, taxotere	NCT02111577
RCC	II	Allogeneic DCs (Intuvax)	Active, not yet recruiting	DCs+Sunitinib	NCT02432846
	II	Autologous DCs loaded with tumor cell lysate	Active, not yet recruiting	DCs+CIKs	NCT02487550
	III	Autologous DCs	Terminated	DCs+Sunitinib	NCT01582672
Uveal melanoma	III	Autologous DCs + autologous tumor RNA	Active, recruiting	DCs+adjuvant	NCT01983748

CIK, cytokine-induced killer; CRC, colorectal carcinoma; DC, dendritic cell; GMB, glioblastoma multiforme; GM-CSF, granulocyte macrophage colony-stimulating factor; HHP, high hydrostatic pressure; IL-2, interleukin 2; NSCLC, non-small cell lung carcinoma; RCC, renal carcinoma; TAA, tumor associated antigen.

Nevertheless, the correlation with clinical responses remains unclear, and larger studies will be needed to evaluate the efficacy of this therapy. Clinical trials of anti-DEC205-NY-ESO-1 are currently ongoing in acute myeloid leukemia (NCT01834248), ovarian cancer (NCT02166905) and melanoma (NCT02129075). The advantage of such an approach is that maturation stimuli

activate only DCs targeted by the antibodies, thereby preventing any toxicity or undesirable systemic activation (13).

A different approach of targeting DCs *in vivo*, called GVAX, involved engineering irradiated gene-transfected tumor cells to secrete GM-CSF to stimulate the recruitment and activation of APCs (114). One phase II trial testing an allogeneic pancreatic cell line that secretes GM-CSF in combination with/without recombinant live attenuated *L. monocytogenes* engineered to secrete mesothelin (CRS-207) and low dose cyclophosphamide resulted in the recruitment of T cells into the TME and improved overall survival in patients with advanced pancreatic cancer (115, 116). However, a phase IIB study failed to show improved overall survival in patients treated with the combination or CRS-207 alone compared with the survival of patients on chemotherapy. Importantly, two different phase III clinical trials to evaluate the therapeutic efficacy of GVAX in prostate cancer patients were conducted. The VITAL-1 trial comparing GVAX to docetaxel plus prednisone in castration-resistant prostate cancer was terminated after showing low efficacy by interim analysis. VITAL-2 comparing GVAX in combination with docetaxel vs. docetaxel in combination with prednisone was also terminated based on interim results showing an increased risk of death in the GVAX arm compared to the control group (117). In this line, promising results showing that FMS-like tyrosine kinase 3 ligand (FLT3L) administration enhanced anti-tumor immunity and limited the tumor cell growth in mouse models (118), are currently followed by clinical trials (NCT01811992, NCT01976585, NCT02129075, and NCT02839265).

DC-BASED THERAPY OF AUTOIMMUNE DISEASES

The current treatment of most autoimmune diseases involves lifelong administration of systemic immunosuppression drugs coupled to anti-inflammatory therapies and hormone replacement. In addition, systemic immunosuppression is inevitably associated with undesirable side effects. Thus, the main goal of autoimmune disease treatment would be the long-term reinduction of self-tolerance. With respect to autoimmune disorders, cell therapy based on autologous tolDCs generated from peripheral blood monocytes following *ex vivo* generation in GM-CSF and IL-4 cell culture medium might be beneficial over standard immunosuppressive treatment in terms of its complex effect on the immune system and the possibility to restore long-term antigen-specific tolerance while avoiding generalized immunosuppression.

In order to achieve the best *in vivo* tolDC efficacy, all the parameters of tolDC therapy, namely, optimal dose, administration route, and frequency of tolDC administration, have to be properly defined as we believe they could dictate what kinds of immune responses are activated to modulate autoreactive T-cells and induce immune tolerance. To date, the best route of tolDC administration is still not known and several challenges remain to allow tolDCs to migrate into draining lymph nodes for T cell encounter or to reach the site of inflammation. In most clinical trials, tolDCs have been administered subcutaneously or intradermally proximal to the inflammatory site to increase tolDC migration to draining lymph nodes where autoreactive T cells predominate and to reach the site of inflammation (119). At the same time, intranodal application and direct administration into the intestinal lesions of tolDCs has also been tested in phase I clinical trials in patients with MS and Crohn's disease, respectively (120). In MS, however, tolDC shuttle across the blood brain barrier seems to be required for the efficient treatment of MS. Recent data suggest that the introduction of de *novo* CCR5 expression using mRNA electroporation into tolDCs might facilitate migration od tolDCs into the inflamed central nervous system and improve the treatment outcome in MS (121). Moreover, the ability of tolDCs to modulate T cell responses might be influenced by the current clinical status of the patient. Indeed, we documented in our studies that hyperglycemia reduces the ability of tolDCs to induce stable Tregs from naive T lymphocytes that can suppress antigen-specific T-cell anergy (122, 123). In that case, metabolic control, we believe, might be relevant for refining the inclusion criteria for clinical trials involving patients with DM1 and the maintenance of a tight metabolic control seem to be beneficial in patients considered for tolDC therapy.

Similar to DC-based cancer vaccines, a number of *in vivo* studies have documented that tolDCs require pulsing with relevant antigens to reach efficient clinical responsiveness following tolDC therapy (124). However, in some instances, antigen loading tolDCs leads to a worse condition and a higher incidence of autoimmune disease (125, 126). In contrast, different *in vivo* studies have suggested that the presence of autoantigen is not necessary for tolDC preparation as tolDCs may upload relevant autoantigens once injected *in vivo* and induce antigen-specific tolerance (127). Moreover, autoimmune diseases are not commonly defined by one universal autoantigen. Suitable disease-specific autoantigens such as insulin and glutamic acid decarboxylase 65 (GAD65) or transgenic myelin oligodendrocyte glycoprotein (MOG) or myelin basic protein have been defined in DM1 and MS, respectively (128, 129). However, in some autoimmune disorders, the specific autoantigen remains unidentified despite significant effort. In addition, not all patients display a uniform autoantigen pattern as antigen spreading, posttranslational modification, and development of neoantigens usually occur during the progression of the disease and complicate the search for the target antigens of the autoimmune response (128). A possible strategy seems to be to use a surrogate "universal" antigen, e.g., HSPs that are ubiquitously expressed in different types of inflammatory tissues (130).

EX-VIVO DC-BASED VACCINES

The ex vivo generation of stable, maturation-resistant tolDCs followed by their adoptive transfer represents novel immunotherapy for the antigen-specific treatment of autoimmune disorders. TolDCs can be established from monocytes from a patient's blood cultured using various pharmacological agents Vitamin D (VitD) and its analogs, dexamethasone, rapamycin, salicylates, and NF-κB inhibitors,

a cocktail of immunomodulatory cytokines (IL-10, TGF-β), growth factors (GM-CSF, M-CSF), and pathogen products and with the use of apoptotic cells or genetic engineering (131). All of these approaches generally suppress the maturation or activation of DCs and reduce the ability of DCs to produce IL-12p70 through different mechanisms (131). Additional activation of tolDCs by lipopolysaccharide (LPS) or its non-toxic analog monophosphoryl lipid A (MPLA) has been shown to improve the antigen-presenting capacity and migratory ability of tolDCs (132). Common features of tolDCs include low antigen presentation capacity combined with the loss or reduction of costimulatory signals, expression of inhibitory molecules, and an anti-inflammatory cytokine profile. Generated tolDCs can be loaded with one or more antigens to confer specificity. To do so, suitable disease-associated antigens such as preproinsulin peptides or GAD65 for DM1, basic myelin proteins for MS or thyreoglobulin for autoimmune thyroiditis are necessary. Once injected *in vivo*, tolDCs are expected to induce antigen-specific tolerance through various mechanisms, such as induction of autoreactive T cell anergy, induction of apoptosis, and induction of various types of Tregs and Bregs (133).

The first clinical study on tolDC therapy was conducted in 2011 in adult patients suffering from autoimmune DM1. TolDC therapy was safe, and some patients exhibited increased blood levels of $B220^{+}CD11c^{+}$ B cells together with evidence for C-peptide reactivation posttreatment (134). To date, further phase I/II clinical studies have been completed or are currently in progress in DM1, RA, MS, and Crohn's disease (**Table 3**) (135). A Rheumavax study on tolDCs from RA patients established by NF-κB inhibitor and pulsed with citrullinated peptides documented decreased numbers of effector T cells, decreased levels of proinflammatory cytokines and chemokines and reduced DAS 28 score (**Table 3**) (136). Another study tested the safety, feasibility, and acceptability of dex-VitD3-treated tolDCs pulsed with autologous synovial fluid as a source of autoantigens (AutoDecRa study) or tolDCs generated in the presence of TNF-α and relevant disease peptides (CreaVax study) in patients with RA. Both studies indicated tolDC therapy to be safe and showed signs of clinical improvement (137). Intraperitoneal administration of Dex/VitD-treated tolDCs in Crohn's disease revealed clinical improvement in some patients associated with an increase in Tregs and reduction in IFN-γ levels (138). Recently, Zubizarreta and colleagues reported the safety, feasibility, and signs of efficacy of tolDC therapy in patients suffering from MS and neuromyelitis optica. Indeed, i.v. administration of peptide-loaded tolDCs led to a significant increase in the production of IL-10 in PBMCs stimulated with the peptides as well as an increase in the frequency of regulatory IL-10-producing Tregs (139, 140). Additionally, follow-up studies testing the safety of VitD3 or dexamethasone-treated tolDCs loaded with relevant disease peptides are currently recruiting patients with MS (135).

IN VIVO DC TARGETING

Ex vivo-generated tolDCs have certain disadvantages, such as laborious, patient-specific, tailored-made preparation and high cost. To overcome these limitations, new approaches are being conducted to establish tolDCs *in vivo. One* possibility is the selective antigen-specific targeting of the DC-restricted endocytic receptor DEC205 with monoclonal antibodies in the absence of maturation stimuli to promote immunological tolerance (141). Another approach exploits coadministration of free autoantigens or autoantigens encapsulated with nanoparticles, microparticles, or liposomes bearing tolerogenic factors that are delivered specifically to DCs (142) or infusion of early-stage apoptotic cells that possess immunomodulatory properties and should prevent autoimmunity or even treat ongoing inflammatory processes (143). Another strategy is based on the non-inflammatory natural process of clearance of red blood cells by splenic APCs. Indeed, transfusion of engineered erythrocytes with covalently attached autoantigenic peptides was documented to induce antigen-specific immune tolerance via the uptake and processing of apoptotic cellular carriers for tolerogenic presentation by host splenic APCs in DM1 and SLE (144).

DCVAC, AN IMMUNOTHERAPY APPROACH HARNESSING DCs TO TREAT BOTH CANCER AND AUTOIMMUNITY

DCVAC, an investigational immunotherapy treatment based on a new active cellular immunotherapy platform, aims to treat cancer or autoimmune diseases by inducing or suppressing patients DCs, respectively. The unique capacity of DCs to induce both immune activation and tolerance under distinct circumstances is widely used for the preparation of several immunotherapy products currently tested in multiple phase I clinical trials in patients with autoimmune diseases and phase II and III clinical trials in cancer patients. The most advanced immunotherapy treatment in the oncology field is designed for prostate (DCVAC/PCa), ovarian (DCVAC/OvCa) and lung (DCVAC/LuCa) cancer patients. Based on theoretical assumptions and experimental data, cancer immunotherapy has the greatest potential when applied at the early stages of the disease or to patients following a radical surgical intervention after a removal of a large amount of tumor tissue (145). In contrast, in advanced stages of the disease, cancer immunotherapy might have a limited impact on malignant cell eradication due to the establishment of tumor-induced immunosuppression (68, 146). Moreover, preclinical and clinical testing supports the fact that the goal of immunotherapy in the late disease stages is not necessarily complete eradication of the tumor but rather the establishment of an equilibrium state between the host immune system and malignant cells (147). Therefore, it is beneficial to combine immunotherapy with other treatment options, for instance, chemotherapy or radiotherapy (145, 148). The concept of combined chemo-immunotherapy explores the fact that cytostatic treatment might not only eradicate the tumor mass but also neutralize tumor-induced immunosuppression, thus facilitating the effect of the concurrent immunotherapy, as discussed previously in detail elsewhere (146, 149–151). Therefore, numerous phase II clinical trials are ongoing to evaluate the potential of DCVAC in patients at various stages of disease (**Table 2**). The DCVAC technology in cancer therapy has been focused on a number of principles. First,

TABLE 3 | Clinical trials currently testing the therapeutic efficacy of dendritic cell-based immunotherapy in autoimmune disorders.

Disorder	Trial phase	Vaccine generation	Antigen	Status	ClinicalTrial.gov identifier
DM1	I	Antisense oligonucleotides against CD40, CD80 and CD86		Completed	NCT00445913
	I	VitD3	Proinsuline peptide	COMPLETED	NTR5542
Rheumatoid arthritis	I	NF-κB inhibitor, Bay 11-7082	Citrullinated peptides of vimentin, collagen type II and fibrinogen α and β chain	Completed	NCT00396812
	I	Dex, $VitD_3$, MPLA activated	Autologous synovial fluid	Completed	NCT01352858
Crohn's disease	I	Dex, VitA, activated with IL-1β, IL-6, TNF-α, PGE2		Completed	NA
Multiple sclerosis	I	Dex	Myelin peptides	Completed	NCT02283671
	I	VitD3	Myelin peptides	Active/recruiting	NCT02618902

Dex, dexamethason; DM1, diabetes mellitus 1; MPLA, lipopolysaccharide analog monophosphoryl lipid A;NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PGE2-prostaglandin E2; TNF-α, tumor necrosis factor alpha; Vit, vitamin.

DCVAC technology using high hydrostatic pressure (HHP)-treated allogenic tumor cell lines is used to activate patients' DCs by a broad range of tumor antigens to induce a complex anti-tumor immune response. The major advantages of this method are that (A) multiple epitopes can be presented on MHC molecules of different haplotypes, thus having the potential to induce both $CD4^+$ and $CD8^+$ T cell responses to a wide spectrum of antigens and (B) for the time it takes for antigen processing results in prolonged antigen presentation. Second, the concept of combination therapy is also being investigated in patients with advanced cancer in combination with multiple treatment modalities, including chemotherapy and hormone therapy, to produce synergistic effects and to improve the clinical outcome. Third, long-term activation of the immune response is achieved. DCVAC is applied in several doses over a prolonged period, which leads to enhanced stimulation of the anti-tumor immune response in the patient. DCVAC/PCa, DCVAC/OvCa, and DCVAC/LuCa immunotherapy is manufactured from monocytes harvested from patient leukapheresis (**Figure 3**). Monocytes are differentiated *ex vivo* into iDCs in the presence of IL-4 and GM-CSF for 6 days (152–154). iDCs are subsequently loaded with tumor cell lines of the appropriate origin based on overlap with the expression profiles of tumor-associated antigens (**Figure 3**) (155, 156). A particular way to enhance the immunogenicity of tumor cells used in the protocol is to induce immunogenic cell death (ICD) and increase the exposure/release of DAMPs to enhance DC maturation. HHP is a potent inducer of ICD, as documented both *in vitro* and *in vivo* (157–162). Moreover, HHP treatment of tumor cells can be easily standardized and performed in good manufacturing practices (GMP) conditions to allow its incorporation into the manufacturing protocol. The patient's own DCs engulf the dying tumor cells and, once activated using TLR3 ligand polyI:C, present tumor antigens on their surface (152). The resulting product is frozen, stored in liquid nitrogen and shipped to the treatment site. The first dose is administered to the patient ∼4 weeks after leukapheresis. A single leukapheresis yields up to 15 doses of DCVAC, which is sufficient to treat the patient for more than 1 year. After being thawed and diluted, DCVAC is administered subcutaneously at various treatment intervals, depending on the trial design. After administration, mature DCs migrate to the draining lymph nodes and activate a tumor-specific immune response (163, 164). Similar to boosting the immune system in cancer patients, DCVAC technology might be exploited to regulate unwanted autoimmune processes and induce long-term antigen-specific tolerance in patients suffering from autoimmune disease, such as DM1. DCVAC aimed at the immunotherapy of patients with DM1 consists of tolDCs generated *in vitro* from peripheral monocytes isolated from patient leukapheresis (**Figure 3**). First, iDCs are generated from monocytes in the presence of GM-CSF and IL-4, similar to DCVAC for cancer patients. Then, in contrast to DCVAC, tolerogenic factors (dexamethasone and VitD2) are introduced to the culture at the indicated days to induce the tolerogenic phenotype of DCs. As antigen loading might decrease the disease-protective effect of tolDCs in animal models of DM1, diabetogenic antigens are not introduced into the manufacturing protocol (125, 165). Finally, tolDCs are activated with the MPLA to improve tolerogenic properties as reported previously (132). Ultimately, tolDCs maintain a semimature phenotype and exhibit tolerogenic properties even under strong inflammatory conditions (166). Overall, DCVAC active cellular immunotherapy represents a personalized treatment of prostate, ovarian, and lung cancers and potentially also autoimmune diseases. The aim of the ongoing phase I to phase III clinical trials is to evaluate the efficacy and confirm the safety of this approach in order to offer new treatments for cancer malignancies and autoimmune disorders.

CONCLUSIONS

DC vaccination has proven to be safe and feasible in multiple clinical trials, as shown over the past two decades. Vaccination strategies involving DCs have been designed with regard to the unique capacity of these cells to coordinate innate and adaptive immune responses. The main aim of DC therapy is therefore to induce tumor-specific effector T cells that can reduce the tumor growth and induce immunological memory to control tumor relapse in cancer patients. In contrast, the main aim of

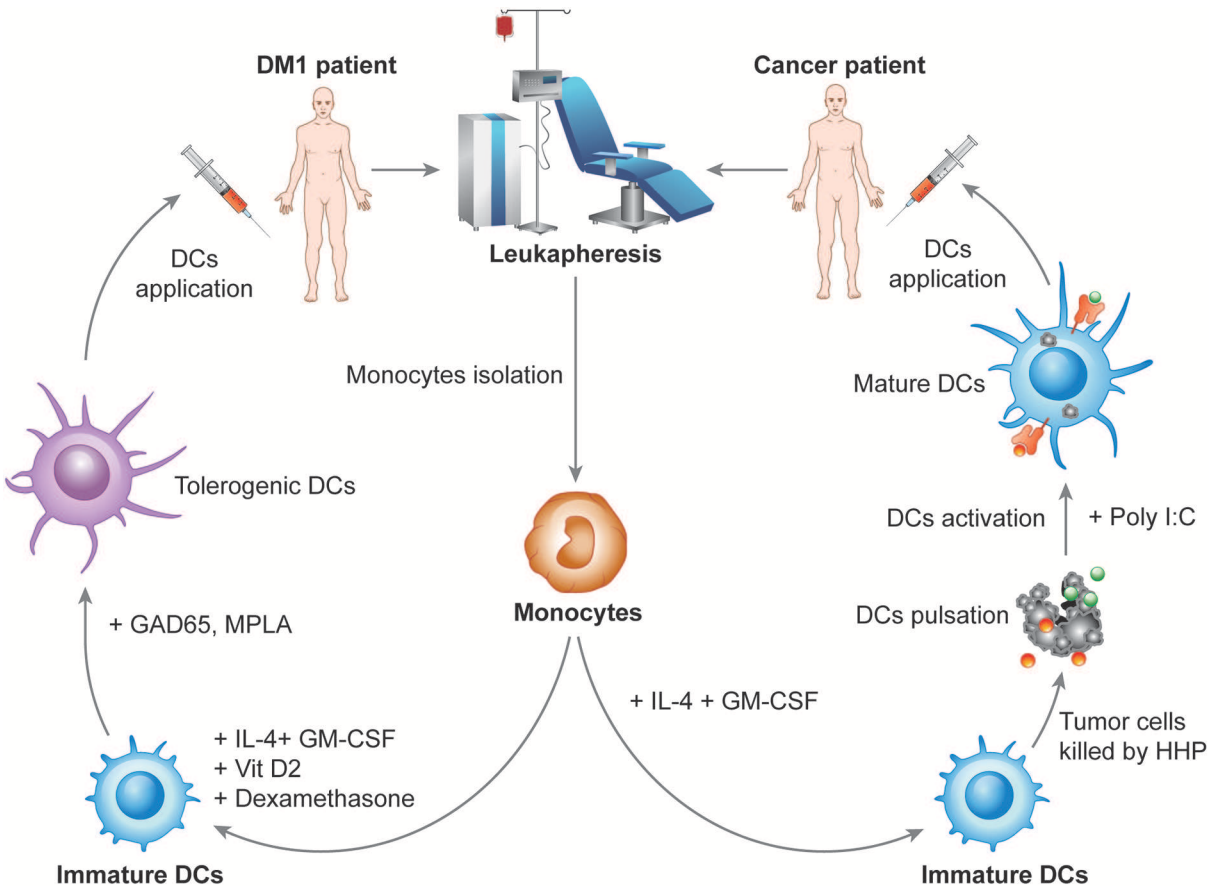

FIGURE 3 | Design of the manufacturing of DCVAC immunotherapy for cancer and autoimmune disorders. In the manufacturing of DCVAC, monocytes are harvested from patient blood by leukapheresis. For cancer patients, monocytes are differentiated into immature DCs (iDCs) in the presence of IL-4 and GM-CSF cytokines for 6 days in a GMP facility. iDCs are subsequently incubated with tumor cell lines treated with high hydrostatic pressure to induce immunogenic cell death of the tumor cells. Finally, DCs are activated using TLR3 ligand polyI:C. Aliquots of DCVAC DC-based immunotherapy are frozen in liquid nitrogen and shipped to the treatment sites. After being thawed and diluted, DCVAC is administered subcutaneously at various treatment intervals depending on the trial design. Similarly, for DM1 patients, monocytes are differentiated into iDCs in the presence of IL-4 and GM-CSF. In contrast, tolerogenic factors (dexamethasone and VitD2) are introduced to the culture at the indicated days to induce the tolerogenic phenotype of DCs. Tolerogenic DCs are finally activated with the lipopolysaccharide analog monophosphoryl lipid A (MPLA), aiming to improve the tolerogenic properties of the DCs.

DC therapy in autoimmune disorders is to expand and induce T cells, usually Tregs, that suppress immunity. Significant advances have been achieved in the last 20 years, and DC vaccines are continuously being optimized. The contemporary view on the potential role of DCs in cancer and autoimmune therapy has expanded remarkably, moving from *ex vivo* generated DC-based vaccines to a broad array of therapeutic options. However, we still need to learn more about potential combination therapy which could promote the efficacy of established cancer therapies and the identification of reliable biomarkers that can predict the propensity of cancer patients to benefit from DC-based immunotherapy.

AUTHOR CONTRIBUTIONS

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

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Photodynamic Modulation of Type 1 Interferon Pathway on Melanoma Cells Promotes Dendritic Cell Activation

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The immune response against cancer generated by type-I-interferons (IFN-1) has recently been described. Exogenous and endogenous IFN-α/β have an important role in immune surveillance and control of tumor development. In addition, IFN-1s have recently emerged as novel DAMPs for the consecutive events connecting innate and adaptive immunity, and they also have been postulated as an essential requirement for induction of immunogenic cell death (ICD). In this context, photodynamic therapy (PDT) has been previously linked to the ICD. PDT consists in the administration of a photosensitizer (PS) and its activation by irradiation of the affected area with visible light producing excitation of the PS. This leads to the local generation of harmful reactive oxygen species (ROS) with limited or no systemic defects. In the current work, Me-ALA inducing PpIX (endogenous PS) was administrated to B16-OVA melanoma cells. PpIX preferentially localized in the endoplasmic reticulum (ER). Subsequent PpIX activation with visible light significantly induced oxidative ER-stress mediated-apoptotic cell death. Under these conditions, the present study was the first to report the *in vitro* upregulation of IFN-1 expression in response to photodynamic treatment in melanoma. This *IFN-α/β* transcripts upregulation was concurrent with IRF-3 phosphorylation at levels that efficiently activated STAT1 and increased ligand receptor (cGAS) and ISG (CXCL10, MX1, ISG15) expression. The IFN-1 pathway has been identified as a critical molecular pathway for the antitumor host immune response, more specifically for the dendritic cells (DCs) functions. In this sense, PDT-treated melanoma cells induced IFN-1-dependent phenotypic maturation of monocyte-derived dendritic cells (DCs) by enhancing co-stimulatory signals (CD80, MHC-II) and tumor-directed chemotaxis. Collectively, our findings showed a new effect of PDT-treated cancer cells by modulating the IFN-1 pathway and its impact on the activation of DCs, emphasizing the potential relevance of PDT in adoptive immunotherapy protocols.

Keywords: photodynamic therapy, IFN-1, dendritic cells, melanoma, immunotherapy

INTRODUCTION

Cutaneous melanoma is the neoplasm originated from the melanocytes of the epidermis, and, although it corresponds to only 4% of skin related cancers, it is the causal agent for 80% of deaths from dermatological cancer (1). Unlike other tumor types, melanoma incidence and its mortality rate increased each year, an event associated with excesses in sun exposure and the progressive loss of the ozone layer (2). When surgical excision is performed on tumors with early diagnosis, the average survival rate at 10 years is 80%. However, in the case of metastatic melanoma, survival decreases to <10% (3). Therefore, the major challenge focuses on designing new therapies to treat melanoma in advanced stages with systemic dispersion. In this sense, immunotherapy emerges as a promising therapeutic option that involves therapeutic strategies with the common aim of enhancing the strengthens of the patient's immune system to advance upon tumors (4, 5). These systemic treatments for melanoma, approved or in experimental phase, include the administration of cytokines and other non-specific immunostimulatory molecules (IL-2, IFN-α2), active immunization (vaccination) with tumor cells, dendritic cells (DCs) or other molecules (recombinant antigens), adoptive transfer of T lymphocytes and monoclonal antibodies against immune checkpoint inhibitors (anti-CTLA-4, anti-PD1, anti-PDL1) (6).

Type I interferons are pleiotropic polypeptides classified according to the activity, structure and type of receptor to which they are bound in IFN-α, β, κ, ω, τ, and ε. Among them, IFN-α and IFN-β are the best characterized in terms of the stimulation of innate and adaptive immunity induced through autocrine and paracrine binding to the common IFNAR1/2 receptor. Previous reports indicated that type I IFNs (IFN-1) have an essential role in both basal and therapeutic-induced immune responses to cancer (7).

Clinical studies showed that high-dose IFN-α2 treatment was favorable for prolonging patient survival, therefore the exogenous administration of this was authorized as an adjuvant treatment for melanoma in 1996 (8). Unfortunately, high-dose treatment is also linked with adverse effects that can be reduced with lower doses, but they do not offer the same therapeutic good outcome (9). On the other hand, IFN-β treatment demonstrated limited efficacy and high toxicity for the treatment of metastatic melanoma (10, 11).

Studies about treatment of melanoma with recombinant type I IFN are ongoing and aim to develop more efficient methods of administration, design optimal treatment regimens, and identify the patient populations that are most likely to benefit. Nonetheless, given their antitumor immune-promoting activity, a variety of stimuli that induce the endogenous expression of IFN-1 are currently evaluated as promising adjuvants in vaccines. In fact, contrasting with the traditional adjuvants like aluminum compounds, which mainly promote humoral immune responses, IFN-α/β is a very effective tool to enhance cell-mediated immunity (12). Therefore, complementary efforts have focused on developing and identifying novel stimuli capable of promoting the IFN-1 pathway.

In this context, the molecular mechanisms subjacent the promotion of an immunogenic modality of cell death, that is, immunogenic cell death (ICD) have been elucidated. ICD includes spatiotemporally coordinated changes in the cell surface and the secretion of soluble mediators. Such signals are recognized by innate receptors expressed by dendritic cells to stimulate the antigenic presentation to T cells. These exposure/released danger signals, called damage-associated molecular patterns (DAMPs), include, but are not limited to, several innate immune stimulators, such as surface-exposed "eat me" signals (e.g., calreticulin, CRT), "find me" signals (e.g., ATP) and other factors (e.g., HMGB1) (13–15). Recently, IFN-1 signaling has been postulated as an essential requirement for ICD (16).

In the last decade, several investigations have analyzed the ability of conventional antitumor to promote ICD, in order to optimize their clinical use and to rationalize their application instead of more immunosuppressive drugs (17). In this context, photodynamic therapy (PDT) has been previously linked to the ICD. PDT is a well-known two-stage procedure. First, non-toxic photosensitizer drug (PS) is administrated and accumulates in tumor sites. After administration of the photosensitizer agent (PS), tumor loci are irradiated with a PS-exciting light of specific wavelength. None of these are independently toxic, but together produce a photochemical reaction, turning molecular oxygen into reactive oxygen species (ROS), which act directly on tumor cells or indirectly by damaging tumor-associated vasculature (18–22).

PDT has been associated with some of the main DAMPs involved in immunogenic cell death (23), such as CRT (24, 25), ATP (26), and HMGB1 (24). However, the relevance of PDT-mediated tumor cell death and its relationship with the IFN-1 pathway remain to be determined.

In the current study, we demonstrated that photodynamic treatment of melanoma cells *in vitro* resulted in IFN-α/β upregulation. Correspondingly, DCs co-cultured with PDT-treated tumor cells showed a potent IFN-1-dependent phenotypic and functional maturation. Taken together, these results delineate a novel photomodulated mechanism with potential application to prepare vaccines using *ex vivo* stimulated DC cultures with photosensitized tumor cells, which ultimately could lead to more effective immunotherapeutic interventions.

MATERIALS AND METHODS

Reagents and Plasmids

LPS from Escherichia coli 055:B5, Methyl-aminolevulinic acid (Me-ALA), Doxorubicin, N-acetyl-L-cysteine (NAC), and BAPTA-AM were from Sigma Aldrich. The plasmid pEYFP-Mito (mitochondrial marker) (27) was from Clontech. The plasmid pEYFP-C1-KDEL-GFP (28) (endoplasmic reticulum marker) was kindly provided by Dr. Sergio Grinstein (University of Toronto, Canada). The plasmid pCRT-EGFP (29) (Green fluorescent protein-tagged calreticulin) was kindly provided by Dra. Marta Hallak (CIQUIBIC, Argentina).

Cell Culture

B16-OVA murine melanoma cells were grown, as previously described, "in complete medium DMEM (Dulbecco's modified Eagle medium high glucose 1X, Gibco) supplemented with 10% v/v fetal bovine serum (FBS) (PAA Laboratories), 1% v/v glutamine (GlutaMAXTM 100X Gibco), 1% v/v antibiotic (Penicillin 10,000 units/mL–streptomycin 10,000 μg/mL Gibco) and 1% v/v of sodium pyruvate 100 mM (Gibco). Cells were maintained in 5% CO_2 and 95% air at 37°C in a humidified incubator. Stock cultures were stored in liquid nitrogen and used for experimentation within 5–7 passages" (30).

Animals

C57BL/6 were purchased from Universidad Nacional de La Plata (Buenos Aires, Argentina) and IFNAR1$^{-/-}$ were kindly provided by CIBICI-UNC (Cordoba, Argentina, purchased from Jackson Laboratory) (31). Animals were maintained under specific pathogen-free conditions at the Animal Resource Facility of Facultad de Ciencias Exactas, Físico-Químicas y Naturales (Universidad Nacional de Río Cuarto) in accordance with the experimental ethics committee guidelines. Experiments were in compliance with the Guide for the Care and Use of Laboratory Animals published by the NIH and approved by the Comité de Ética de la Investigación (COEDI) from Universidad Nacional de Río Cuarto.

Photodynamic Treatment

As previously described, B16-OVA cells monolayers "were washed twice with PBS to remove all traces of FBS and then incubated with 5-methylaminolevulinic acid (Me-ALA, Sigma) in medium without FBS for 4 h to allow the endogenous generation of the photosensitizer PpIX. After Me-ALA incubation, tumor cells were irradiated at room temperature with monochromatic light source (636 ± 17 nm) using a MultiLED system (coherent light). The fluence rate was 0.89 mW/cm^2, as measured by Radiometer Laser Mate-Q. Drug solution was then removed and replaced with fresh medium" (30).

Photosensitizer Localization Assay

B16-OVA cells were seeded on glass coverslips in a 24-well plate and allowed to attach overnight. Next, cells were transfected with pEYFP-Mito (mitochondrial marker) (27) or pEYFP-C1-KDEL-GFP (endoplasmic reticulum marker) (28). Transient transfections were performed using FuGENE® HD Transfection Reagent (Roche) according to the manufacturer's instructions (32). The following day, cells were washed twice with PBS to remove all traces of FBS and then incubated with 5-methylaminolevulinic acid (1 mM) in medium without FBS for 4 h to allow the endogenous generation of the photosensitizer PpIX. Next, they were fixed with paraformaldehyde (PAF) 4% for 20 min, and the cell nuclei were stained with Hoechst (HÖ) for visualization. The fluorescence of PpIX (red), organelles (green) and nuclei (blue) was observed by confocal microscopy (Olympus FV1000 Spectral confocal microscope, CIQUIBIC-UNC-CONICET). The co-localization is evidenced in yellow color. The analysis of the images was carried out using the free ImageJ 1.42q software (plugging Coloc 2), and the correlation was quantified through the Pearson coefficient (r).

Calreticulin (CRT) Localization Assay

B16-OVA cells were seeded in a 24-well plate and allowed to attach overnight. Next, cells were transfected with pCRT-EGFP (29) (Green fluorescent protein-tagged calreticulin). Transient transfections were performed using FuGENE® HD Transfection Reagent (Roche) according to the manufacturer's instructions (32). The following day, cells were washed twice with PBS to remove all traces of FBS and then incubated with 5-methylaminolevulinic acid (0.3 mM) in medium without FBS for 4 h to allow the endogenous generation of the photosensitizer PpIX. After Me-ALA incubation, tumor cells were irradiated with a light dose of 0.5 J/cm^2. The localization of CRT was observed 1 h after treatment on an inverted Carl Zeiss fluorescence microscope (UNRC) coupled to a high resolution monochromatic digital camera. The analysis of the images was carried out using the free ImageJ 1.42q software.

Cell Viability Assay

As previously described, "cell viability was evaluated by 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) assay, which is reduced by mitochondrial dehydrogenases of viable cells to non-water-soluble violet formazan crystals. Twenty-four hours post-PDT, MTT solution (5 mg/ml in phosphate buffer saline, PBS) was added for 3 h (dilution rate: 1/10). Then, dimethyl sulfoxide (DMSO) was added to lyse the cells and solubilize the precipitated formazan product. Optical density of the resulting solution of formazan salt was read at 540 nm using ELISA reader plate (Thermo Scientific, Multiskan FC) (33).

Analysis of Apoptosis Rate by Annexin V-FITC/PI Assay

Twenty-four hours after treatment, the percentage of apoptotic cells was assessed using a standard flow cytometry Annexin-V-FITC binding assay (BD Pharmingen). Briefly, cells were disaggregated by trypsin digestion and washed with PBS. The pellet was incubated at room temperature with 5 μg/ml Annexin V-FITC, 5 μg/ml propidium iodide (PI) and binding buffer for 15 min in the dark. Annexin V and PI fluorescence were measured using a Millipore Guava Easycyte 6 2L cytometer. According to the manufacturer's instructions, "cells that stain positive for FITC Annexin V and negative for PI are undergoing apoptosis. Cells that stain positive for both FITC Annexin V and PI are either in the end stage of apoptosis, are undergoing necrosis, or are already dead. Cells that stain negative for both FITC Annexin V and PI are alive and not undergoing measurable apoptosis." Data was analyzed using FlowJo 10.0.7 software.

Quantitative Real Time RT-PCR

Total RNA was extracted using Trizol Reagent (Life Technologies) and M-MLV reverse transcriptase was used to generate cDNA (Promega). Target transcripts were quantified by real time qRT-PCR (Stratagene Mx3000PRO) using the

Mx3000P software (34). Experiments were performed using SYBR Green PCR Master Mix (Applied Biosystems) (35). The gene-specific primers were designed with the Primer BLAST software: GAPDH: Forward: TGCACCACCAACTGCTTAG–Reverse: GGATGCAGGGATGATGTTC; IFN-α: Forward: TCTGATGCAGCAGGTGGG–Reverse: AGGGCTCTCCAGACTTCTGCTCTG; IFN-β: Forward: GCACTGGGTGGAATGAGACT–Reverse: AGTGGAGAGCAGTTGAGGACA; RIG1: Forward: AAGAGCCAGAGTGTCAGAATCT–Reverse: AGCTCCAGTTGGTAATTTCTTGG; TLR3: Forward: GTGAGATACAACGTAGCTGAACT–Reverse: TCCTGCATCCAAGATAGCAAGT; MDA5: Forward: AGATCAACACCTGTGGTAACACC–Reverse: CTCTAGGGCCTCCACGAACA; cGAS: Forward: GAGGCGCGGAAAGTCGTAA–Reverse: TTGTCCGGTTCCTTCCTGGA; ISG15: Forward: GGTGTCCGTGACTAACTCCAT–Reverse: TGGAAAGGGTAAGACCGTCCT; CXCL10: Forward: AGTGCTGCCGTCATTTTCTG–Reverse: ATTCTCACTGGCCCGTCAT; MX1: Forward: AGACTTGCTCTTTCTGAAAAGCC–Reverse: GACCATAGGGGTCTTGACCAA. Specificity was verified by melting curve analysis. Fold change in gene expression was calculated according to the $2^{-\Delta\Delta Ct}$ method. Each sample was analyzed in triplicate. No amplification was observed in PCR reactions containing water.

Western Blot

As previously described, "total cell lysates were extracted with lysis buffer containing 20 mM HEPES pH 7.5; 1.5 mM KCl; 1 mM EDTA; 1 mM EGTA; 0.15% Triton-X100; 1 mM PMSF; 1 mM DTT; and a cocktail of protease inhibitors (Sigma). The protein content of the lysate was measured using BCA protein assay reagent (Pierce). Aliquots containing an equal amount of protein (30 μg) were separated by SDS-PAGE and then transferred onto PVDF membranes (Sigma). Blots were blocked with 5% non-fat dry milk in PBS Tween 0.1% (PBST) and then incubated with primary antibodies overnight" (30): anti-phosphoIRF3 antibody (Cell Signaling−4947), anti-phospo-STAT1 (Cell Signaling−9167), anti-STAT1 (Cell Signaling−9172), anti-α-Tubulin (Cell Signaling−2144). Next, blots were incubated with corresponding horseradish peroxidase–conjugated IgG secondary antibody (anti-rabbit or anti-mouse, Cell Signaling). Detection of immunoreactive bands detection was carried out using the enhanced chemiluminescence (ECL) kit (Amersham) according to the manufacturer's instructions.

Dendritic Cell Differentiation From Bone Marrow Precursors

Dendritic cells (DCs) were obtained from bone marrow of C57BL/6 and IFNAR1$^{-/-}$ mice as described previously (36). Briefly, isolated bone marrow cells from femurs and tibiae were cultured for 7 days at a density of 3×10^6 per 10-cm dish (10 ml) in RPMI medium supplemented with 10% FBS (PAA Laboratories), 1% v/v glutamine (GlutaMAXTM 100X Gibco), 1% v/v antibiotic (Penicillin 10,000 units/mL–streptomycin 10,000 μg/mL Gibco), 1% v/v of sodium pyruvate 100 mM (Gibco) and GM-CSF (10% J558-conditioned medium v/v), hereafter termed "complete differentiation medium," in 5% CO_2 and 95% air at 37°C in a humidified incubator. On day 3, floating cells were discarded and fresh complete differentiation medium was added. Cells were further differentiated for an additional 4 days. Floating and attached cells were separately examined for their surface marker expressions, and we obtained attached cells in this study by scraping after gently washing the culture plates with warm PBS twice. More than 80% of harvested cells were immature dendritic cells (imDCs) CD11c$^+$.

Transwell Migration Assays

WT or IFNAR$^{-/-}$ CD11c$^+$ imDCs (2×10^5 cells) were loaded in their own "complete differentiation medium" in the upper chamber of a Transwell apparatus (5-μm pore size; Cornings, Lowell, MA), while B16-OVA (TCs) or PDT-treated B16-OVA (PDT-TCs) (3×10^4 cells) were seeded in the lower chamber. After 16 h at 37°C, DCs that have migrated through the membrane toward the tumor stimuli and attached on the underside of the membrane were stained with Hoechst dye for 1 h. After that, epifluorescence images were taken using an inverted Carl Zeiss fluorescence microscope (UNRC) and migrating cells were counted in different fields of view (37).

Dendritic Cell Maturation

For dendritic cells maturation analysis, WT or IFNAR$^{-/-}$ imDCs were co-cultured with B16-OVA (TCs) or with PDT-treated B16-OVA (PDT-TCs) for 24 h at a 1:1 ratio. As positive control, imDCs were exposed to LPS (0.5 μg/mL) for 24 h. CD86 and MHC-II were used as DC maturation markers evaluated on the CD11c$^+$ population (36).

Flow Cytometry

Surface staining of single-cell suspensions of dendritic cells was performed using standard protocols (31) and analyzed on a Millipore Guava Easycyte 6 2L cytometer. Data analysis was conducted using FlowJo 10.0.7 software. The following were obtained from BioLegend: anti-CD11c-APC (147309), anti-MHC-II-PerCP-Cy5.5 (107626), and anti-CD86-PeCy5 (105014).

Statistics

Data handling, analysis and graphic representation (all shown as mean ± SEM) were performed using Prism 7.0 (GraphPad Software). Statistical data are informed in the corresponding figure legend.

RESULTS

ER-Associated Cell Death Promoted by Photodynamic Therapy

By definition, "ICD inducers must be cytotoxic and provoke cell death above a minimal threshold level" (15). Therefore, we initially examined the ability of PDT to elicit melanoma cell death. In this context, B16-OVA were incubated during 4 h with the prodrug Me-ALA to allow the generation of the photosensitizer PpIX. Upon red-light activation (0.5 J/cm^2), variable cell toxicity dependent on the pro-drug concentration (0.1–0.35 mM) was observed (**Figure 1A**). No damage was

induced by the prodrug Me-ALA *per se* or by red-light irradiation alone (**Figure 1A**). As expected, the antioxidant NAC (30) reversed the cytotoxic effect of high-dose PDT (**Figure 1B**). To determine the organelles in which PpIX was located, we performed co-localization experiments with mitochondria and endoplasmic reticulum markers. We observed that PpIX displayed preferential endoplasmic reticulum (ER) localization (**Figure 1C**). Pre-incubation of melanoma cells with the calcium chelator BAPTA-AM (38) inhibited PDT-induced cell death (**Figure 1D**), indicative of ER stress associated with photodynamic effect. The ER response to stress is accompanied by translocation of danger signals to the cell surface (38). CRT is the most abundant protein in the ER lumen which translocates to cell surface in response to stress-mediated dying cells (39). Here, 72.3% of photosensitized melanoma cells exhibited the typical "patches" (40) of anterograde intracellular transport of CRT, suggesting that PDT also modulates CRT mobilization (**Figure 1E**).

Enhancement of IFN-1 Expression Mediated by Photodynamic Treatment

Until now, PDT had been associated with CRT (24, 25), ATP (26), and HMGB1 (24) exposition and/or release, but there was no evidence for type I interferon pathway regulation. Having shown that CRT was translocated through photodynamic stimuli (**Figure 1E**) and given the connection between this chaperone and the modulation of IFN-1 (41), levels of *ifn-α/β* mRNA were measured in dying cells undergoing anticancer PDT. B16-OVA were exposed to Me-ALA (0.1, 0.2, and 0.3 mM) and then irradiated with red light (0.5 J/cm^2). Interestingly, a significant increase in IFN-1 transcription was detected in melanoma cells as early as 5 h following high-dose photosensitization (**Figure 2A**). In addition, as the doses of Me-ALA increased, the frequency of apoptotic cells (both early and late apoptotic cells) (**Figures 2B,C**) and the expression of IFN-α/β also augmented (**Figure 2A**), suggesting that an autocrine effect of IFN-1 could be playing a role in inducing apoptosis. Next, we exposed melanoma cells to a lethal dose of PDT (Me-ALA 0.3 mM + 0.5 J/cm^2) or doxorubicin (30 μM), a relevant chemotherapeutic agent bona fide ICD inducer (16, 42), analyzing IFN-1 regulation in a time-course experiment. Notably, PDT was a strong IFNα/β inducer 5 h post-PDT; in contrast the significant upregulation of IFN-α/β was absent in those subjected to doxorubicin (**Figure 2D**). Overall, we provide here experimental data regarding specific *in vitro* apoptotic lethal conditions of PDT that strongly induced IFN-1 in B16-OVA cells.

Photodynamic Autocrine Modulation of Type 1 Interferon Pathway

Type I IFNs are cytokines of major importance for the innate antiviral response that have been recently associated to ICD (16). They are produced after recognition of nucleic acids by toll-like receptors (TLR3-7-8) or by cytoplasmic proteins, such as RIG-I like receptors (RIG-1, MDA-5) or the cyclic GMP-AMP synthase (cGAS), which activate adaptor proteins that culminate in IRF3 phosphorylation. IRF3 is a transcription factor that leads the expression of Type 1 interferons. After their secretion, IFN-α/β bind to their cognate receptor IFNAR1/2, triggering the phosphorylation of STAT transcription factors, and the consequent induction of hundreds of interferon-stimulated genes (ISGs) in the responding cells (7). Based on our findings (**Figure 2**), we decided to explore the mechanisms underlying the photodynamic modulation of IFN-1. After 14 h of PDT-stimulation, an upregulation of cGAS receptor, but not MDA-5, TLR3, or RIG-1, was detected (**Figure 3A**). Interestingly, a significant increase in the transcription of ISGs CXCL10, ISG15, and MX1 was observed (**Figure 3B**). As expected, induction of interferon regulatory factor (IRF) related genes was paralleled by phosphorylation of IRF3 0.5 h after photodynamic treatment (**Figures 3C,D**). The type I IFN autocrine loop was also manifested in our experimental setting, since STAT1 phosphorylation was evidenced 1 h after the initial PpIX photoactivation on tumor cells (**Figures 2E,F**). Collectively, these data suggest that Me-ALA-based PDT stimulates the production of type I IFN and this can act autocrinally augmenting the transcription of several interferon stimulated genes.

IFN-1-Dependent Activation of Dendritic Cells Induced by PDT-Treated Melanoma Cells

The spatiotemporally coordinated emission of specific DAMPs promotes the recruitment of DCs to sites of ongoing ICD and their capacity to prime an adaptive immune response (13). For this reason, we next examined whether IFN-1 detected in PDT-melanoma tumor cells (PDT-TCs) could act in a paracrine fashion on DC migration. Immature WT and IFNAR$^{-/-}$ DCs were loaded into the upper chamber of transwells with growth media (Control), B16-OVA (TCs) or photosensitized B16-OVA (PDT-TCs) in the lower chamber. Although the absence of IFNAR did not affect the basal migration of dendritic cells or in response to untreated tumor cells, WT DCs migrated toward PDT-TCs in much greater numbers than IFNAR$^{-/-}$ DCs (**Figures 4A,B**). The expression of cell-surface co-stimulatory molecules that are involved in DC maturation, such as CD86 and MHC-II, was assessed by flow cytometry after 24 h of DCs-TCs co-culture. Untreated imDCs were used as negative control (DCs control) and imDCs stimulated by lipopolysaccharide (DCs + LPS) were used as positive control. Interestingly, PDT-TCs were capable *per se* of significantly enhancing the maturation of WT DCs, which was partially abrogated when IFN-1 receptor was absent in DCs. Similar results were observed with the positive control of LPS treatment (**Figures 4C–G**). Taken together, these results indicated that apoptotic PDT on melanoma cells induces the production of type I IFNs, which in turn can promote an improvement in DC function.

DISCUSSION

The success of cancer treatments fundamentally relies on the synergic interaction between dying/dead cancer cells and immune cells. The ideal cancer therapeutic strategy should

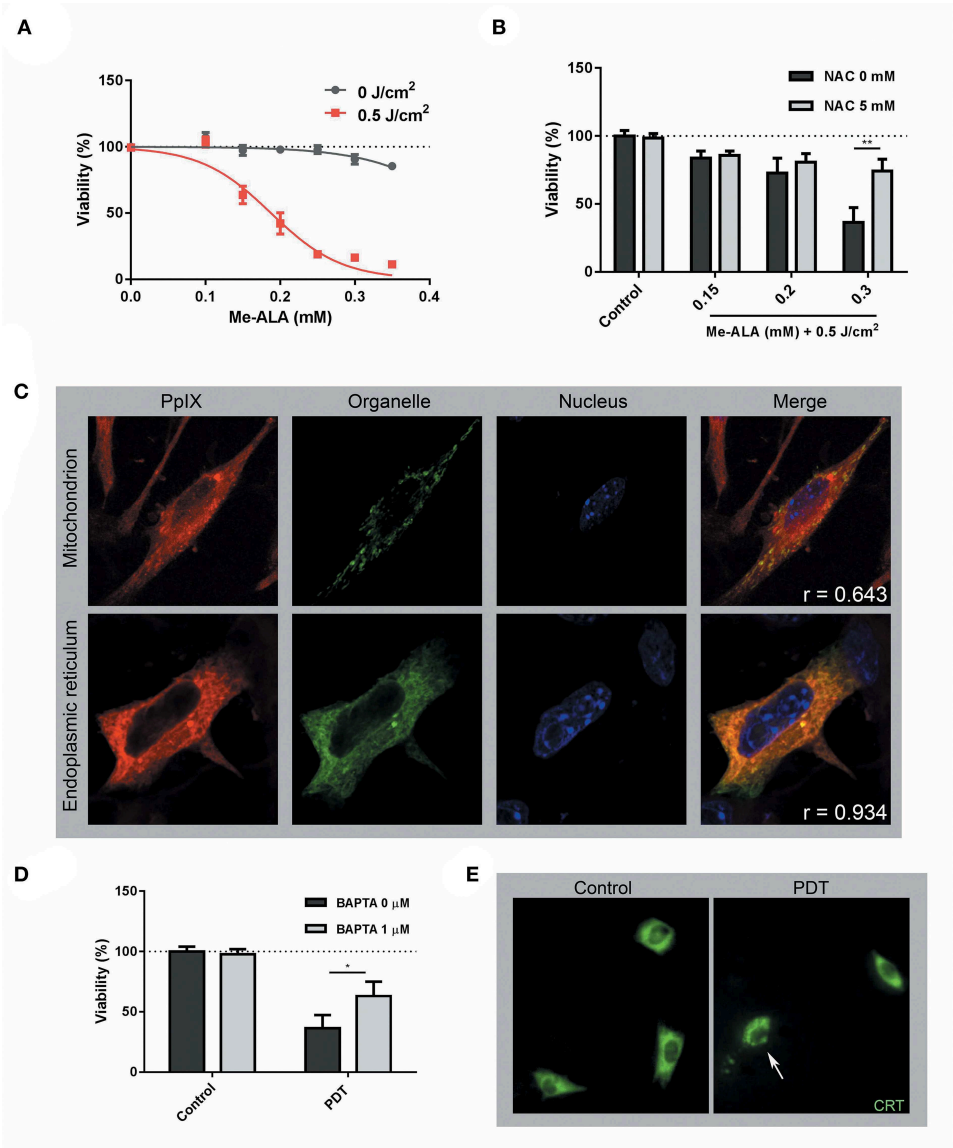

FIGURE 1 | Photodynamic Therapy induced ER-associated cell death and CRT mobilization on melanoma cells. **(A)** B16-OVA cells were incubated with increasing concentrations of the pro-drug Me-ALA (0–0.35 mM) for 4 h and then were irradiated with visible light (λ: 635 ± 17 nm, light dose: 0.5 J/cm^2). At 24 h post-treatment, cell viability was evaluated through the MTT assay and expressed as a percentage with respect to the non-treated control (dotted line: 100% viability). **(B)** B16-OVA cells were incubated with Me-ALA (0.1, 0.2, and 0.3 mM) in the presence or absence of NAC (5 mM) for 4 h and then exposed to irradiation (0.5 J/cm^2). Viability was evaluated by MTT assay 24 h post-PDT and referred to non-treated conditions (dotted line: 100% viability). Data are mean ± SEM of three independent experiments. $^{**}p < 0.01$ vs. control group (NAC 0 mM, gray bars), Two-Way ANOVA Bonferroni post-test. **(C)** B16-OVA cells transfected with pEYFP-Mito (mitochondrial marker) and pEYFP-C1-KDEL-GFP (endoplasmic reticulum marker) were incubated for 4 h with the Me-ALA drug (1 mM). Next, they were fixed with paraformaldehyde (PAF) 4% for 20 min and the cell nuclei were stained with Hoechst (HÖ) for visualization. The fluorescence of PpIX (red), organelles (green), and nuclei (blue) was observed by confocal microscopy. The co-localization is evidenced in yellow color. The analysis of the images was carried out using the free ImageJ 1.42q software (plugging Coloc 2) and the correlation was quantified through the Pearson coefficient (r). **(D)** B16-OVA cells were subjected to high dose PDT (Me-ALA 0.3 mM + 0.5 J/cm^2) in the presence or absence of BAPTA-AM (1 μM) for 4 h. Viability was evaluated by MTT assay 24 h post-PDT and referred to non-treated conditions (dotted line: 100% viability). Data are mean ± SEM of three independent experiments. $^{*}p < 0.05$ vs. control group (BAPTA 0 μM, gray bars), Two-Way ANOVA Bonferroni post-test. **(E)** B16-OVA cells transfected with pCRT-EGFP [green fluorescent protein-tagged calreticulin (CRT)] were subjected to high dose PDT (Me-ALA 0.3 mM + 0.5 J/cm^2). The fluorescence of CRT (green) was observed by epifluorescence microscopy 0.5 h post-treatment. CRT translocation is marked with a narrow. The analysis of the images was carried out using the free ImageJ 1.42q software.

involve both direct cytotoxic action on tumor cells and immunostimulatory effects based on the immune recognition of molecular antigenic determinants on dying cells. However, to cause an immune response against malignant cells, the presence of tumor antigens is not enough. Also, such cells must emit danger signals, such as danger-associated molecular

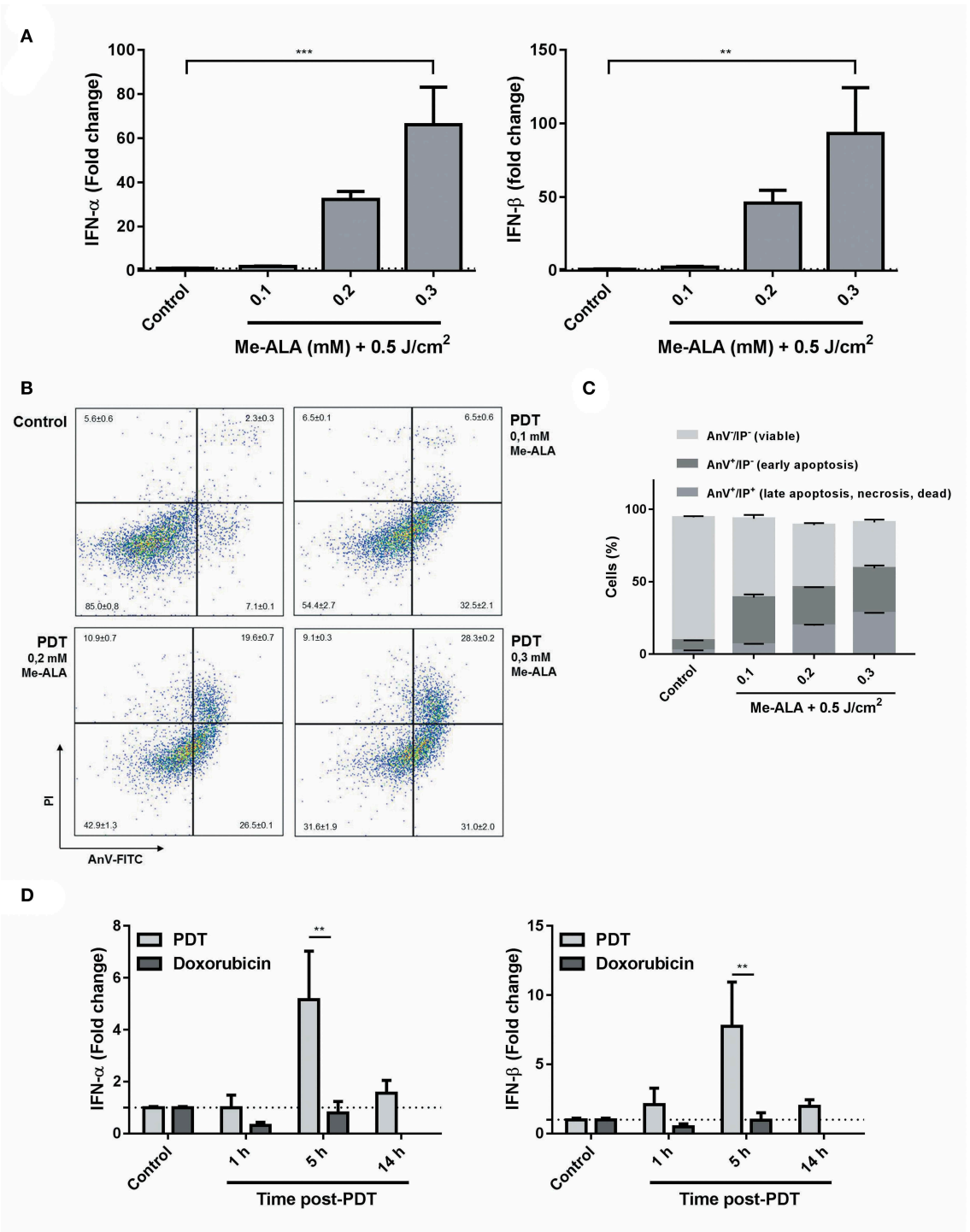

FIGURE 2 | Photodynamic therapy as a novel inductor of IFN-1 expression on melanoma cells. **(A)** B16-OVA cells were incubated with Me-ALA (0.1, 0.2, and 0.3 mM) for 4 h and then were irradiated with visible light (0.5 J/cm^2). Quantification of mRNA expression of IFN-1α (right) and IFN-β (left) was performed 5 h after treatment by RTqPCR and normalized with respect to the non-treated control (dotted line: 1). Data are mean ± SEM of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.05$ vs. control group (untreated cells), One-Way ANOVA Bonferroni post-test. **(B)** Type of cell death was evaluated using Annexin V/PI staining by flow cytometry. The data generated by flow cytometry were plotted in two-dimensional dot plots in which PI is represented vs. Annexin V-FICT. **(C)** Viable cells (Annexin V$^-$/PI$^-$), undergoing (early) apoptotic cells (Annexin V$^+$/PI$^-$) and dead, necrotic or late (end-stage) apoptotic cells (Annexin V$^+$/PI$^+$) were quantified using FlowJo 10.0.7 software. **(D)** B16-OVA cells were subjected to high dose PDT (Me-ALA 0.3 mM + 0.5 J/cm^2) or doxorubicin (30 μM). Quantification of mRNA expression of IFN-1-α (right) and IFN-β (left) was performed 1, 5, and 14 h after treatment by RTqPCR and normalized with respect to the non-treated control (dotted line: 1). Data are mean ± SEM of three independent experiments. $^{**}p < 0.01$ vs. PDT group (gray bars), Two-Way ANOVA Bonferroni post-test.

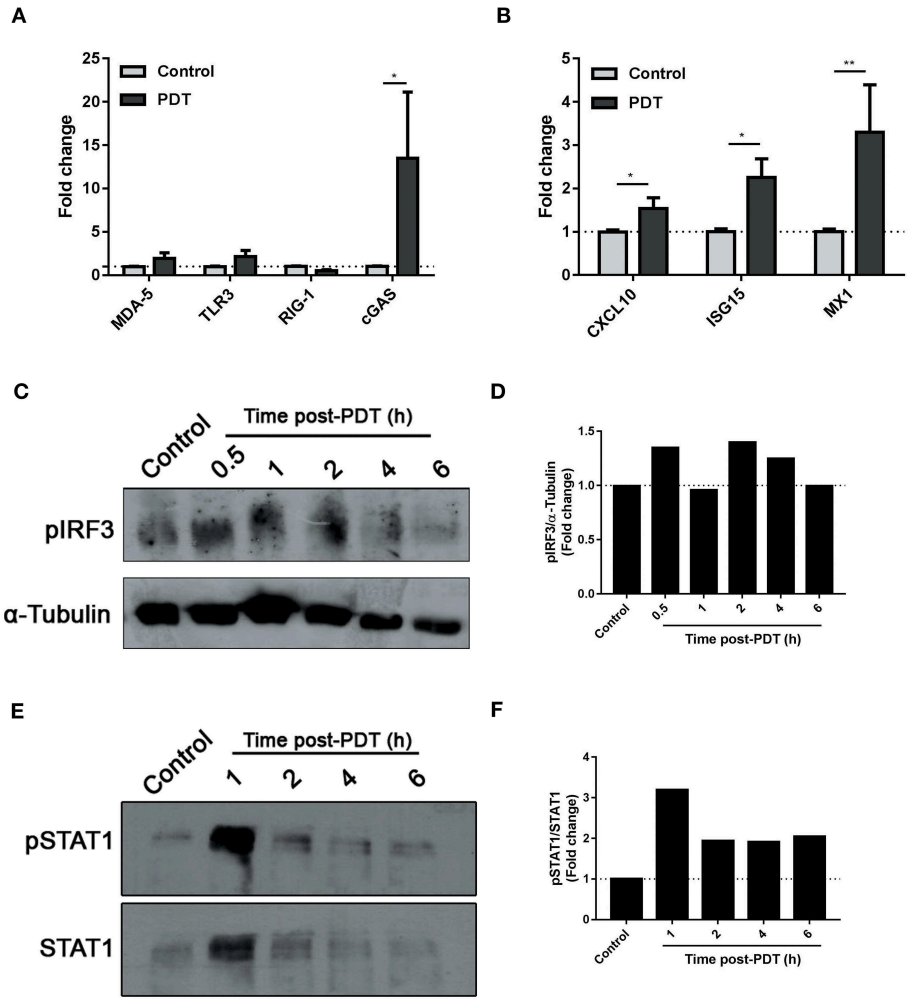

FIGURE 3 | Modulation of IFN-1 pathway by photodynamic therapy. B16-OVA cells were incubated with Me-ALA (0.3 mM) for 4 h and then were irradiated with visible light (λ: 635 ± 17 nm, light dose: 0.5 J/cm^2) (PDT). Non-treated cells were used as "Control." **(A)** Quantification of mRNA expression of receptors MDA-5, TLR3, RIG-1, and cGAS was performed 14 h after treatment by RTqPCR and normalized with respect to the non-treated control (dotted line: 1). Data are mean ± SEM of three independent experiments. *$p < 0.05$ vs. control group (untreated cells, gray bars), Two-Way ANOVA Bonferroni post-test. **(B)** Quantification of mRNA expression of ISGs CXCL10, ISG15, and MX1 was performed 14 h after treatment by RTqPCR and normalized with respect to the non-treated control (dotted line: 1). Data are mean ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.01$ vs. control group (untreated cells, gray bars), Two-Way ANOVA Bonferroni post-test. **(C)** Western blot was performed to detect phospho-IRF3 at 0.5–6 h post-treatment. The same membrane was stripped and reblotted for α-Tubulin as loading control. **(D)** Densitometric analysis performed with the ImageJ software represented the signal intensity of phospho-IRF3; the signal was normalized α-Tubulin intensity. **(E)** Western blot was performed to detect phospho-STAT1 at 1–6 h post-treatment. The same membrane was stripped and reblotted for total STAT1 as loading control. **(F)** Densitometric analysis performed with the ImageJ software represented the signal intensity of phospho-STAT1; the signal was normalized total STAT1 intensity.

patterns (DAMPs) that work as adjuvants (43). In this context, several successful antitumor agents have demonstrated to be highly efficient in stimulating the emission of DAMPs by cancer cells, thus inducing ICD (15). Two categories have been proposed in order to classify ICD inducers based on their direct or indirect ability to cause endoplasmic reticulum (ER) stress leading to apoptotic cell death. The majority of ICD inducers, such as chemotherapeutic agents (oxaliplatin mitoxantrone, doxorubicin, and cyclophosphamide), shikonin, vorinostat, cardiac glycosides, among others, are categorized as type I ICD inducers that primarily target cytosolic proteins, plasma membranes, or nucleic proteins. They also induce ER stress via collateral effects. On the other hand, type II ICD inducers, such as hypericin-based PDT and coxsackievirus B3, preferentially target the ER. The quality and/or quantity of ER stress induced by ICD, also associated with oxidative stress, may define the ICD inducer properties, and it was demonstrated that type II ones are more efficient in terms of immunological antitumor ability (44).

In the last decades, several investigations have been devoted on the search of particular stress agents capable of provoking ICD in cancer cells. Photodynamic therapy

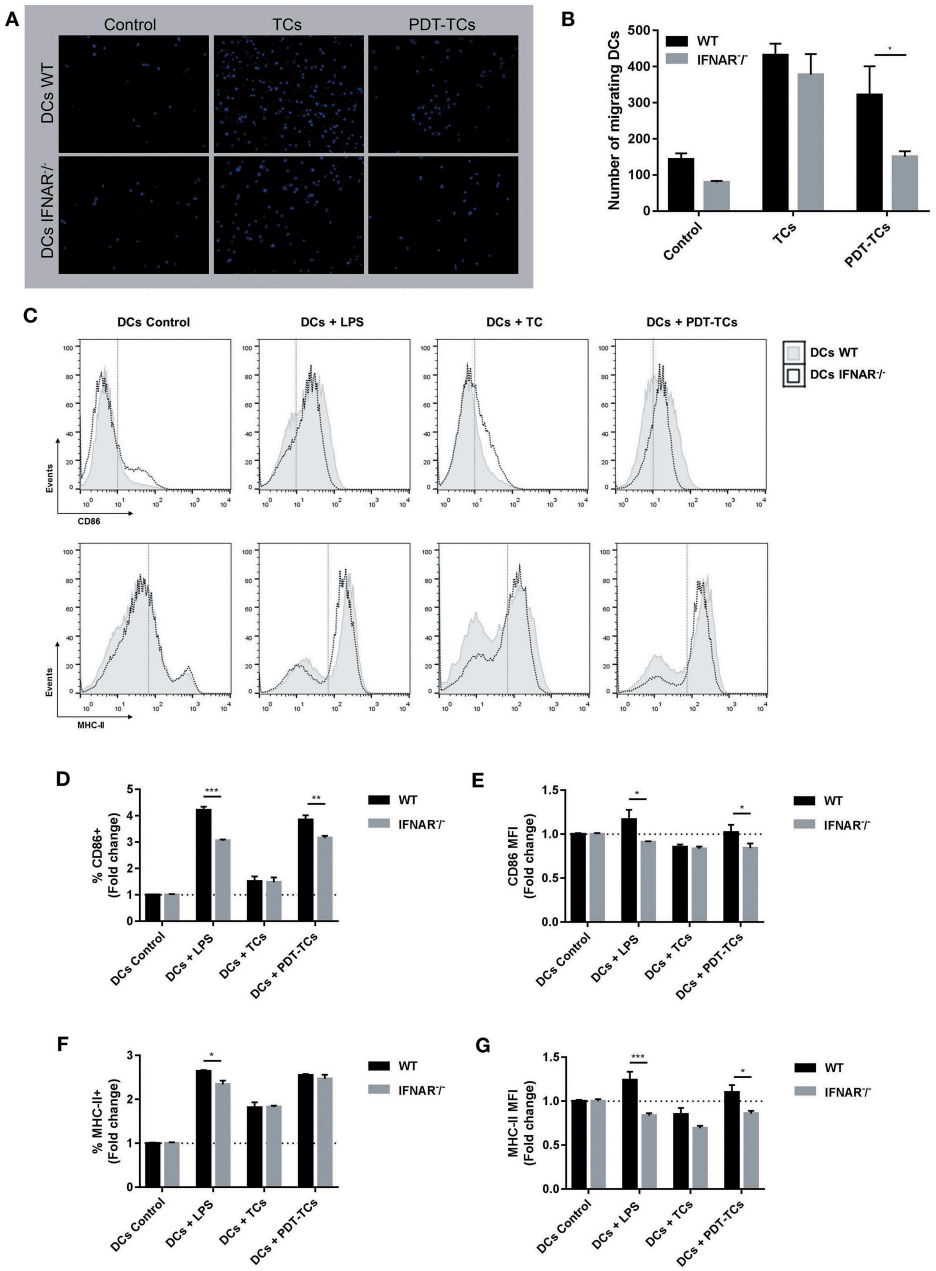

FIGURE 4 | Phenotypic and functional maturation of dendritic cells mediated by IFN-1 upregulation on PDT-subjected melanoma cells. WT or IFNAR$^{-/-}$ DCs were in the upper chamber of a Transwell apparatus while B16-OVA (TCs) or with PDT-treated B16-OVA (PDT-TCs) were seeded in the lower chamber. Complete growth media was used as "control." **(A)** After 16 h at 37°C, DCs that have migrated through the membrane toward the tumor stimuli and attached on the underside of the membrane were stained with Hoechst dye for 1 h, and epifluorescence images were taken. **(B)** Migrating cells were counted in different fields of view. Data are mean ± SEM of three independent experiments. *$p < 0.05$ vs. control group (WT dendritic cells, black bars), Two-Way ANOVA Bonferroni post-test. **(C)** WT (solid line, gray filled) or IFNAR$^{-/-}$ DCs (dotted line, non-filled) were co-cultured with B16-OVA (TCs) or with PDT-treated B16-OVA (PDT-TCs) for 24 h in a 1:1 ratio. As positive control, DCs were exposed to LPS (0.5 μg/mL) for 24 h. CD86 and MHC-II were used as DCs maturation markers. Representative flow cytometry histograms were performed with FlowJo 10.0.7 software. **(D)** CD86^{+} cells were referred to untreated imDCs used as negative control (DCs Control, dotted line: 1). Data are mean ± SEM of three independent experiments. **$p < 0.01$, ***$p < 0.001$ vs. control group (WT dendritic cells, black bars), Two-Way ANOVA Bonferroni post-test. **(E)** CD86 expression intensity of CD86^{+} cells was indicated by geometric mean (MFI, mean fluorescence intensity) referred to untreated imDCs used as negative control (DCs Control, dotted line: 1) Data are mean ± SEM of three independent experiments. *$p < 0.05$ vs. control group (WT dendritic cells, black bars), Two-Way ANOVA Bonferroni post-test. **(F)** MHC-II^{+} cells were referred to untreated imDCs used as negative control (DCs Control, dotted line: 1) Data are mean ± SEM of three independent experiments. *$p < 0.05$ vs. control group (WT dendritic cells, black bars), Two-Way ANOVA Bonferroni post-test. **(G)** MHC-II expression intensity of MHC-II^{+} cells was indicated by geometric mean (MFI) referred to untreated imDCs used as negative control (DCs Control, dotted line: 1). Data are mean ± SEM of three independent experiments. *$p < 0.05$, ***$p < 0.001$ vs. control group (WT dendritic cells, black bars), Two-Way ANOVA Bonferroni post-test.

(PDT), a regulatory approved cancer treatment, has the ability of inducing immunogenic apoptosis (45, 46). Here, we demonstrated that oxidative stress induced by PDT promoted apoptotic cell death (**Figures 1**, **2B,C**). Following PDT, ROS exhibit a short half-life, thus they exert their effect close to their site of generation. Consequently, the precise subcellular localization of the PS within the cell influences the degree and the type of photodamage. The knowledge of PS localization is therefore important for choosing the most effective PS for each purpose (47). For this reason, we decided to evaluate the precise location of PpIX. Under our experimental conditions, PpIX localized in ER (**Figure 1C**), suggesting this organelle as its major target. In addition, ER-stress was associated with photodynamic effect (**Figure 1D**). These data postulated Me-ALA based-PDT as a potential Type-II ICD inducer.

ICD is a death mechanism in which specific stimuli lethally damage cancer cells while producing the spatiotemporally emission of immunogenic signals (15). Previous reports demonstrated the photodynamic mobilization of some of the main DAMPs involved in ICD, such as ATP (26), HMGB1 (24), and CRT (24, 25). In this sense, in the current work, we observed a significant translocation of CRT from ER to plasma membrane (**Figure 2E**). In an ICD context, this translocation of CRT occurs in a pre-apoptotic stage (before translocation of phosphatidylserine to the outer side of the plasma membrane) (39, 40). The ecto-CRT serves as a potent "eat me" signal for local patrolling DCs (39). For immunogenicity to be detected, dying cells must emit signals in addition to CRT. In fact, recently the capacity of surface-exposed CRT to initiate type I IFN-dependent anticancer immunity was shown (41). The immune response against cancer generated by type-I-interferons (IFN-1) has recently described. Exogenous and endogenous IFN-α/β have an important role in immune surveillance and control of tumor development. Accordingly, the role of TLR agonists as cancer therapeutic agents is being revisited with the idea of stimulating the production of endogenous type I IFN inside the tumor (31, 34, 36).

In addition, type-I-interferons (IFN-1) have recently emerged as novel DAMPs for the sequential events bridging innate and cognate immunity (16, 48). Both IFN-1 as well as ISGs had been activated *in vitro* and *in vivo* following anthracycline-based chemotherapy. It was described how the cancer autocrine axis of TLR3 > IFNs-I > IFNAR affects immunogenicity of anthracycline-mediated tumor cell death (14, 16). Based on these findings, IFN-1s are now classified as a Class IIIA DAMPs: endogenous native molecules operating as inducible DAMPs (49). Interestingly, to the best of our knowledge, the present study is the first to report the *in vitro* upregulation of IFN-1 expression in response to photodynamic treatment in melanoma (**Figure 2**). Our data suggest that Me-ALA based PDT stimulate the production of IFN-α/β and related ISGs through an autocrine molecular pathway (**Figure 3**).

For a successful immunogenic cell death promotion, the concomitant DAMPs must have activating effects on dendritic cells (DCs). DCs are mobile cells, and this feature is crucial for their antitumor action *in vivo* for the proper detection and capture of tumor antigens in peripheral tissues. Next, DCs upregulate the expression of co-stimulatory molecules, in order to cross-present and activate antigen-specific T cells. DAMPs recognition is an essential requirement for the activation of immature DCs associated with the expression of co-stimulatory T cell molecules (50). Here, we demonstrated that photosensitized melanoma promotion of both DCs migration to tumor site and DCs maturation was dependent on IFN-1 signaling (**Figure 4**). Taken together, our results show that cancer cells subjected to oxidative stress due to ER-associated pro-apoptotic PDT could potentiate antitumor immunity through an autocrine/paracrine activation of IFN-1 pathway.

In recent years, anticancer vaccination success has been correlated with the immunogenic potential of dead/dying cells used as antigen/adjuvant source. The danger signals-dependent efficacy of ICD-based DC vaccines has recently been shown (17, 51–54). However, chemical ICD inducers are not desirable for production of DC-based vaccines because they either leave residual drug concentrations behind or may exert cytotoxicity against DCs. For that reason, appropriate preselection of ICD should be critically considered (55–57). In this sense, as the prodrug Me-ALA is not toxic *per se* (**Figure 1A**), and given the immune stimulation properties observed (**Figure 4**), the PDT conditions here tested could represent a promising approach in the design of ICD-based DCs vaccines.

Collectively, our findings showed the effects of a novel danger signal released by PDT-treated cancer cells on the activation of DCs, emphasizing the potential relevance of PDT in adoptive/personalized immunotherapy protocols.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Comité de Ética de la Investigación de la Universidad Nacional de Río Cuarto (CoEdI).

AUTHOR CONTRIBUTIONS

ML: formal analysis, investigation, methodology, writing—original draft, funding acquisition, and project administration. FM, ER, and PA: formal analysis, investigation, and methodology. VR: conceptualization, supervision, resources, funding acquisition, and project administration. MM: conceptualization, formal analysis, resources, supervision, and writing—review and editing. NR: conceptualization, formal analysis, resources, supervision, funding acquisition, project administration, and writing—review and editing.

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REVIEW
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Role of Tumor-Mediated Dendritic Cell Tolerization in Immune Evasion

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The vast majority of cancer-related deaths are due to metastasis, a process that requires evasion of the host immune system. In addition, a significant percentage of cancer patients do not benefit from our current immunotherapy arsenal due to either primary or secondary immunotherapy resistance. Importantly, select subsets of dendritic cells (DCs) have been shown to be indispensable for generating responses to checkpoint inhibitor immunotherapy. These observations are consistent with the critical role of DCs in antigen cross-presentation and the generation of effective anti-tumor immunity. Therefore, the evolution of efficient tumor-extrinsic mechanisms to modulate DCs is expected to be a potent strategy to escape immunosurveillance and various immunotherapy strategies. Despite this critical role, little is known regarding the methods by which cancers subvert DC function. Herein, we focus on those select mechanisms utilized by developing cancers to co-opt and tolerize local DC populations. We discuss the reported mechanisms utilized by cancers to induce DC tolerization in the tumor microenvironment, describing various parallels between the evolution of these mechanisms and the process of mesenchymal transformation involved in tumorigenesis and metastasis, and we highlight strategies to reverse these mechanisms in order to enhance the efficacy of the currently available checkpoint inhibitor immunotherapies.

Keywords: dendritic cell tolerance, cancer immunotherapy, immune checkpoint inhibition, metastasis, epithelial-to-mesenchymal transition, exosomes, myeloid-derived suppressor cells, dendritic cell immunotherapy

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INTRODUCTION

During tumorigenesis, the process of malignant transformation occurs concurrently with evasion of the host immune system (1, 2). The ability of tumors to evolve mechanisms to manipulate their local immune microenvironment is also a key component of metastatic progression to distant tissue sites. Given their critical role in orchestrating tumor-targeted immune responses, cancers facilitate their escape from immune recognition and subsequent progression by subverting the functions of antigen presenting cells (APCs) known as dendritic cells (DCs). This process of DC tolerization involves the genetic reprogramming of DCs to ultimately disable immune recognition of developing malignancies (3–6). As the field of immuno-oncology has been primarily focused on directly enhancing the activation of effector T cells, the process of tumor-mediated DC tolerization is comprised of many unexplored opportunities for therapeutically enhancing anti-tumor immunity at earlier stages of the tumor immunity cycle. Herein, we review the processes by which cancers actively drive DC tolerization, how these mechanisms may influence responses to modern immunotherapy, and how these processes can be therapeutically manipulated to improve patient outcomes.

DENDRITIC CELL TOLERIZATION IN CANCER

DCs represent the functional transition point between the innate and adaptive immune systems and tumor-infiltrating DCs have been described across multiple histologies (7, 8). They have the ability to process antigens derived from the environment and cross-present these antigens to major histocompatibility (MHC) class I-restricted $CD8^+$ T cells (9, 10). These DCs further serve to direct the functional programming of the activated T cell, thereby dictating their capacity to defend the host from cancer progression (11, 12).

The phenotypically and functionally distinct subsets of DCs including the plasmacytoid (pDC), conventional (cDC1 or cDC2), and inflammatory DC (moDC), have been extensively reviewed previously (13). Specifically, murine $CD8a^+CD103^+BATF3^+CLEC9A^+XCR1^+$ cDC1s have been demonstrated to have a critical role in the cross-presentation of tumor antigens and are generally thought to be indispensable in the development of host anti-tumor immune responses (14–17). Human cDC1s are necessary for $CD8^+$ T cell cross priming and are identified by expression of CD141 (BDCA3) (18) in addition to CD8a, BATF3, XCR1, and CLEC9A (DNGR1) (19–22). In human melanoma samples from the Cancer Genome Atlas (TCGA), the presence of $BATF3^+$ DCs was correlated with enhanced $CD8^+$ T cell infiltration and T cell homing chemokines CXCL9 and CXCL10 (17). Antigen cross-presentation defects, such as loss of *Batf3*, *Clec9a*, or *Wdfy4* results in a restrained $CD8^+$ T cell repertoire and an inability to reject tumors (23–25). In mouse models lacking $BATF3^+$ DCs, IL-12 production and natural killer (NK) cell mediated control of metastasis is impaired while *BATF3* and *IRF8* expression have been associated with improved relapse-free survival in breast cancer patients (26). These data exemplify the importance of DC antigen processing and cross-presentation in the immunologic control of cancer.

Tumors condition the pre-metastatic niche to develop a favorable immune microenvironment and progressively adapt to immune pressure during dissemination (**Figure 1)** (27). Therefore, DCs represent logical targets for the evolution of tumor-mediated suppressive mechanisms to facilitate their local and metastatic progression and it is these mechanisms which drive DC tolerization. Despite the advances in our understanding of DC subsets, it remains unclear whether there are unique phenotypic identifiers of tolerized DCs and whether there are multiple subtypes of tolerized DC populations that utilize different modalities to drive immune suppression. To date, investigators have largely utilized the functional conversion of naïve $CD4^+$ T cells to the immune suppressive $CD4^+FoxP3^+$ regulatory T cell population (Tregs) coupled with an impaired ability to induce the activation of effector $CD8^+$ T cells as their defining features (24, 25, 28).

The recent literature has provided some emerging examples of these immunosuppressive DC subsets contributing to tumor progression and suggests some markers that may identify them. For example, expression of macrophage galactose N-acetyl-galactosamine-specific lectin 2 (MGL2; CD301b; or CLEC10A) was previously described in dermal populations of DCs that promote Th2 differentiation in the draining lymph nodes (29). More recently, in an orthotopic model of pancreatic cancer that metastasizes to the liver, Kenkel et al. described an immunosuppressive subset of hepatic $MGL2^+PD\text{-}L2^+CD11b^+F4/80^-$ DCs that accumulate in metastatic loci. These DCs promoted Treg development *in vivo* and *in vitro*, and the development of metastasis was hindered by anti-PD-L2 or $MGL2^+$ cell depletion (5). In an ovarian cancer model, tumor-driven *Satb1* overexpression in terminally differentiated DCs results in a tolerant, pro-inflammatory state as evidenced by the secretion of Galectin-1 and IL-6, promoting tumor growth and immune evasion (30). Additionally, tumor draining lymph nodes from a Lewis Lung carcinoma model harbor DCs with elevated cyclooxygenase-2 (COX-2) while inhibition of COX-2 results in diminished Tregs and reduced lymph node metastasis suggesting that COX-2 may also promote and be a marker of DC tolerization (31). Experiments performed in a p53-inducible metastatic model of ovarian cancer revealed an $MHCII^{lo}CD40^{lo}PD\text{-}L1^{hi}$ subset of DCs which suppressed $CD8^+$ T cell proliferation and failed to induce IFN-γ and Granzyme B production, an effect attributed to TGFβ and prostaglandin E2 (PGE2). The investigators also identified an increasing population of these tolerogenic DCs with metastatic progression and further found that depletion of DCs later in tumor progression using a CD11c-DTR (diphtheria toxin receptor) system impaired tumor growth, suggesting the activation of a phenotypic switch driving DC tolerization during cancer progression (32). Others have also identified tumor-derived PGE2 and TGFβ as being capable of inducing a $CD11c^{lo}CD11b^{hi}$ arginase-expressing DC subset which impairs T cell activation, while additional studies have defined a $CD11c^{hi}CD11b^+MHC\ II^+$ DC population that inhibits $CD8^+$ T cell responses in several murine tumor models in an arginase-dependent manner (33, 34).

Plasmacytoid DC (pDCs) subsets, defined as $CD11c^+PDCA\text{-}1^+$ in mice and $CD11c^-CD123^+CLEC4C^+$ in humans, have been implicated in the maintenance of peripheral tolerance, as well as the control of anti-viral immunity via the production of type I interferons, exemplifying their functional plasticity (3, 35). pDCs have broadly been associated with poor prognosis across multiple tumor types, perhaps due to their ability to promote Th2 differentiation via the expression of OX40L and ICOSL (3). Further studies have indicated that the more rapid turnover of surface MHC II:Ag complexes on pDCs relative to conventional DCs contributes to their preferential ability to drive Treg development (36). In addition, ovarian cancer-associated pDCs have been characterized as expressing less IFN-α and stimulating higher levels of IL-10-expressing $CD4^+$ T cells compared to their circulating counterparts (37). Indeed, the stromal-derived factor-1 (CXCL12) chemokine has been implicated in the recruitment of pDCs to ovarian cancer epithelial tissues to generate an immunosuppressive microenvironment (38). Importantly, Munn and Sharma et al. have described an IDO1-expressing pDC subset in the tumor draining lymph node that is capable of inducing Treg generation, T cell anergy, and potently suppressing T cell response to tumor antigens (39, 40). More work is needed to understand the diversity of pDCs in cancer and

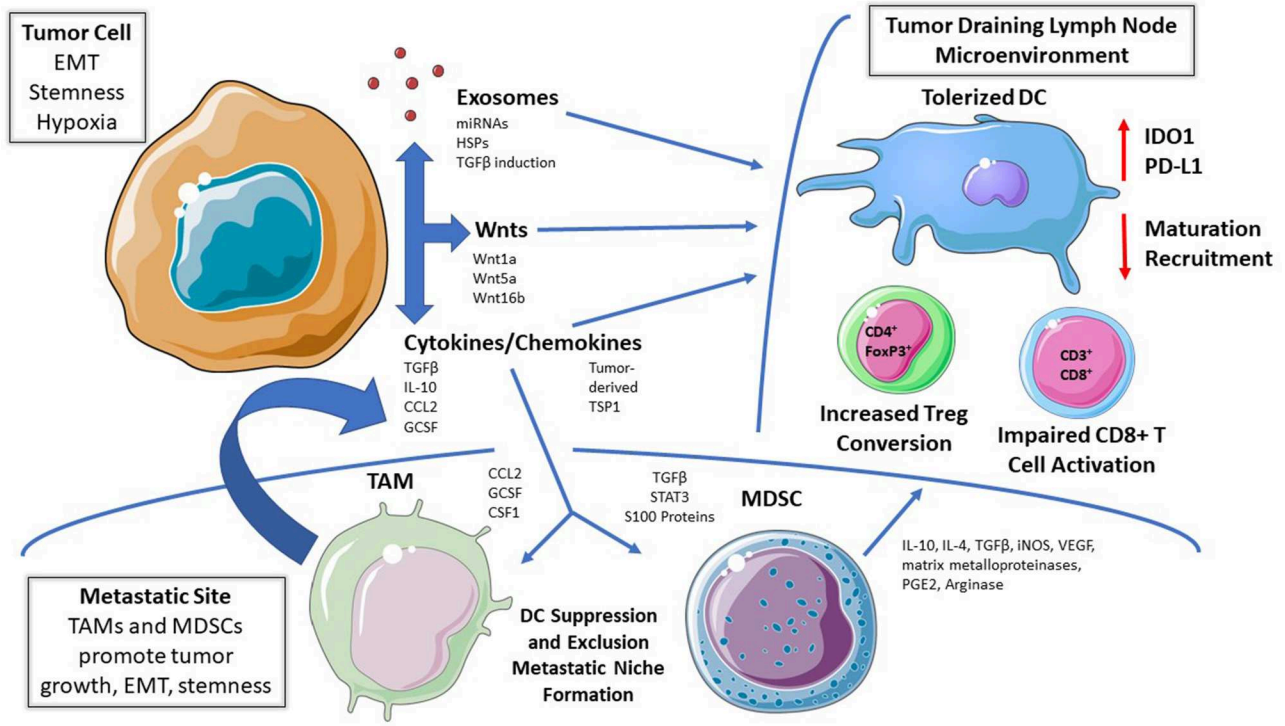

FIGURE 1 | Mechanisms of DC Tolerization in the Tumor Microenvironment. Dendritic cells (DCs) residing within tumor beds, tumor-draining lymph node tissues, or within more distant metastatic sites can be functionally tolerized by tumor-derived soluble mediators, tumor-derived exosomes, and/or via the recruitment of other immunosuppressive cell populations. This process suppresses DC-mediated effector T cell responses while promoting DC-dependent regulatory T cell (Treg) differentiation; thereby facilitating cancer progression and metastasis. EMT, epithelial-mesenchymal transition. TAM, tumor-associated macrophage; MDSC, myeloid-derived suppressor cell; IDO, indoleamine 2,3-dioxygenase; RA, retinoic acid; Arg, arginase; TSP1, thrombospondin-1.

to define their individual roles in tumor development, metastasis, and immune regulation.

How these tolerized DC populations are related and how they vary between different tumor types remains unclear. Further studies are warranted to improve our understanding of these associations and determine the functional relevance of these DC markers in human malignancies. In addition, an improved understanding of the relationships between specific oncogenic signaling pathways, the mechanisms driving metastatic progression, and the induction of DC tolerization in the tumor microenvironment promises to ultimately lead to the development of novel strategies for enhancing the efficacy of immunotherapy. Examining the tumor-derived soluble mediators of DC tolerization represents an important step in developing this higher order understanding necessary for translating these findings into clinical trials.

SOLUBLE MEDIATORS OF DC TOLERIZATION IN CANCER

Cytokines and Chemokines

DC development and migration are both significantly altered by paracrine mediators in the microenvironment. Tumors can manipulate this to their advantage to promote metastatic behavior and therapeutic resistance, in part through DC tolerization (**Table 1**) (56). In one of the earliest reports of tumor-mediated DC tolerization, progressive melanoma tissues refractory to a chemoimmunotherapy regimen were shown to inhibit DC-dependent T cell proliferation via the IL-10 cytokine (57). In a murine breast cancer model, TAMs were the primary source of IL-10 and IL-10R was expressed at high levels on DCs leading to the suppression of the anti-tumor cytokine IL-12. Blockade of IL-10 restored DC function and IL-12 production, and when combined with CSF-1 inhibition, reduced metastatic burden and improved the efficacy of paclitaxel chemotherapy in a manner dependent upon DC production of IL-12 (58). Tumor production of PGE2 impairs recruitment of NK cells responsible for CCL5 and XCL1 production, ultimately reducing intra-tumoral cDC1 migration leading to immune evasion and metastatic progression (59). Other paracrine mediators, such as the release of the tumor cell death factor high-mobility group box-1 (HMGB1), have been shown to bind to TIM-3 on DCs and impair their ability to orchestrate anti-tumor immune responses (33). Various mechanisms leading to β-catenin activation in tumors have also been implicated in the suppression of DC function via enhanced paracrine IL-10 signaling and inhibition of $BATF3^{+}$ $CD103^{+}$ dendritic cell recruitment via CCL4 downregulation (17, 60, 61).

In addition to tumor cells, other cell populations within the tumor microenvironment also express cytokines and chemokines

TABLE 1 | Tumor-derived Factors Inducing DC Tolerization.

Tumor derived factor	Mechanism of DC tolerance	DC marker(s) induced	References
PGE2	↓DC-mediated $CD8^+$ T cell proliferation and cytotoxicity	$MHCII^{lo}CD40^{lo}PD\text{-}L1^{hi}$ $CD11c^{lo}CD11b^{hi}$ Arg^+	(32)
TGF-β*	↓DC-mediated $CD8^+$ T cell proliferation and cytotoxicity	$MHCII^{lo}CD40^{lo}PD\text{-}L1^{hi}$ $CD11c^{lo}CD11b^{hi}$ Arg^+	(33, 34)
	↑DC-mediated Treg generation	↓CD86 and CD80, ↓IL-12	(41)
	↑pDC-mediated Treg generation	↑IDO1	(42)
	↓Recruitment of $Batf3^+$ DCs	N/A	(43, 44)
Wnt5a	↑DC-mediated Treg generation	β-catenin activation, ↑IDO1,↓IL-12,↓IL-6	(45, 46)
Wnt16b, Wnt1	↑DC-mediated Treg generation	β-catenin activation, ↓CXCL9	(47, 48)
HMGB1	↓DC-mediated $CD8^+$ T cell activation	↑TIM3	(33)
CXCL12	↑pDC Recruitment	N/A	(38)
GCSF	↓cDC1 lineage development	↓ IRF8	(6)
CCL2	↑Treg development	HLA-DR, PD-L1	(49)
VEGF	↓DC Maturation	↓MHCII, ↓CD40, ↓CD86, ↓IL-12	(50–53)
Tumor-derived Exosomes	Arginase-1 Delivery	↑Arg-1	(54)
	mir-212-3p Delivery	↓MHCII	(55)

*May also be derived from TAMs, CAFs, MDSCs.

that influence DC-dependent immunity. Stromal production of immunosuppressive chemokines CCL2, which promote tumor metastasis and M2 macrophage recruitment, have also been described (62, 63). CCL2 has been demonstrated to cooperate with Lipocalin-2 to induce Snail-dependent epithelial-to-mesenchymal transition (EMT) in tumors and to generate immunoregulatory DCs which exhibit decreased levels of HLA-DR expression, upregulated PD-L1 expression, and that functionally induce Treg differentiation (49). Collectively, these findings demonstrate that soluble cytokines and chemokines in the tumor microenvironment play an important role in tumor immune evasion by manipulating DC function.

IFN-γ is also well-known to induce the expression of compensatory regulators, including PD-L1, suppressor of cytokine 2 (SOCS2), and IDO1. Previous studies have described PD-1 and PD-L1 as markers of immunosuppressive DCs that proportionally increase as tumors progress (64, 65). In addition, constitutive IFN-γ signaling in metastatic human melanomas has been associated with an upregulation of the protein suppressor of cytokine signaling-2 (SOCS2) in DCs, which limits their ability to prime T cells, and may serve as a marker of "exhausted" regulatory DCs (66). Finally, granulocyte colony stimulating factor (G-CSF) suppresses cDC1 lineage development via Irf 8, leading to impaired anti-tumor immunity in breast and pancreatic cancer mouse models. Interestingly, fewer relative cDC1 cells in the bone marrow of breast and pancreatic cancer patients have also been associated with poor clinical outcomes (6). The ability of select soluble proteins to promote DC tolerance and contribute to cancer progression have been investigated more extensively and are discussed in the following sections.

Transforming Growth Factor-β

TGF-β, a paracrine mediator and principal driver of EMT in cancers, has also been implicated in DC tolerance. Co-culture studies of human DCs with lung carcinoma cells resulted in the generation of TGF-β-producing DCs, which exhibit decreased expression of CD86 and IL-12 and an increased ability to generate Tregs (41). TGF-β also promotes the conversion of tumor associated pDCs into a suppressive phenotype by inhibiting IFN-α and MHCI expression in cells activated by the toll like receptor 9 (TLR9) agonist, CpG. Mice lacking pDCs exhibit impaired tumor growth and Treg recruitment, and *in vivo* treatment with anti-TGF-β led to control of tumor growth and diminished recruitment of Tregs (42). Tumor-derived TGF-β suppresses CD80 and CD86 costimulatory molecule expression by DCs and promotes the development of a PD-L1-expressing immunosuppressive DC subset capable of inhibiting $CD8^+$ T cell activity in a metastatic ovarian cancer model (32, 33, 67). Loss of the type III TGF-β receptor (TGFβR3) negatively regulates the TGF-β signaling pathway in soluble form following its surface cleavage and suppresses metastatic progression. This process is accompanied by enhanced TGF-β signaling in local DC populations, resulting in IDO1 upregulation in pDCs and CCL22 production in cDCs, both resulting in the accumulation of Tregs and the suppression of anti-tumor immunity (68). These data indicate an overlap between TGFβ-mediated EMT and tumor-associated DC-mediated immunosuppression. TGF-β is known to contribute to an overall immunosuppressive microenvironment, promoting cross-talk in the tumor with pathways of stemness such as Wnt/β-catenin, which is correlated with impaired recruitment of $BATF3^+$ DCs (43, 44). Both small and large TGF-β inhibitors are currently being combined with anti-PD-1/anti-PD-L1 antibodies in ongoing clinical trials in a variety of solid tumor types (**Table 2**).

Vascular Endothelial Growth Factors

Vascular endothelial growth factors (VEGFs), are abundant in the tumor microenvironment where they play critical roles in angiogenesis, lymphangiogenesis, and metastatic progression. VEGF promotes recruitment of immunosuppressive myeloid cells, impairs cDC maturation, and facilitates a tolerant lymph node microenvironment (50–53). VEGF binds to neuropilin-1 during lipopolysaccharide-dependent DC maturation, resulting in downregulation of MHC II, CD40, and CD86 as well as diminished production of pro-inflammatory cytokines such as IL-12 (69). In a murine ovarian cancer study, tumor-derived β-defensin, in cooperation with VEGF, also recruits a $CD34^-CD8\alpha^-$ $MHC\text{-}II^{lo}CD11c^{hi}CD11b^{hi}$ DC subset which promotes tumor neovascularization and T cell exclusion (70). Given the plethora of therapeutics directed toward VEGF and potential combinatorial opportunities with immunotherapy, a

TABLE 2 | Clinical trial protocols that may impact DC tolerization.

Agent(s)	Mechanism of action	Registration number
Bemcentinib	Inhibition of Axl	NCT03184571
Pexidartinib ARRY-382 Cabiralizumab	Inhibition of CSF1R	NCT02777710 NCT02880371 NCT03336216 NCT03599362
M7824	Dual anti-PD-L1 blockade and TGFβ Trap	NCT03620201
Galunisertib	Type I TGFβ receptor serine/threonine kinase inhibitor	NCT02423343
SAR439459	Pan-TGFβ neutralizing antibody	NCT03192345
Regorafenib Ramucirumab Bevacizumab	Inhibition of VEGF (TKI or mAb)	NCT03406871 NCT03712943 NCT02337491 NCT02999295
LGK974 CGX1321 ETC-1922159	Blockade of Wnt Ligand Secretion via PORCN Inhibition	NCT01351103 NCT02675946 NCT02521844
MK-1454	STING agonism	NCT03010176
Epacadostat NLG919 BMS986205	Selective IDO1 inhibitor	NCT03006302 NCT03414229
Indoximod	Tryptophan Mimetic	NCT02073123
APX005M ABBV-927	CD40 agonism	NCT02706353 NCT03123783 NCT03502330 NCT02988960

better understanding of the role of VEGF in DC tolerization and the modulation of anti-tumor immunity could be generated based on immune monitoring studies accompanying these clinical trial protocols (**Table 2**).

Wnt Ligands

A role for the Wnt-β-catenin signaling pathway in the genetic re-programming involved in DC tolerization was originally described in 2007, where activation of β-catenin was demonstrated to promote IL-10-expressing $CD4^+$ T cells and generate tolerance in a model of experimental autoimmune encephalitis (71). Consistent with this, further work revealed that intestinal DCs required β-catenin to express immunosuppressive factors such as IL-10 and TGF-β and drive DC-dependent Treg differentiation in the gut (72). Based on these findings, we hypothesized that tumors may evolve mechanisms for stimulating the activation of the DC β-catenin signaling pathway to generate an immunotolerant microenvironment more conducive to disease progression. This line of investigation led to the discovery that the melanoma-derived WNT5A ligand both promotes the expression and supports the enzymatic activity of IDO1 in local DCs by inducing the synthesis of its required heme prosthetic group, protoporphyrin IX (45, 46, 73). In addition, by promoting β-catenin-dependent fatty acid oxidation in DCs, WNT5A further diminishes IL-6 and IL-12 pro-inflammatory cytokine expression. These alterations culminate in the development and accumulation of Tregs both *in vitro* and *in vivo* and are dominate over other TLR-dependent maturation stimuli. Illustrating the importance of this pathway, these studies also showed that the genetic silencing of *Wnt5a* in melanoma resulted in a significant influx of activated tumor antigen-specific $CD8^+$ T cells (46). In addition, the activation of β-catenin in DCs has been associated with the inhibition of antigen cross-presentation via a mechanism dependent upon a mTOR/IL-10 signaling pathway as well as the enhanced synthesis of retinoic acid capable of also promoting DC-dependent Treg differentiation (74–76). Other Wnt ligands such as WNT16B have been demonstrated to promote DC-mediated Treg development *in vitro* while *WNT1* overexpression in human and mouse lung cancers results in cDC1 β-catenin-dependent downregulation of the T cell-recruiting chemokine, CXCL9 (47, 48).

Wnt ligand signaling is perceived as being primarily limited to local and nearby cell populations. Indeed, the ability of many soluble protein-dependent mechanisms to alter distant DC function such as in draining lymph node tissues is more limited. The recent realization that tumor-derived exosomes are capable of genetically altering distant immune cell populations implies that these extracellular vesicles and their molecular cargo are likely to be very important players in DC tolerization and tumor-mediated immune evasion (54, 55, 77).

Tumor-Derived Exosomes

Over the past decade, a remarkable amount of evidence has emerged demonstrating the role of extracellular vesicles (EVs) in promoting tumor progression and metastasis (78). Exosomes are a sub-class of EVs ranging in size from 30 to 150 nm that primarily function as a vehicle to deliver nucleic acids and proteins (79–81). During tumor progression, tumor cells release exosomes that transit to distant lymphoid tissues and organs where they promote the formation of a tumor supporting microenvironment called the "pre-metastatic niche" (82). Several studies have demonstrated the capacity for exosomes to promote metastasis. For example, in 2011, Hood et al. demonstrated that melanoma exosomes home to the sentinel lymph node where they induce global gene expression changes in the lymph node microenvironment leading to the recruitment and proliferation of tumor cells (83, 84). Additionally, in a landmark study, Peinedo et al. demonstrated that through the delivery of MET tyrosine kinase, melanoma exosomes drive bone marrow progenitor cells toward a phenotype that promotes melanoma metastasis to the lung (85). In pancreatic ductal adenocarcinoma (PDAC) models, PDAC exosomes were found to carry macrophage migration inhibitory factor (MIF), which induces TGF-β signaling in Kupffer cells in the liver resulting in extracellular matrix (ECM) remodeling, a recruitment of bone marrow-derived macrophages and increased metastasis. Importantly, this phenomenon can be inhibited by blocking MIF (86). These pioneering findings were critical to establishing the role of exosomes and other EVs at promoting metastatic progression and immune evasion, however the effects of EVs on DC-mediated T cell activation remains unclear.

Due to the important role of DCs in activating adaptive immune responses, as well as the established capacity for tumor EVs to induce immune suppression, it is logical to anticipate that EVs can function in part by manipulating DC phenotype. Indeed, a recent report has demonstrated that EVs from ovarian cancers transit over long distances to the draining lymph node where they deliver arginase-1 to DCs resulting in a suppression of $CD4^+$ and $CD8^+$ T cell proliferation (54). In a separate study, Shen et al. report that tumor-derived exosomes induce immune suppression via the delivery of heat shock proteins (HSP72 and HSP105) to DCs leading to increased IL-6 production. IL-6 subsequently led to STAT3 activation and MMP9 expression in tumor cells, enabling increased metastatic invasion (87). In addition to the EV-mediated delivery of protein cargo to DCs, studies have also identified multiple miRNAs that play an important role in DC functions during tumor progression. For example, Ding et al. demonstrated that pancreatic cancer exosomes deliver miR-212-3p to DCs resulting in silencing of the transcription factor regulatory factor-X associated protein (RFXAP), a critical transcription factor for the expression of the MHC II genes (55).

While tumor cells produce a large amount of the EVs in circulation and in the tumor microenvironment during tumor progression, EVs of other cellular origin also can influence the DC phenotype. For example, Mittlebrunn et al. found that T cells can transfer miRNAs to APCs, including DCs, across the immune synapse, which can alter gene expression (88). Additionally, one study demonstrates that Tregs can transfer miRNAs (primarily miR-150-5p and miR-142-3p) to DCs resulting in the induction of a tolerogenic pathway including increased production of IL-10 and decreased IL-6 (89). While the importance of these mechanisms in the context of cancer and other diseases still remains unclear, the ability of EVs to manipulate DC gene expression via the delivery of miRNAs and protein cargo likely has repercussions in cancer immunity.

A large number of soluble mediators that include chemokines and cytokines, developmental and EMT-associated signaling molecules such as the Wnt ligands and TGF-β, as well as tumor manufactured exosomes have been implicated in the processes of DC tolerization and tumor progression. **Table 1** summarizes these varied mechanisms and how they modulate DC function. The extracellular nature of these mediators which rely on ligand-receptor interactions, represent a fortuitous area for drug development and potentially biomarker discovery. While numerous, the tumor-derived extracellular factors are likely to constitute only a fraction of the mechanisms leading to DC tolerance. Indeed, other cell populations and biological processes within the tumor microenvironment are also likely to influence DC tolerance.

OTHER COMPONENTS OF THE TUMOR MICROENVIRONMENT THAT DRIVE DC TOLERIZATION

Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) play pleiotropic roles in cancer cell progression, metastasis, and recurrence while contributing to immunotherapy resistance by shaping the tumor microenvironment and metastatic niche (90–94). MDSCs are generally categorized into two sub-populations, monocytic myeloid suppressor cells (M-MDSCs) and granulocytic or polymorphonuclear MDSCs (PMN-MDSC) (91, 95). In the mouse, M-MDSCs are phenotypically identified as $Ly6c^+Ly6G^-CD11b^+$ cells and PMN-MDSCs as $Ly6G^+Ly6C^{int}CD11b^+$ (95). In humans, M-MDSCs can be identified as $CD11b^+CD14^+CD15^-HLA\text{-}DR^{lo}$ and PMN-MDSCs as $CD11b^+CD14^-CD15^+HLA\text{-}DR^-$ (95). PMN-MDSCs and M-MDSCs are capable of utilizing several different mechanisms of immunosuppression both in the primary tumor bed as well as within distant sites of metastatic disease. This includes the ability of MDSCs to promote tumor growth and metastasis by inhibiting the maturation and antigen presentation function of DCs while secreting immunosuppressive mediators including IL-10, TGF-β, iNOS, VEGF, matrix metalloproteinases, and PGE2 (90, 96–98). In the pre-metastatic niche, MDSCs secrete IL-10 and IL-4, which may prime DCs for tolerance prior to tumor cell seeding (99).

Myeloid progenitors can also be shifted toward MDSC differentiation and away from DCs and macrophages by tumor-derived soluble factors that induce STAT3 activation, leading toward the MDSC phenotype by suppression of protein kinase C βII (100, 101). Tumor-derived S100A9 activates the NF-κB pathway in myeloid cells and suppresses differentiation toward DCs. S100A proteins can also be produced by MDSCs themselves in a STAT3-dependent manner, representing a potential positive feedback loop to suppress the DC lineage in the setting of a malignancy (102, 103). Other groups have also shown that Inhibitor of Differentiation-1 (ID1) is upregulated in DCs by melanoma-derived TGF-β, shunting DCs to differentiate toward an immature MDSC population. ID1 overexpressing bone marrow-derived DCs have also been implicated in the promotion of tumor growth and lung metastasis (104). Additional studies describing the effect of MDSCs on DCs in cancer are necessary to fully clarify the role of MDSCs in immune evasion.

Structural Components of the Tumor Microenvironment

While less well-described, the ECM may also have a significant role in impairing DC-mediated tumor rejection. Tumor-associated mucins, such as MUC1, promote metastasis formation and interfere with DC function. Mucins can mask TLRs on APCs, and bind to siglecs and galectins on immature DCs, facilitating IL-10 and TGF-β upregulation and reduced IL-12 and costimulatory molecule expression (105). Other ECM components such as Versican (VCAN) correlate with $CD8^+$ T cell exclusion and tumor-intrinsic β-catenin nuclear translocation in colorectal cancer, while proteolysis of VCAN into versikine reverses this effect through the recruitment of $CD103^+$ $MHCII^{hi}$ $BATF3^+$ DCs via IRF8 (106). Additionally, cancer associated fibroblasts (CAFs) are common cells in the tumor microenvironment that can produce the previously discussed suppressive soluble mediators TGF-β, IL-6, VEGFs, as well as express tryptophan 2,3-dioxygenase (TDO) leading to impaired DC maturation, costimulatory molecule expression, and antigen presenting function (107, 108). Furthermore, Cheng

TABLE 3 | Tumor intrinsic signaling pathways inducing DC tolerization or suppressing DC recruitment.

Intrinsic signaling pathway	Mechanism of DC tolerance	DC marker(s) induced	References
Snail-TSP1	↑DC-mediated Treg generation	↓MHCII, ↑IDO1	(118)
Loss of TβRIII	↑pDC-mediated Treg generation ↑cDC-mediated Treg recruitment	↑IDO1, ↑CCL22	(68)
Intrinsic β-catenin Activation	↓CCL4 ⇒ ↓BATF3$^+$ DC recruitment	n/a	(17, 61)
Tumor stemness	↓antigenicity, ↑ Immunosuppressive Cytokine production (IL-4, IL-10, TGFβ, CXCL12)	n/a	(119, 120)

et al. have shown that DCs co-cultured with CAFs upregulate IDO expression, downregulate costimulatory molecules, and facilitate Treg generation while exhibiting impaired antigen presentation in an IL-6-STAT3-dependent manner (109). Further study of the structural components within the tumor microenvironment as well as non-tumor and non-immune cells during metastasis may reveal additional therapeutic avenues for understanding and overcoming DC tolerization.

Tumor EMT and DC Tolerization

The adroit cancer cell invokes developmental pathways of wound healing that lead to mesenchymal transformation or EMT. EMT is a malleable dedifferentiated state during which tumors migrate from their primary site of development to other organs, even co-opting pathways utilized by immune cells for lymphatic trafficking (110–114). In healthy tissues, developmental processes like EMT are not active, however in pathological states like chronic inflammation, wound healing, and cancer, it plays a pivotal role. EMT has been associated with cancer stemness, immune evasion, and therapeutic resistance and is regulated by a network of transcription factors (TF), extrinsic factors such as hypoxia and nutrient deprivation, microRNAs (miRNAs), and long non-coding RNAs (lncRNAs) which contribute to metastatic progression. However, the effects of these specific elements of EMT on immunosuppression, namely local DC populations, is poorly understood (99, 115–117) (**Table 3**). When the EMT TF Snail was overexpressed in B16F10 mouse melanoma cells, CD4$^+$FoxP3$^+$ Tregs were generated via MHCII^{lo} IDO-expressing regulatory DCs that developed in response to tumor production of thrombospondin-1 (TSP1). Snail-overexpressing melanomas were resistant to peptide-pulsed DC vaccines while both intra-tumoral Snail-specific siRNA and neutralization of TSP1 restored T cell infiltration (118).

Cancers both shape and are molded by the myeloid compartment of their microenvironment, and the process of EMT both recruits and is enhanced by tumor-associated macrophages (TAMs) and MDSC populations. During EMT, tumor cells produce CSF1 which recruits TAMs that are able to produce a diverse array of growth factors, facilitating the formation of a metastatic niche (121). TAMs promote tumor progression via stimulation of cancer cell proliferation, as well as through secretion of IL-10 and TGF-β which impair effector T cells and inhibit DC maturation (58, 122). MDSCs also induce EMT via TGF-β and HGF in a mouse model of melanoma, whereby depletion of MDSCs reversed the EMT process (123). Therefore, suppressive myeloid populations are likely to play important roles in EMT and metastatic niche formation. However, it remains unclear how much the process of DC tolerization contributes to these processes.

Tumors may activate well-conserved stem cell pathways along with EMT, allowing for metastatic seeding, immune evasion, and therapeutic resistance (99, 116, 117, 119, 124). Hypoxia-inducible factors including HIF1α in the tumor microenvironment trigger both stemness and EMT programs in the tumor, while impairing DC mediated anti-tumor immunity (125, 126). Additionally, cancer stem cells produce the immunosuppressive cytokines IL-4, IL-10, IL-13, TGF-β, and express higher levels of PD-L1, B7-H3, CD47, and IDO1 (119, 120), enabling these stem cell populations to manipulate DC function. These cancer initiating cells also express CXCR4 and produce its ligand CXCL12, which leads to recruitment of regulatory DCs. These DCs produce CXCL12 themselves, representing a potential feed-forward mechanism where tolerant DCs recruited by cancer stem cells also maintain their stemness (127, 128).

Our understanding of how stem-like, mesenchymal tumor cells interact with and manipulate DC function is in its infancy. Given the tumor-initiating potential of these cells, determining their mechanisms of immune escape could lead to therapeutic strategies capable of suppressing metastatic progression. Understanding the underlying mechanisms involved in these tumor cell-DC and stromal cell-DC interactions will be critical for overcoming resistance to current immunotherapies.

DC TOLERIZATION AND CHECKPOINT INHIBITOR IMMUNOTHERAPY

While immune checkpoint blockade (ICB) has demonstrated durable efficacy across multiple tumor types, most patients do not respond (129–132). DCs have been shown to be critical for generating responses to anti-PD-1 antibody therapy (17, 133, 134). Indeed, a subset of BATF3$^+$ IRF8$^+$ cDC1s are not only required for T cell trafficking, but are also necessary for the generation of effector T cell responses to anti-PD-1 therapy (17, 135). Importantly, processes of metastasis co-opted by tumors such as EMT also influence DC maturation, migration, and phenotype, and are associated with ICB resistance in both melanoma and bladder cancer (136, 137). These studies highlight the importance of DCs in ICB and suggest that targeting DCs to reverse tolerogenesis may sensitize previously unresponsive patients to ICB.

Various strategies utilizing DCs to enhance anti-tumor immunity have been attempted but have so far met with limited success in the clinic, highlighting the need for novel approaches. Progress to date on *ex vivo* generated DC-based

vaccines is discussed elsewhere (138). Below we will review selected strategies to enhance anti-tumor immunity by indirect or direct reversal of DC tolerization mechanisms *in situ*. Selected clinical trials that deploy these agents to enhance responses to ICB are listed in **Table 2**.

Targeting the Tumor

Axl, a Tyro3, Axl, and MerTK family receptor tyrosine kinase implicated in the process of EMT, tumor progression, and metastasis, was found to be upregulated in patients with melanoma who do not respond to ICB (136). In a murine model of ovarian cancer, inhibition of Axl promotes tumor infiltration of $CD8^+$ T cell and $CD103^+$ DCs associated with an upregulation of the T cell recruiting chemokines CXCL9 and CXCL10. Axl inhibition enhanced anti-PD-1 ab responses, suggesting that Axl may have promise as a therapeutic target (139).

TAMs produce IL-10, suppressing DC production of IL-12, contributing to immune escape and metastatic progression. Pharmacologic modulation of the tumor's ability to recruit TAMs cia CSF1 has shown efficacy preclinically (58). It remains to be seen whether CSF1/CSF1R targeting will be effective in a clinical setting although clinical trials are underway. Other mechanisms for diminishing TAM recruitment by the tumor or re-polarizing TAMs to the M1 phenotype are also being investigated, including antagonism of CCL2 and/or CCL5 or their receptors (140). Recently, Panni et al. demonstrated in a murine pancreatic cancer model that a partial agonist of $CD11b^+$ repolarized TAMs and reduced MDSC infiltration while enhancing intratumoral $CD103^+$ DC populations, rendering previously resistant murine pancreatic tumors responsive to checkpoint blockade (141). These findings highlight the potential of modulating the myeloid compartment as a therapeutic approach in improving DC-mediated tumor rejection.

Augmenting the Cytokine and Chemokine Milieu

Manipulating the pro-tumorogenic cytokines and chemokines in the microenvironment also holds promise for sensitizing tumors to ICB. Our group has demonstrated TGF-β to promote IDO1 expression in plasmacytoid DCs, thus facilitating local Treg differentiation within the tumor microenvironment (68). We have further demonstrated that the inhibition of TGF-β enhances anti-CTLA-4 antibody treatment in an autochthonous melanoma model, and that delayed inhibition of TGF-β, but not initial combinatorial therapy, improves anti-PD-1 antibody responses by reversing adaptive resistance (142). TGF-β inhibitor clinical trials are underway (143, 144), and our data indicate these agents could be particularly effective in anti-PD-1 antibody-refractory tumors. As described above, previous studies have shown VEGF to suppress DC maturation. Bevacizumab, an anti-VEGF blocking monoclonal antibody, has been shown to decrease immature myeloid progenitor cells in the peripheral blood of breast, lung, and colorectal cancer patients and enhance IL-12 production (53). Combinations of anti-PD-1 and regorafenib or ramucirumab, both of which target VEGF receptor 2, have shown activity in gastrointestinal malignancies and are proceeding into later stage development (145, 146).

Targeting the DC

A variety of therapeutic avenues that aim to reverse tumor-induced tolerogenesis or to promote DC licensing and maturation have been pursued, and may ameliorate the shortcomings of DC-based vaccines and/or support the generation of clinical responses to ICB. As we previously discussed, IDO1 is active in regulatory DCs, but is also expressed by other cells including tumors. Preclinical data demonstrated anti-tumor activity with IDO1 inhibition (147–149). However, targeting IDO1 utilizing the selective inhibitor Epacadostat led to disappointing results in combination with pembrolizumab in the Phase III ECHO-301/KEYNOTE-252 trial (150). Potential reasons for this trial's failure are discussed elsewhere (151), however other methods of targeting the Tryptophan (Trp)—Kynurenine (Kyn)—aryl hydrocarbon receptor (AhR) pathway, including the Trp mimetic, Indoximod (152–154), dual IDO1 and tryptophan-2,3-dioxygenase (TDO) inhibition (155), and AhR inhibition (148) are all under investigation. IDO1 has also been reported to be upregulated by both hypoxia and adenosine, which are typical components of the tumor microenvironment encountered by DCs (156, 157). The HIF1a pathway is activated in tolerogenic DCs, and drugs targeting HIF1a have begun to move into the clinical setting (125). While adenosine has been demonstrated to drive a tolerogenic DC phenotype, importantly by the downregulation of IL-12, and has been shown to diminish $CD103^+$ DC recruitment in murine models, human data and the effect of adenosine inhibition on DCs in the setting of anti-tumor immunity is yet to be determined (158, 159). CD40 is expressed on APCs including DCs where it interacts with CD40L resulting in the upregulation of co-stimulatory molecules, MHC molecules, and the release of stimulatory cytokines including IL-12. Agonism of CD40 has been shown in preclinical models to enhance both vaccines and anti-PD-1 antibody treatment, and has moved into several clinical trials (160–162). Myeloid development into DCs is impaired by STAT3 signaling, and inhibition of JAK2/STAT3 has been shown to enhance anti-tumor responses through the promotion of DC maturation in preclinical models (101).

Finally, we have shown that inhibition of the Wnt/β-catenin pathway using an anti-Fzd receptor antibody or a Wnt ligand trap enhances anti-tumor immunity in autochthonous melanoma and Lewis lung carcinoma mouse models. These agents suppressed primary tumor growth and the formation of lung metastasis, and led to improved antigen-specific T cell responses over anti-PD-1 antibody treatment alone (163). We have further demonstrated that small molecule inhibitors of the PORCN acyltransferase enzyme, which effectively block Wnt ligand release, synergistically enhances the efficacy of anti-CTLA-4 antibody immunotherapy in pre-clinical models of melanoma. Others have also shown that deletion or pharmacologic inhibition of the Fzd co-receptors, LRP5/6, in DCs promoted their anti-tumor effects, further highlighting the therapeutic potential of targeting this pathway and a possible method of enhancing DC-based vaccines (164). Clinical trials examining the PORCN inhibitors in combination with anti-PD-1 antibody checkpoint inhibitor therapy are currently ongoing.

Other Strategies for Reversing DC Tolerance

A myriad of other approaches are also early in therapeutic development. Blockade of the CXCL12/CXCR4 axis utilizing an oncolytic viral vector or small molecule inhibitor impaired tumor stemness and enhanced DC activation (127, 128). Effects of these approaches on metastasis remains unclear, however. Type 1 interferons have been shown to be essential in murine models for DC-mediated anti-tumor immunity and can be induced through several mechanisms including toll-like receptor (TLR) and Stimulator of Interferon Genes (STING) agonists, both of which can impact DC maturation (165). TLRs bind bacterial cell wall components as well as danger-associated molecular patterns, CpG motifs, and ssRNA or dsRNA released during cell death, while the STING pathway is activated in response to cytosolic DNA. In addition to inducing Type 1 IFNs, co-stimulatory molecules (CD80, CD86, CD80) are also upregulated and targeting these pathways may assist in overcoming tumor-induced DC tolerance, particularly when designing therapies that manipulate the DC directly such as vaccines (166). This is exemplified in the development of CDX-1401 which contains a DC receptor (DEC-205, CD205) specific monoclonal antibody to deliver the conjugated tumor antigen NY-ESO-1 in combination with TLR7/8 agonists (167), and is now in clinical trials combined with IDO1 inhibition (NCT02166905). Other groups, in both mouse models and patients, have utilized radiation to release tumor antigens combined with a TLR3 agonist and Flt3L to expose DCs to antigen and foster DC maturation, resulting in immune-mediated tumor elimination at distant sites (known as the abscopal effect) (168). *In situ* DC targeting utilizing viral vectors can potentially provide tumor antigens, co-stimulatory, and maturation (i.e., Flt3L) signals (169), enabling DCs to overcome tumor-induced tolerance. Owing to their complexity, the development of viral vectors and other *in situ* methods to specifically target DCs are likely to require significant time before clinical outcomes are demonstrated (170). Other unique strategies, such as loading DCs with cancer stem cell lysates and the implantation of TLR 7/8 or STING agonists post-operatively to convert the surgical bed into an anti-tumor microenvironment have also been investigated (171–173).

Effectively reversing DC tolerization will likely require therapeutic approaches tailored to the individual tumor type, if not the patient. An improved understanding of tumor-induced DC tolerization mechanisms promises to streamline the selection of more novel, higher yield approaches for the development of future combinatorial immunotherapy strategies.

CONCLUSIONS, FUTURE DIRECTIONS, AND REMAINING KEY QUESTIONS

Herein, we have described those studies implicating an important role for DC tolerization in tumor-mediated immune evasion and immunotherapy resistance. While the field of tumor immunology has made significant advances in the clinic, the majority of our cancer patients still do not benefit from immunotherapy. A significant fraction of the ongoing effort to maintain this momentum in immuno-oncology remains focused on pharmacological and/or genetic manipulation of the effector phase of the anti-tumor immune response. However, we believe that it will be those approaches that effectively combine these strategies targeting cytolytic T cell function in the effector phase with those strategies designed to modulate DC functionality in the priming phase that will ultimately generate clinically meaningful responses in a broader population of cancer patients. Several critical unanswered question relevant to this area remain (**Box 1**). Technological advancements in single cell technologies promise to help identify populations of tolerogenic and tumor-promoting DCs, elucidating their defining features and perhaps therapeutic targets. We believe this understanding will be enhanced by a renewed focus on the tumor draining lymph node microenvironment and how tumors condition DCs within these tissues to induce immune tolerance. Collaborations between clinicians, translational investigators, and basic scientists will be critical in obtaining patient specimens in order to build upon the progress and promise of immunotherapy—to prevent and treat metastatic cancer, prolonging the lives of those affected.

BOX 1 | Unanswered questions about the role of tumor-mediated dendritic cell tolerance during immune evasion.

What dendritic cell markers define a tolerized DC and are there markers that define more nuanced phenotypes by the functional mechanism of suppression?

How do tumors suppress DC-mediated antigen cross-presentation and do these mechanisms vary by cancer type?

What are the tumor-intrinsic metastasis-initiating events that lead to DC tolerance in both the tumor microenvironment and the draining lymph node?

What role do tumor-derived exosomes play in the induction of DC tolerance, and can they be used as biomarkers and/or as therapeutic vectors?

What role do DCs play in resistance to immune checkpoint blockade and how can we modulate these DCs to enhance current immunotherapeutic strategies in a patient-specific manner?

What strategies will be most effective for modulating DC function in vivo?

AUTHOR'S NOTE

The figure was produced using Servier Medical Art.

AUTHOR CONTRIBUTIONS

ND and BH conceptualized the project. ND, MP, BT, and BH wrote and edited the manuscript and approved the final draft for publication.

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REVIEW
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Wnt Signaling Cascade in Dendritic Cells and Regulation of Anti-tumor Immunity

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Dendritic cells (DCs) control the strength and quality of antigen-specific adaptive immune responses. This is critical for launching a robust immunity against invading pathogens while maintaining a state of tolerance to self-antigens. However, this also represents a fundamental barrier to anti-tumor immune responses and cancer immunotherapy. DCs in the tumor microenvironment (TME) play a key role in this process. The factors in the TME and signaling networks that program DCs to a regulatory state are not fully understood. Recent advances point to novel mechanisms by which the canonical Wnt signaling cascade in DCs regulates immune suppression, and the same pathway in tumors is associated with the evasion of anti-tumor immunity. Here, we review these recent advances in the context of the pleiotropic effects of the Wnts in shaping anti-tumor immune responses by modulating DC functions. In addition, we will discuss how Wnt/β-catenin pathway in DCs can be targeted for successful cancer immunotherapy.

Keywords: Wnt, dendritic cells, beta-catenin (β-catenin), tumor microenvironment (TME), immunotherapy, anti-tumor immunity, immunotherapy

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INTRODUCTION

Dendritic cells (DCs) control the strength and quality of the adaptive immune response (1, 2). This is critical for launching robust immunity against invading pathogens while maintaining a state of tolerance to self-antigen (3, 4). This dichotomy assumes a particular significance in tumor immune surveillance, as tumors actively suppress immune response through multiple mechanisms by creating tolerance to their own antigen (2, 4). This also represents a fundamental barrier to successful cancer immune therapy (2, 4). Accumulating evidence suggest that DCs play a fundamental role in driving immune suppression against tumor-associated antigens (TAAs) (5–7). We now know that there are multiple subpopulations of DCs that differentially regulate anti-tumor immune responses, and that these subsets display tremendous functional plasticity in response to instructive signals from the tumor microenvironment (TME) and tumor vaccines (5–7). Although there has been much progress in understanding DCs-driven immune suppression, signaling networks and transcription factors within DCs that regulate these responses are not fully understood. Thus, understanding the molecular mechanisms by which DCs fine tune the anti-tumor immunity will be useful in the rational design of therapies against various tumors. Emerging studies suggest a fundamental role for the Wnt signaling cascade in shaping the functions of immune cells in the TME, particularly DCs, in this process.

In addition, recent studies have shown that tumor cell-intrinsic Wnt signaling plays a key role in the evasion of anti-tumor immunity in several human cancers. Here, we review these studies, highlight unanswered questions, and offer a conceptual framework for understanding the Wnt-signaling-mediated control of anti-tumor immunity.

Wnts IN THE TME

The TME is a distinctive niche that contains not only malignant cells but also cells of the immune system, the tumor vasculature, lymphatics, fibroblasts, perivascular stromal cells, and extracellular matrix components (8). Wnts are secreted lipid-modified cysteine-rich glycoproteins, and the TME contains high levels of the Wnt family of ligands (Wnts) (9). In the TME, malignant cells, stromal cells, DCs, and macrophages secrete Wnts (10). In humans, there are at least 19 different Wnt proteins all within 350–400 amino acids in length and 10 different cognate Frizzled (Fzd) receptors (9). The composition of Wnt proteins varies depending on the types of tumors. For example, Wnt3a and Wnt5a are expressed at higher levels in melanoma, whereas Wnt1 is highly expressed in lung adenocarcinoma. Wnts can exert autocrine effects on tumor cells and paracrine effects on immune cells. Although there has been much progress in understanding the role of the Wnt pathway in tumor development, progression, and metastasis, the role of this pathway in regulating anti-tumor immunity is only beginning to become better defined in the past few years.

Wnt ligands bind to Fzd receptors and activate multiple signaling pathways that includes the canonical and non-canonical pathways (9). Co-receptors Low-density lipoprotein receptor-related protein 5 (LRP5) and LRP6 are critical for canonical Wnt signaling and β-catenin, a multifunctional protein, is a central component of this pathway (9). In the absence of Wnt-signaling, β-catenin is sequestered by adenomatus polyposis coli (APC)/Axin complex, leading to its phosphorylation by glycogen synthase kinase 3β (GSK-3β), which targets it for degradation via the ubiquitin–proteosome pathway (**Figure 1**). Wnt-signaling inactivates APC/Axin complex, resulting in the accumulation and translocation of unphosphorylated free β-catenin to the nucleus. In the nucleus, β-catenin interacts with T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors and regulates transcription of several target genes (**Figure 1**) (9). In addition, Wnts can activate a number of non-canonical pathways, such as the planar cell polarity pathway and the Wnt-Ca^{++} pathways that activates several transcription factors, such as NFAT, AP1, JUN, CREB by β-catenin-independent mechanisms (**Figure 1**) (9). Recent reports show that tumor-intrinsic Wnt/β-catenin signaling facilitates immune evasion, whereas immune-cell intrinsic Wnt/β-catenin signaling drives immune suppression. However, the role of non-canonical Wnt signaling in regulating anti-tumor immune responses remains largely unexplored.

DCs IN THE TME

DCs also play a fundamental role in maintaining the balance between immunity and tolerance (1, 3). DCs can be classified into distinct subsets, based on their phenotype, microenvironmental localizations, and functions. A detailed discussion of DC subsets and their influence on adaptive immunity is outside the scope of the present review, and the reader is referred elsewhere (5, 6). Briefly, in mice, multiple subsets of DCs exist in both lymphoid ($CD8\alpha^+$ DCs; pDCs; $CD11b^+$ DCs) and non-lymphoid tissues ($CD103^+$ DCs; $CD11b^+$ DCs; pDCs). The TME contains all these major DC subsets. $CD8\alpha^+/CD103^+$ are the migratory DCs that excel in transporting tumor-associated antigens (TAAs) to the lymph nodes in a CCR7-dependent manner (7, 11–13). They also display enhanced ability to prime and cross-present tumor antigens to $CD8^+$ T cells. In general, $CD11b^+$ DCs are more effective at driving $CD4^+$ helper T cell responses; however, the role of these DCs in tumor immunity remain largely unexplored (14). The human counterparts of $CD8\alpha^+/CD103^+$ and $CD11b^+$ DCs have been identified, and they are $CD141^+$ and $CD1c^+$ DCs (14). Moreover, these DC subsets produce chemokines that are important for recruitment of CTLs to tumors (5, 7). Tumors also contain pDCs that are capable of producing high levels of type I interferons (IFN-I) in response to viral infection (5, 7, 15). Emerging studies have shown pDCs can drive effective anti-tumor specific immune responses where certain TLR ligands are used as tumor vaccine adjuvants (16). However, their role in anti-tumor immunity is still unclear and debatable.

MODULATION OF DC FUNCTIONS BY Wnts WITHIN TUMORS

Robust anti-tumor immune response is dependent on several factors, such as the degree of maturation and activation of DCs, their ability to capture tumor cells and tumor-associated (TAAs), them trafficking to tumors and tumor draining lymph nodes (TDLNs), and type of factors they produce in the TME. However, DCs within the TME are often perceived as tolerogenic or immunosuppressive. In general, it is believed that instructive signals within the TME program DCs to a tolerogenic or immunosuppressive state rather than to an inflammatory state. A key issue is the nature of the instructive signals and molecules within the TME that promote regulatory DCs. Emerging studies have shown that canonical Wnt signaling plays a key role in shaping anti-tumor immune responses by modulating DC functions (**Table 1**; **Figure 2**). Here, we will discuss how Wnts shape key functions of DC in the TME that influence robust T cell- mediated cancer immunity.

Regulation of DC Maturation and Activation by Wnts

DC maturation and activation is an important step in inducing robust immune response against tumors. Immature or tolerogenic DCs facilitate tolerance toward tumors whereas immunogenic/inflammatory DCs facilitate robust

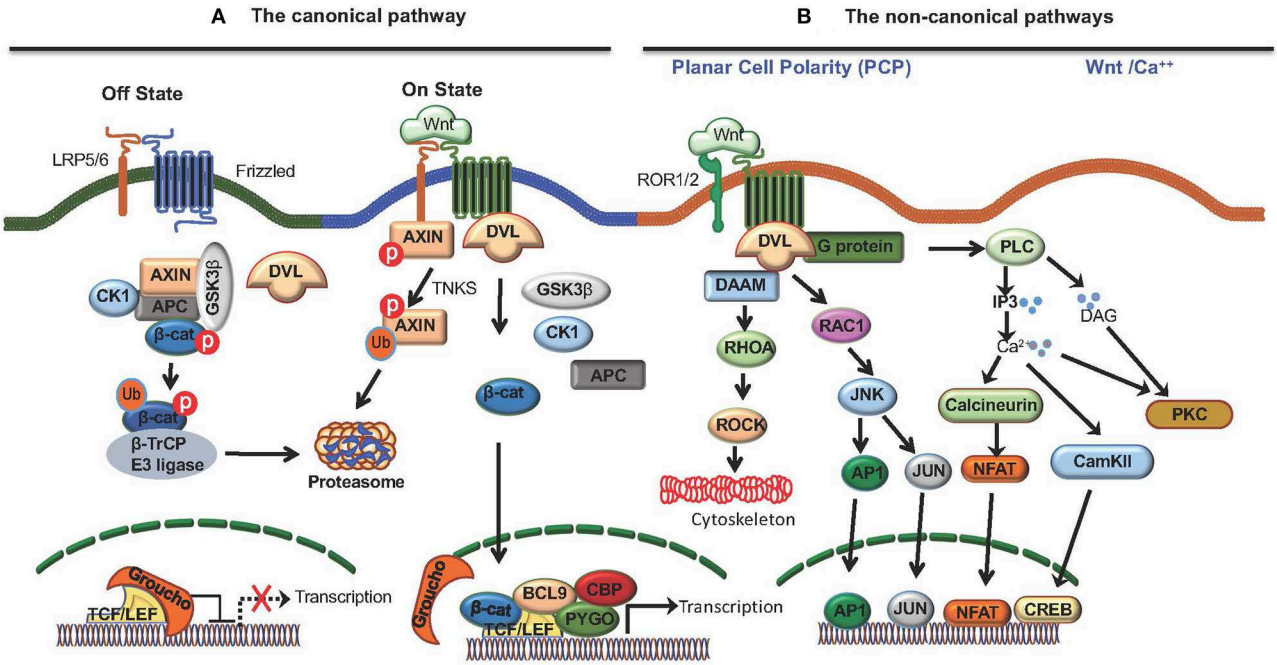

FIGURE 1 | The Wnt signaling pathways. Wnt ligands bind to Frizzled (Fzd) receptors and activate the canonical Wnt pathway that is dependent on co-receptors LRP5/6 and β-catenin, and the non-canonical Wnt pathway that is independent of β-catenin. **(A)** The canonical Wnt pathway. In the absence of Wnt signaling (off state), free β-catenin in the cytoplasm is sequestered by adenomatus polyposis coli (APC)/Axin/CK1α/GSK3β complex, leading to its phosphorylation by glycogen synthase kinase 3β (GSK-3β) which targets it for degradation via the ubiquitin–proteosome pathway. Wnts binding Fzd receptors and LRP5/6 co-receptors (on state) recruits DVL resulting in disassembly of (APC)/Axin/CK1α/GSK3β complex, and the accumulation and translocation of unphosphorylated β-catenin to the nucleus. β-catenin translocation to nucleus results in the displacement of the co-repressor Groucho on the TCF/LEF transcription factor and the recruitment of co-activators, such as BCL9, CBP, and PYGO resulting in the transcription of target genes. Moreover, cytoplasmic TNKS ubiquitinates Axin, targeting it for proteasomal degradation and causing disassembly of the β-catenin destruction complex. **(B)** Wnts also activate non-canonical pathways, such as the planar cell polarity pathway and the Wnt-Ca^{++} pathway. The planar cell polarity pathway regulates cytoskeletal organization through Rhoa and Rock whereas the Wnt-Ca^{++} pathway activates several transcription factors, such as NFAT, AP1, JUN, CREB by β-catenin-independent mechanisms.

anti-tumor responses (1, 49). Marked differences were observed in maturation and activation of status between tolerogenic/regulatory DCs and immunogenic/inflammatory DCs (1, 3). DCs in the TME have been shown to exhibit immature state or tolerogenic state (7, 49). Immature DC or regulatory DCs express markedly lower levels of costimulatory molecules (CD80, CD86, and CD40) whereas the immunogenic or inflammatory DCs express markedly higher levels costimulatory molecules (1, 3). DCs recognize a diverse array of microbial structures through multiple receptors collectively known as pattern recognition receptors (PRRs) (50). In addition, DCs can recognize damage-associated molecular patterns (DAMPs) and other endogenous ligands that are released from dying tumor cells through PPRs (51–53). PRRs include Toll-like receptors (TLRs), C-type lectin like receptors (CLRs), RIG-I like receptors (RLRs), and Nod-like receptors (NLRs) (50). TLR ligands have gained great interest in cancer immunotherapy in the recent years for their potential use as vaccine adjuvants (16, 54). PRR-mediated signaling controls DC functions, such as antigen uptake, antigen presentation, activation, and cytokine production that are critical for anti-tumor immunity (50). In general, PRR engagement potently activates DCs by upregulating the surface expression of maturation markers, such as MHCII, CD80, CD83, and CD86 (50). Even though, PRR ligands are there in the TME (51, 52), tumor-associated DCs display markedly decreased expression of co-stimulatory molecules (49). Emerging studies have shown an important role for Wnts in regulating maturation and activation of tumor-associated DCs. Earlier *ex vivo* studies on human and murine DCs have shown that exposure to Wnt1, Wnt3a, and Wnt5a that activates β-catenin can program DCs to a regulatory state with decreased expression of co-stimulatory molecules (17, 20, 21). Similar observations were made with murine and human DCs upon blocking GSK3β activation (a negative regulator of β-catenin) or activating β-catenin in DCs (55, 56). Such Wnt-conditioned regulatory DCs failed to upregulate co-stimulatory molecules even in response to TLR ligands (10, 57). Further, mechanistic studies have shown that the canonical Wnt signaling can negatively regulate the inflammatory pathways, such as the NF-kB and MAPK pathways, which are critical for DC activation (58). Accordingly, tumor DCs lacking LRP5/6 or β-catenin isolated from knockout mouse models displayed increased activation with upregulated expression of co-stimulatory molecule and decreased expression of co-inhibitory molecules (PD-L1, PD-L2) (21, 22). Furthermore, studies using small molecule inhibitors of canonical Wnt signaling in tumor bearing mice showed an

TABLE 1 | Evidence for involvement of the Wnt/β-catenin pathway in regulating immune suppression and immune cell exclusion.

Observations	References
Wnt signaling regulating DC function	
Wnt/β-catenin signaling regulates differentiation, maturation, and activation of DCs	(17–20)
Like tumor DCs, Wnt-conditioned DCs are programmed to a regulatory state to induce Tregs	(19)
Tumor DCs-deficient in LRP5/6 or β-catenin is more potent in capturing and cross-presenting TAAs to $CD8^+$ T cells	(21–24)
Tumor DCs lacking LRP5/6 or β-catenin are programmed to induce Th1/Th17 cells	(21, 22)
Active Wnt/β-catenin signaling affects trafficking of DCs to tumors and TDLNs	(17)
Active Wnt/β-catenin signaling in tumor DCs regulates metabolic pathways involving FAO, vitamin A, and tryptophan to induce regulatory T cell (Treg) response	(21–26)
Wnt-signaling in tumor DCs suppresses chemokines that are critical of recruitment and accumulation of CTL in the TME	(17, 27, 28)
Wnt signaling in T cells	
Wnt/β-catenin signaling in Tregs promotes its survival, activity and infiltration	(29–31)
Wnt3a/β-catenin signaling suppresses effector T cell differentiation	(28, 32)
Wnt/β-catenin-signaling limits the expansion of tumor-antigen specific $CD8^+$ T cells and is important in the maintenance of stemness of memory $CD8^+$ T cells	(28, 32)
Wnt signaling in $CD4^+$ T cells favors Th17 cell differentiation	(33, 34)
Wnt signaling in macrophages	
Wnt-β-catenin signaling regulates macrophages functions, such as adhesion, migration and tissue recruitment	(35, 36)
Wnt-b-catenin signaling promotes M2-like polarization of TAMs resulting in tumor growth, migration, metastasis, and immunosuppression	(35, 37, 38)
Wnts produced by macrophages drive contribute to tumor cell invasiveness and tumor growth	(35, 37, 38)
Wnt signaling in MDSCs	
The MUC1-β-catenin pathway regulates MDSC-mediated immune suppression in the TME	(39)
The PLCγ2-β-catenin pathway in MDSCs promotes tumor progression	(40, 41)
Wnt signaling in NK cells	
Wnt signaling in NK cells regulates maturation and effector functions	(42)
Wnt signaling in tumor cells	
Tumor growth, migration, and metastasis	(43–45)
Immune cell exclusion	(17, 27, 28, 46–48)

augmented DCs activation with an increased expression of co-stimulatory molecules and decreased expression of co-inhibitory molecules (21, 22, 56). Collectively, these studies show that Wnt/β-catenin signaling interferes with DC maturation and activation in the TME.

Regulation of DC Trafficking by Wnts

The migration of DCs is essential for tumor immune surveillance (5, 6). This involves, DCs migrating to tumor tissues, capturing and endocytosing dead tumor cells or cellular debris, and transporting TAAs to TDLNs where they prime and activate tumor-specific T cells (7, 11–13). This is dependent on the expression of specific chemokine receptors on DCs and its cognate chemokine ligand expression within the TME and TDLNs. The migration of DCs to TDLNs requires CCR7 expression whereas the recruitment of DCs to the TME is dependent on chemokines, such as CCL4, CCL5, and XCL1 (5). However, only a small fraction of DCs end up migrating to tumor tissue and subsequently to TDLNs. This is due to factors in the TME that control the expression of chemokine receptors and chemokines. Recent studies have shown that Wnts in the TME regulate DC trafficking by regulating chemokine receptors and chemokines via two different mechanisms. First, DC-intrinsic Wnt-signaling regulates its migration to tumors and TDLNs. Evidence supporting this come from studies showing that conditional deletion of either LRP5/6 or β-catenin in DCs in mice lead to marked increase in the number of DCs in TDLNs and TME. Furthermore, similar observations were made upon treating tumor-bearing mice with pharmacological inhibitors of the Wnt-β-catenin pathway (21, 22). Tumor DCs also produce chemokines that are critical for accumulation of T cells within the TME (5). A recent study on lung adenocarcinoma (LUND) has shown that tumor DC-intrinsic Wnt signaling plays a key role in blocking T cell infiltration into the tumors and driving cross-tolerance to tumor antigens (17). Mechanistically, Wnt1-mediated β-catenin signaling in tumor DCs resulted in transcriptional silencing of CC/CXC chemokines that are critical for recruiting effector T cells to the TME (17). In line with these observations, other studies have shown that the pharmacological blocking of the canonical Wnt signaling in DCs result in increased accumulation of effector T cells within the TME (21, 22). Second, tumor-intrinsic Wnt-signaling regulates the evasion of anti-tumor immunity by regulating the expression of chemokines that are critical for recruitment and accumulation of DCs in the TME (10). In this context, it was shown that an active Wnt-β-catenin signaling in melanoma cells suppresses production of CCL4 (C-C motif chemokine ligand 4) and consequently reduces migration and accumulation of $CD103^+$ DCs in the TME (46). $CD103^+$ DCs play an important role in recruitment of CTLs in TME through their production of CXCL9 (C-X-C motif chemokine ligand 9) and CXCL10 (46, 47). Collectively, these studies suggest that Wnt/β-catenin signaling regulates trafficking of DCs and T cells to the tumor tissue by regulating the expression of chemokine receptors and chemokines.

Regulation of Antigen Delivery and Presentation by Wnts

Effective cross-priming of tumor-specific $CD8^+$ T cells by DCs involves efficient capture and cross-presentation of tumor-associated antigens (2, 4, 5). However, DCs within the TME are less efficient in cross-priming $CD8^+$ T cells (2, 4, 5). In addition

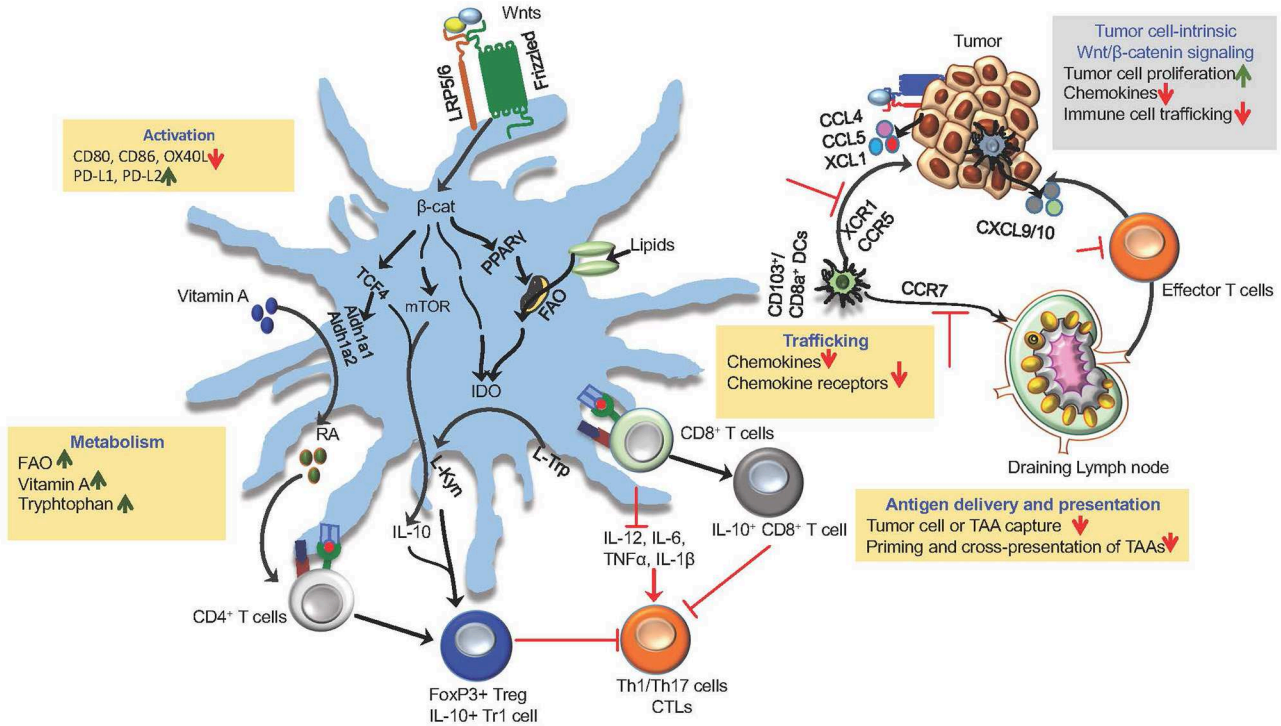

FIGURE 2 | Wnt/β-catenin signaling in DCs and tumors in driving immune suppression and immune evasion. The canonical Wnt signaling shape anti-tumor immune responses by modulating DC functions, such as activation, trafficking, capturing and cross-presenting TAAs and expression of immune regulatory factors (RA, IL-10, IDO, TGF-β). Activation of β-catenin and its down-stream mediators mTOR and TCF4 in tumor DCs through Wnt-LRP5/6 signaling leads to the induction of anti-inflammatory factors RA, IDO, and IL-10 that are critical for promoting Treg response to tumor antigens. Wnts regulate trafficking of DCs to tumor and tumor-draining lymph nodes (TDLNs) by regulating the expression of chemokine receptors CCR5, XCR1, and CCR7. Within the TME, DC-intrinsic Wnt signaling limits the recruitment of CTLs by suppressing the expression of chemokines CXCL9/10. Wnt signaling leads to metabolic alterations in DCs that programs them to a regulatory state. Wnt-β-catenin-PPAR**γ**-mediated signaling shifts DC metabolism from glycolysis to FAO by upregulating the expression of the carnitine palmitoyltransferase-1A (CPT1A) fatty acid transporter. In addition, Wnt-β-catenin-TCF signaling in tumor DCs promote vitamin A metabolism through the induction of enzymes involved in RA synthesis and tryptophan metabolism through the induction of IDO. The canonical Wnt signaling pathway in tumor cells promotes tumor growth and immune evasion via chemokines (CCL4, XCL1, CCL5) that are critical for the recruitment and accumulation of DCs within the TME.

TABLE 2 | Some knowledge gaps in understanding how Wnts regulate anti-tumor immunity.

The role of the canonical Wnt signaling in shaping the innate immune functions of DC subsets ($CD103^+/CD8a^+$ DCs vs. $CD11b^+$ DCs vs. pDCs). How Wnt signaling shape the innate immune functions of migratory vs. non-migratory DCs in the TME?
Immune cell type-specific differences in Wnt/β-catenin signaling in the TME (e.g., DCs vs. TAMs vs. MDSCs vs. Tregs vs. CTLs)
The role of the canonical Wnt signaling in intercellular cooperation in the TME and their relative contributions to anti-tumor immunity induction in a variety of tumor settings
Tumor-specific differences in Wnt-signaling and its impact on anti-tumor immunity (e.g., Melanoma vs. intestinal cancer vs. liver cancer vs. breast cancer)
The role of the non-canonical Wnt pathways in regulating the induction and maintenance anti-tumor immunity
How are signals from dying cells (chemotherapy), DAMPs, and endogenous TLR ligands integrated with signals from Wnts, and their effect on anti-tumor immunity? Consequence of Wnt inhibitors plus TLR vaccine adjuvants on anti-tumor immune responses
The role of Wnts in regulating anti-tumor immunity to immune checkpoint inhibitors (ICI), such as anti-PD1, anti-CTLA4, or anti-PD-L1. Consequence of blocking the Wnt/β-catenin (inhibitors as possible adjuvants) plus ICI on anti-tumor immune responses

to regulating DC trafficking, recent studies with fluorescently labeled proteins have shown that in the absence of the canonical Wnt signaling, DCs are more efficient in capturing TAAs and actively transporting them to the TDLNs (21, 22). These findings are further supported by observations that LRP5/6- or β-catenin-deficient tumor DCs are more potent in capturing TAAs and are robust in priming and cross-presenting TAAs to $CD8^+$ T cells (23–25, 59). Similar observations were made upon pharmacologically blocking canonical Wnt signaling in tumor DCs (17, 23–25, 27, 48, 59). In contrast, Wnt-conditioned DCs or tumor DCs expressing a constitutively active form of β-catenin is less potent in capturing and cross-presenting TAAs (17, 21, 22, 27, 48). Collectively, these studies show that the activation of canonical Wnt signaling pathway in tumor DCs suppresses efficient capture of tumor-associated antigens and cross-priming of $CD8^+$ T cells.

Metabolic Reprogramming of DCs by Wnts

Aberrant Wnt signaling leads to metabolic alterations in cancer cells that are critical for their survival and proliferation (60). Interestingly, Wnt signaling also plays an important role in the metabolic reprogramming of DCs in the TME (60, 61). Cellular metabolic pathways play a critical role in modulating the functions of DCs (61–63). Emerging evidence show that potential metabolic differences exist among DC subsets and also between tolerogenic and immunogenic DCs (60, 62). Immature or tolerogenic DCs show a catabolic metabolism that is manifested by increased oxidative phosphorylation, fatty acid oxidation (FAO), and glutaminolysis (61, 63, 64). In contrast, immunogenic or inflammatory DCs display an anabolic metabolism that is marked by increased glycolysis (61, 63, 64). Accumulating evidence show that tolerogenic response to tumors is also related to the metabolic dysfunction and metabolic reprograming of immune cells within the TME (26, 61, 63, 64). A recent study has shown that Wnt-mediated signaling shifts DC metabolism from glycolysis to FAO by upregulating the expression of the carnitine palmitoyltransferase-1A (CPT1A), an enzyme important for the transport of fatty acids into mitochondria (26). Furthermore, this study also revealed that this metabolic shift is dependent on the activation of β-catenin and PPAR-γ in DCs (26). In addition, other studies have shown that the canonical Wnt signaling in tumor DCs promote the metabolism of vitamin A and tryptophan through the induction of enzymes involved in retinoic acid (RA) synthesis and indoleamine 2,3-dioxygenase-1 (IDO) (22, 25). Collectively, these studies have conclusively demonstrated that Wnt-mediated metabolic shift is critical for programming DCs to the regulatory state in the TME.

Induction of Immune Regulatory Factors by Wnts

The types of cytokines and other factors secreted by DCs program the differentiation of newly primed $CD4^+$ and $CD8^+$ T cells into effector T cell or regulatory T cells (1, 3). The TME contains higher levels of immune regulatory factors, such as IL-10, RA, and TGF-β that actively suppress differentiation and expansion of tumor-specific effector T cells (10, 57). Given the enormous burden of endogenous PRR ligands, DCs associated with the tumors express higher levels of immune regulatory factors that drive Treg response (10, 57). Recent studies have highlighted an important role for the Wnt signaling in tumor-associated DCs in regulating the expression of immune regulatory factors (21–26). Unlike DCs stimulated with microbial products, Wnt-conditioned DCs do not release immunostimulatory cytokines; instead they express IDO, IL-10, RA synthesizing enzymes and TGF-β1 (21–26). In contrast, tumor DCs that are deficient in LRP5/6 or β-catenin expressed markedly higher levels of IL-12, IL-6, IL-23, and TNF-α, and lower levels of immune regulatory factors (21, 22). These observations where further corroborated by studies showing that pharmacological blocking of the Wnt/β-catenin pathway in tumor DCs decreased the expression of RA synthesizing enzymes, IDO, IL-10, and TGF-β1 while markedly increased the expression of inflammatory cytokines (21–26).

Wnt SIGNALING NETWORKS IN DCs THAT DRIVE Treg RESPONSES

The TME conditions DCs to acquire tolerogenic or immunosuppressive properties by activating the immune regulatory pathways (1, 5, 49). Although there has been much progress in understanding the role of DCs in inducing regulatory responses to tumor antigens, we understand very little about the regulatory signaling networks that program tumor DCs to become tolerogenic or immunosuppressive. In this context, there are emerging insights into the roles of the Wnt signaling network in DCs orchestrating tolerogenic responses to tumors.

The Wnt-β-Catenin-RA Signaling Axis

First, a key mechanism by which Wnts in the TME drives immune suppression is through induction of vitamin A-metabolizing enzymes in DC via the β-catenin/TCF pathway (22). TME contains high levels of RA, and APCs are major producers of RA (22, 65). It is well-established that RA, an active metabolite of vitamin A, regulates a broad array of immune responses (66, 67). RA synthesis is a tightly regulated process that includes several key enzymes. Within cells, RA is produced from vitamin A (retinol) via a two-step enzymatic pathway where retinol is first oxidized to retinaldehyde (retinal) by alcohol dehydrogenases (ADH-1, -4, -5), which is next converted to RA by retinal dehydrogenases (Aldh1a1 and Aldh1a2) (50). In contrast to the gut DCs, DCs in the periphery do not express Aldh1a1 and Aldh1a2, but do constitutively express different isoforms of ADH, and hence, they lack the ability to convert vitamin A to RA (68, 69). However, within the TME, DCs express enzymes Aldh1a1 and Aldh1a2 and acquire the ability to metabolize Vitamin A to RA (22). RA produced by tumor DCs acts directly on $CD4^+$ T cell cells, inducing Treg cell response while suppressing T effector cell response. Pharmacological blocking of RA synthesis or RA signaling affected the ability of tumor DCs to induce Treg (22). In line with these observations, DCs isolated form LRP5/6- or β-catenin- conditional knockout mice bearing B16F10, LLC, or EL4 tumors expressed markedly lower levels of vitamin A-metabolizing enzymes, and these DCs were less potent in inducing Tregs in response to tumor antigens (21, 22). Collectively these studies have shown that tumors, through DCs, exploit the LRP5/6-β-catenin-RA pathway as a mechanism of immune suppression by inducing regulatory T-cell responses.

The Wnt-β-Catenin-PPARγ-IDO Signaling Axis

A second key mechanism by which Wnt-β-catenin signaling in DCs promotes immune suppression is through the induction of IDO (25, 26). IDO is an immunoregulatory enzyme that catalyzes the degradation of the essential amino acid tryptophan into kynurenines (59). Previous work has identified that tumor DCs express IDO and depletion of tryptophan dampens T-cell proliferation and the generation of kynurenine drives Treg differentiation (59). However, the signaling pathways in DCs that control IDO expression and its activity remained unknown. In this context, recent studies have shown an important role for the Wnt5a-β-catenin-PPARγ signaling pathway in regulating

IDO expression and activity (25, 26). Furthermore, abrogation or blocking this pathway in a transgenic murine melanoma model markedly reduced IDO expression and activity in DCs with augmented anti-tumor immune responses (25, 26). These data indicate that Wnt5a-conditioned DCs promote the differentiation of Tregs in an IDO-dependent manner and that this process serves to suppress melanoma immune surveillance. Since Wnt5a activates the non-canonical Wnt pathway in DCs (18, 19), further mechanistic studies are necessary to understand how Wnt5a activates β-catenin in DCs within the TME. Also, additional studies are warranted to understand whether β-catenin directly regulates IDO expression in DCs through TCF4 or PPAR**γ**.

The Wnt-β-Catenin-mTOR-IL-10 Signaling Axis

Finally, the Wnt-β-catenin pathway in DCs can drive T cell tolerance to tumors through the induction of IL-10. IL-10 is a key immunosuppressive cytokine that regulates a broad array of immune responses (70). Recent studies have shown that Wnt-β-catenin-dependent activation of mTOR and TCF4 in DCs regulates IL-10 expression (21, 23, 24). IL-10 produced by DCs exert autocrine effects to suppress cross-priming of tumor-specific $CD8^+$ T cells (23). Furthermore, tumor DCs that are deficient in LRP5/6 or β-catenin express markedly lower levels of IL-10 and are more potent in cross-priming $CD8^+$ T cells (21, 23, 24). In line with these observations, selective blocking of LRP5/6, β-catenin, and mTOR activation resulted in markedly reduced IL-10 production by DCs with augmented anti-tumor immune response in mice (21, 23, 24). However, further studies are warranted to understand mechanistically how β-catenin regulates mTOR activation in tumor DCs to induce IL-10. Collectively, these studies illustrate a critical role for the canonical Wnt signaling network in programming tumor DCs to induce regulatory response through induction of immune regulatory factors.

EFFECTS OF Wnts IN MODULATING FUNCTIONS OF OTHER IMMUNE CELLS WITHIN THE TME

The role of the Wnt signaling cascade in other immune cells in promoting tolerogenic response is poorly understood. Beyond the ability to modulate DC functions, Wnts in the TME can directly influence the development and effector functions of various immune cell types. Wnt-mediated immunological tolerance to tumors is often considered to occur by multiple processes. Emerging studies are beginning to provide insights into the mechanisms by which Wnt signaling cascade directly modulates immunological functions of other immune cells, such as macrophages, myeloid-derived suppressor cells (MDSCs), Tregs, $CD4^+$ T cells, $CD8^+$ T cells, and NK cells (71). There are several excellent studies and reviews that discuss extensively how the Wnt signaling pathway shape the functions of other immune cells (43, 44, 60, 71, 72) and will thus be discussed only briefly (**Table 1**). Tumor-infiltrating T cells express markedly higher levels of Wnt3a and β-catenin, and display dysfunctional and exhausted effector memory phenotype (28). Furthermore, Wnt-mediated activation of β-catenin/TCF1 pathway activation suppresses naïve T cell differentiation and terminal effector differentiation of $CD8^+$ T cells (28, 32). Likewise, T cell-infiltrating human hepatocellular carcinoma and colorectal cancer are dysfunctional and show an exhausted effector memory phenotype (43, 44, 60). In line with these observations, Wnt3a neutralization in tumor-bearing mice controls tumor growth by augmenting the expansion of tumor-antigen-specific $CD8^+$ T cells with enhanced effector functions (27). Furthermore, the induction of Wnt signaling is critical for maintaining stemness of memory $CD8^+$ T cells and Treg-intrinsic β-catenin signaling is critical for Treg survival, migration and suppressive functions (29–31). IL-17A-producing $CD4^+$ T (Th17) cells play an important role in the pathogenesis of colorectal cancer (33, 34). In murine colorectal cancer model, forced expression of β-catenin in $CD4^+$ T cells caused increased IL-17A expression that favor tumor progression. Natural killer T (NKT) cells are specialized CD1d-restricted T cells that play a critical role in tumor immune surveillance (73). Cytokines produced by activated NKT cells regulate the functions of other immune cells in the TME. There is ample evidence that Wnts can modulate anti-tumor immune response through NKT cells by suppressing the expression IFN**γ** (42). In addition, the chemokines CCL5 and XCL1 produced by NK cells also contribute to the recruitment and accumulation of DCs, macrophages, and Tregs within the TME (74, 75). However, it is not known whether Wnt signaling in NK cells regulates the expression of these chemokines. Tumor-associated macrophages (TAMs) play an important role in tumor progression and immune suppression (76–78). Wnts regulate macrophage functions, such as adhesion, migration, and tissue recruitment (35). In addition, it is well-documented that Wnts produced by macrophages is critical for tissue development and repair (35). Emerging studies have shown that Wnts produced by macrophages contribute to tumor cell invasiveness and tumor growth (36, 37). Furthermore, active Wnt-β-catenin signaling in macrophages programs to M2-phenotype that drives cancer cell growth, migration, metastasis, and immunosuppression (37, 38). MDSCs are a heterogeneous mix of cells that expand during cancer and potently suppress T cell responses (79, 80). Interestingly, in the murine extraskeletal tumor model, the PLC**γ**2-β-catenin pathway plays an important role in tumor progression, suggesting an anti-tumorigenic role for this pathway in MDSCs (40, 41). Conversely, in EL4 tumor model, the MUC1-β-catenin pathway is critical for MDSC development, suggesting a key role in MDSC-mediated immune suppression instead of tumor progression (39). Collectively, these studies show that in addition to DCs, Wnts in the TME can modulate anti-tumor immunity by directly regulating the effector functions of other immune cells.

TARGETING THE CANONICAL Wnt SIGNALING PATHWAY FOR CANCER IMMUNOTHERAPY

Accumulating evidence from studies involving human cancers suggest that enhanced Wnt signaling is associated with worst

clinical outcomes. Hence, targeting the canonical Wnt signaling pathway is a promising approach to overcome immune evasion by tumors and to augment anti-tumor immunity by potently activating DCs. Pre-clinical studies have shown that the canonical Wnt signaling pathways can be targeted at four different levels to overcome tumor-mediated immune suppression and augment anti-tumor immunity. These strategies include (1) blocking ligand-receptor interaction, (2) blocking Fzd-LRP5/6 signaling (PORCN inhibitors), (3) promoting β-catenin degradation (tankyrase enzyme or TNKS inhibitors), and (4) blocking β-catenin-TCF interaction (β-catenin inhibitors) (**Figure 3**). Pharmacological inhibitors of the Wnt pathway exist and several of them are currently in clinical testing [extensively reviewed in (43–45)]. Here, we will briefly discuss pre-clinical studies related to effects of blocking the canonical Wnt pathway on anti-tumor immunity.

Blocking Wnt Ligand Interaction With Fzd Receptors

Emerging studies indicate a strong correlation between specific Wnt ligand expression based on the type of tumor (43–45). Thus, blocking specific Wnt ligand interaction with cognate Fzd receptors represents a potential strategy to restrain tumor cell proliferation while boosting the anti-tumor immunity (43–45). In this context, a previous study (81) using a murine model of prostate cancer has shown that administration of Wnt3a-neutralizing antibodies restrained tumor growth. However, the impact on anti-tumor immunity was not assessed in this study (81). In another critical study, it was shown that administration of Wnt3a-neutralizing antibodies in tumor bearing mice restrains tumor growth by potently activating DCs and boosting the expansion of tumor-specific $CD8^+$ T cells with improved effector activities (27). Likewise, neutralizing or silencing Wnt1 in lung adenocarcinoma model augmented DC activation, resulting in increased recruitment and accumulation of CTLs in the TME (17). In addition, In addition, monoclonal antibodies (mAbs) that targets the different Fzd receptors are in preclinical stage and early clinical trails in humans (43–45). There are also several recently developed therapeutic agents targeting dickkopf family members (DKK1) that inhibit the binding of Wnts to co-receptors LRP5/6 (43–45). However, further studies are warranted to understand the immune consequence of DKK1 as therapeutic agents in tumor settings.

Blocking Fzd-LRP5/6 Signaling

Using clinically relevant murine tumor models, studies have examined anti-tumor immune responses by blocking Fzd-LRP5/6 signaling. Porcupine (POCRN) is a membrane-bound-O-acetyltransferase enzyme that palmitoylates Wnts, which is critical for its interactions with co-receptors LRP5/6 and Fzd receptors (10). Treatment of mice with established B16-OVA tumors or EL4-OVA tumors with IWP-L6 or C59 delayed tumor growth, and this was due to marked increase in tumor-antigen-specific $CD4^+$ and $CD8^+$ effector T cells

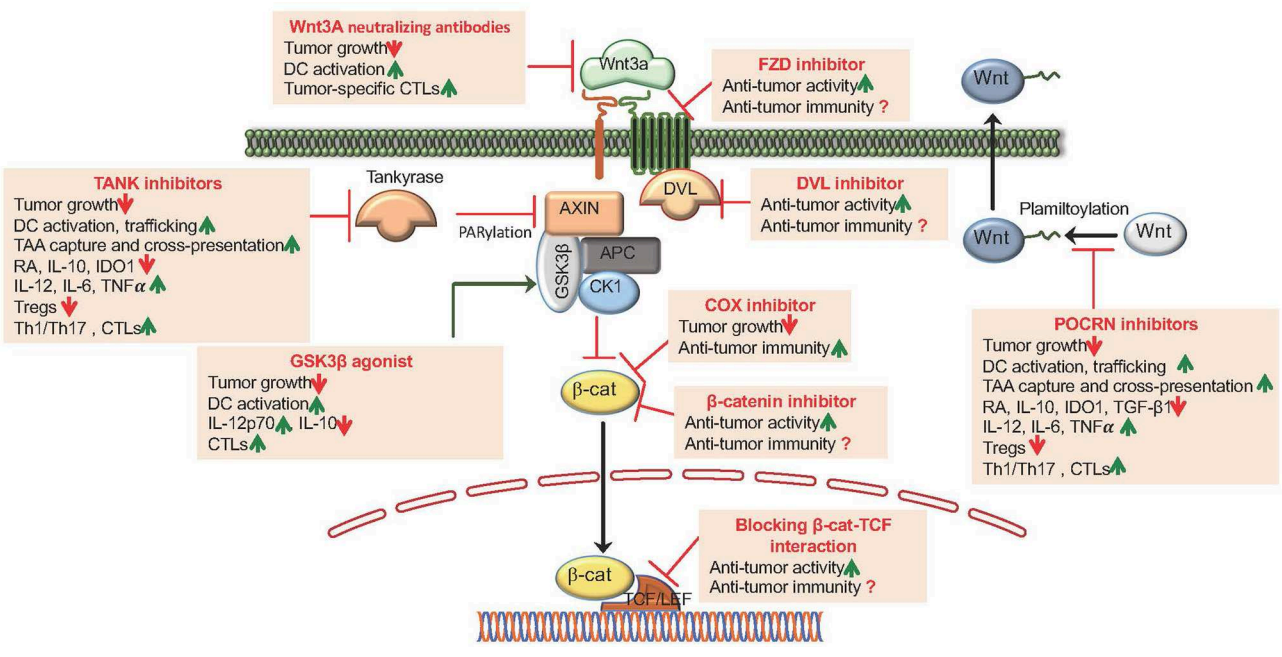

FIGURE 3 | Pharmacological targeting of Wnt/β-catenin signaling augments anti-tumor activity and anti-tumor immune responses. The canonical Wnt signaling pathways can be targeted at four different levels: (1) Blocking Wnt ligand interaction with Fzd receptors by utilizing Wnt ligand antagonists that includes mAbs generated against the Wnts and Fzds and DKK1; (2) Blocking Fzd-LRP5/6 signaling by utilizing POCRN or DVL inhibitors; (3) Promoting β-catenin degradation by utilizing TNKS inhibitors, β-catenin-destruction complex activators that targets the Ser/Thr kinases CK1α or GSK3β and COX inhibitors and (4) Antagonizing β-catenin interaction with TCFs utilizing several small molecule modulators and synthetic inhibitors.

with reduced number of Tregs, IL-10$^+$ Tr1, and IL-10$^+$ CD8 T cells within the tumors (21). Furthermore, IWP-L6 or C59 treatment enhances the ability of DCs to capture and cross-present tumor antigens to CD8$^+$ T cells (71). Similar effect was observed in murine models of melanoma, colorectal, and ovarian cancer using different POCRN inhibitors, RXC004 and WNT974 (82). In addition, POCRN inhibitor in combination with checkpoint inhibitors, such as anti-PD-1, or chemotherapy was also found to enhance anti-tumor immunity (82).

Promoting β-Catenin Degradation

Third strategy to augment anti-tumor immune responses involves promoting β-catenin degradation using TNKS inhibitors (43–45). TNKS enzymes are members of PARP family that regulate the canonical Wnt signaling via PARylation of AXIN, a key component of β-catenin destruction complex. TNKS inhibitors promote β-catenin degradation by increasing the levels of AXIN (43–45). Recent studies have shown that treatment of melanoma or EG7 tumor-bearing mice with TNKS inhibitors XAV939 or JW55 markedly delayed tumor growth with augmented anti-tumor immunity (21, 22, 24). Likewise, recent *ex vivo* study on co-culture of LNCaP and PC-3 prostate cancer, cells with lymphocytes from prostrate cancer patients have shown that lymphocytes treated with XAV 939 are more potent in eliminating LNCaP and PC-3 prostate cancer cells (83). In addition, XAV 939 in combination with vaccines enhances anti-tumor immune responses by potently activating DCs in mice (83). Similarly, RNAi-mediated inhibition of β-catenin resulted in marked increase in anti-tumor immune response with reduced tumor burden in models of B16F10 melanoma, 4T1 mammary carcinoma, Neuro2A neuroblastoma, and renal adenocarcinoma (48). Another potential approach to promote β-catenin degradation is by activating GSK3β (55). In this context, a recent study using murine melanoma model has shown that intratumoral activation of GSK3β improved tumor immune surveillance that is associated with increased DC activation and CD8$^+$ effector response (55).

Blocking β-Catenin-TCF Interaction

Finally, another potential strategy to target the canonical Wnt pathway is blocking the interaction of β-catenin with its downstream transcription factors (43–45). Several small molecule modulators and synthetic inhibitors that antagonize β-catenin interaction with TCFs have been identified and tested on murine tumor models of myeloma, liver, colorectal, and breast cancer (43–45). These studies have shown that blocking the interaction of β-catenin and TCF interaction markedly reduced the tumor growth (43–45). However, the primary focuses of these studies are mostly to test the effect on cancer stem cells and tumor cells, but not on immune cells. In this context, a recent study has shown the peptide-mediated targeted blocking of β-catenin interaction with BCL9 co-factor and its downstream transcription factor shows robust anti-tumor efficacy across multiple murine tumor models (56, 84). Markedly, this treatment approach augmented intratumoral infiltration of cytotoxic T cells by reducing Tregs and increasing DCs and also sensitizing cancer cells to PD-1 inhibitors (84). Further preclinical studies are warranted to understand the effect of other pharmacological inhibitors that targets β-catenin and TCF interaction on anti-tumor immunity.

Thus, pharmacological blocking of the canonical Wnt pathway appears promising in preclinical models. However, the impacts of potential side effects are currently unclear, as this pathway plays an important role in several physiological processes and immune-mediated inflammatory diseases. Recent advances in the understanding of Wnt/β-catenin signaling in DCs and other immune cells present promising new therapeutic opportunities for targeted regulation of this pathway to overcome immune evasion by tumors and to augment anti-tumor immunity. In this context, targeted delivery of Wnt modulators specifically to DCs and/or tumor cells using nanoparticles and antibody drug conjugates represents a promising approach for the development of novel combinatorial anti-cancer immunotherapies. This will aid in overcoming the toxicity and potential side effects associated with Wnt inhibitors.

SUMMARY

The central role of the Wnt pathway in regulating diverse biological processes has been appreciated for a long time. Even immunologists have recognized for decades that aberrant Wnt signaling occurs in several tumors. However, it is only recently that immunologists have begun to explore the cellular and molecular mechanisms by which the Wnt signaling pathway exerts its effects on the innate and adaptive immune systems. As evident from the discussion above, several recent observations have highlighted novel mechanisms by which the canonical Wnt signaling cascade in DCs regulates immune suppression, and the same pathway in tumors is associated with the evasion of anti-tumor immunity. In addition, preclinical studies have shown that targeting the canonical Wnt signaling pathway represents a promising approach to overcome immune evasion by tumors and promote anti-tumor immunity by potently activating DCs. However, several important questions remain unexplored (**Table 2**). For example, how Wnt signaling shape the innate immune functions of migratory DCs vs. non-migratory DCs in the TME? How are signals from dying cells (chemotherapy), DAMPs, and endogenous TLR ligands integrated with signals from Wnts, and what is the consequence on anti-tumor immunity? What are the effects of Wnt inhibitors plus TLR vaccine adjuvants on anti-tumor immune responses? What are the consequences of blocking the Wnt/β-catenin pathway using inhibitors as possible adjuvants plus ICI on anti-tumor immune responses? Furthermore, a major unanswered question is the extent to which the tumor-specific differences in Wnt-signaling impacts the evasion of anti-tumor immunity. Clearly, discovering answers to these questions is likely to unravel molecular mechanisms by which Wnts play a pervasive and central role in regulating anti-tumor immune response.

This in turn is likely to be of great value in the design of immunotherapies against a whole range of human cancers.

AUTHOR CONTRIBUTIONS

AS, MH, PP, and SM have performed bibliographic researches. AS, PP, and SM have drafted the manuscript.

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