TARGETED ANTIGEN DELIVERY: BRIDGING INNATE AND ADAPTIVE IMMUNITY

EDITED BY: Bénédicte Manoury and Piergiuseppe De Berardinis
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TARGETED ANTIGEN DELIVERY: BRIDGING INNATE AND ADAPTIVE IMMUNITY

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Ashurbanipal VII BC, Nineveh, Assyria.
Image: Viacheslav Lopatin/Shutterstock.com.

The most efficient way to mount a sustained immune response is to target antigens to antigen presenting cells that trigger both innate and adaptive immune responses. A comprehensive view of the current approaches to the design of new antigenic formulations will enhance our understanding and perspective of targeted immunotherapy.

The aim of this Research Topic is to provide an overview of the currently adopted targeting strategies by a collection of articles on:

1. Novel approaches of antigen targeting for immunotherapeutic strategies against cancer and/or infectious diseases.

2. Diversity and biology of dendritic cell subsets in human and mouse.

3. Combined strategies for the delivery of antigens and adjuvant molecules that stimulate innate immune responses and their influence on the quality of immune responses.

4. Impact of the receptor mediate intracellular trafficking on antigen presentation.

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EDITORIAL
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Editorial: Targeted Antigen Delivery: Bridging Innate and Adaptive Immunity

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Keywords: targeted delivery, dendritic cells, innate and adaptive immunity, pattern recognition receptors (PRR), immunotherapy

Those who are skilled can hit the target with one arrow. Whether one hits or not is entirely a matter of the two words "talent" and "learning".

Harmony garden Yuan Mei (1716-1798)

Editorial on the Research Topic

Targeted Antigen Delivery: Bridging Innate and Adaptive Immunity

The aim of this topic is to provide an exhaustive as possible overview of currently used or developing targeting strategies for antigen delivery and of emerging approaches for simultaneous delivery of immunogenic molecules that can activate innate and adaptive responses.

To optimize T cell responses, immunologists focused on targeting antigenic proteins to professional antigen presenting cells of the immune system, like Dendritic Cells (DC), which also regulate innate immune responses, expressing various pattern recognition receptors (PRR), like Toll-like receptors, NOD-like receptors and cytosolic DNA and/or RNA sensors. Thus, developing strategies aim to exploit specialized uptake receptors constructing immunogens able to combine PRR ligands to antigen delivery via specific targeting.

This topic is a collection of 3 review, 5 mini-reviews, and 6 research articles which we hope will interest the readers and provide useful information for researchers more directly working on this field.

For their capability to be a bridge between innate and adaptive immunity, targeting of DC is an attractive approach to generate strong protective cellular responses against infectious diseases and cancer.

In order to optimize DC-based vaccine, Antonialli et al. reported the targeting of different DC subsets by using hybrids mAbs able to deliver antigens of interest to DC surface receptors. They found that using anti-DEC-205 or anti-DCIR mAbs in the presence of CPG ODN or bacterial flagellin, when the antigen was targeted to $CD8^+$ DC subset, a specific proliferation of $CD4^+$ T cells was induced able to produce pro-inflammatory cytokines, while targeting $CD8^-$ DC with the same hybrid mAbs promoted specific antibody responses but no detectable pro-inflammatory $CD4^+$ T cell response.

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On a similar path line of DCs targeting, another research article of this issue by Gomes-Neto et al. described a vaccine formulation based on the use of filamentous bacteriophage fd carrying *Trypanosoma cruzi* (*T. cruzi*) antigenic determinants and its efficacy in the absence of exogenous adjuvant administration. Gomes-Neto et al. reported the ability of fd nanoparticles, which were previously demonstrated to be taken up by dendritic cells, to protect against mortality induced by a high inoculum dose of parasite in a mice model of *T. cruzi* infection.

Moreover, the research article by Sartorius et al. showed a further exploitation of bacteriophage fd nanoparticles to deliver immunologically active lipids together with antigenic peptides. It was demonstrated that the delivery of alphaGalactosylCeramide (αGalCer) like bacteriophage fd/αGalCer conjugates was able to repeatedly stimulate iNKT cells *in vitro* and *in vivo*, without inducing anergy. In addition, the authors found that co-delivery of αGalCer and $CD8^+$ T cell antigens to APCs via bacteriophages strongly boosted the adaptive $CD8^+$T cell response, and therapeutic vaccination with these phage conjugates was able to protect mice against subcutaneous tumor engraft.

Targeting of macrophages was also addressed in this topic. In particular, in an original research article van Dinther et al. proposed a strategy to target CD169+ macrophages, which are located in the marginal zone of the spleen and the sub capsular sinus in the lymph nodes. In previous work, the authors demonstrated that antigen delivery to $CD169^+$ macrophages resulted in antigen presentation by DCs and activation of strong $CD8^+$ T cell responses in mice. Here by targeting tumor antigenic proteins or peptides as anti-CD169 antibody-antigen conjugates, they showed induction of strong primary, memory, and recall response. Moreover, a protective immunity against melanoma was generated in mice injected with B16 melanoma cells.

Several review articles are also an important part of this topic providing a compendium of the current and envisaged strategy of antigen delivery and, importantly, an updated dissection of the basic mechanisms, which orchestrate the innate and adaptive response to antigen delivery.

The central role of dendritic cells in the "cross hair for the generation of tailored vaccines" was reviewed in this research topic issue by Gornati et al. Crucial key points as the adjuvant selection, the vehicle design and the choice of membrane receptor molecules to target were discussed in this article. A more precise DCs classification in order to dissect an accurate view of the DCs role and cues for more specific targeting was described. Overall, the complexity of the immune response to cancer was also addressed in order to envisage efficacious personalized therapies.

Since decades, amelioration of DC immunotherapy to fight cancer has been the focus of intensive work. The group of E Lion wrote an extensive review on strategies to increase anti-tumor immunity by interfering with the PD1/PDL-1 pathway such as the used of humanized antibodies, nanobodies, soluble PD1 and RNA interference. On the same line, an original research article by Takeda et al. described the role of a short form of Mycoplasma fermentas-derived diacylated lipoprotein (MALP2) (a TLR2/TLR6 ligand) in inducing tumor rejection when used with anti PDL-1 antibodies therapy. MALP2 was shown to induce tumor DC activation and CTL proliferation.

Also in this topic, a comprehensive review on how to manipulate dendritic cells for effective anti-tumor response in hematological malignancies was written by Cornel et al. This review focused on strategies to potentiate DCs, which can be generated in sufficient quantities from $CD34^+$ hematopoietic and stem cells progenitors, to express tumor associated antigens, to mature, to polarize, to migrate, to cross-present antigens, aiming at improving their potency in anti-tumor response.

In another mini review, Guo et al. described recent advancement in the development of neo-antigen-based cancer vaccines, reporting current strategies for lymphoid organ targeting and on-going efforts in using direct injection of *ex vivo* pulsed dendritic cells vaccines. Moreover, these authors reviewed the use of biomaterials designed for passive delivery via antigen capture *in vivo*. In addition, the authors described the use of bulk nanomaterial made from silica microrod for enhancing cancer vaccines through constructing artificial antigen-presenting niche.

Recently, nanovaccines engineered to express antigens and adjuvants on the same nanocarrier can also be used instead of traditional vaccines (antigen and adjuvant are delivered separately). Bros et al. discussed the biochemical properties and the nature of these nanocarriers, which can affect the uptake and trafficking of nanovaccines, in a mini-review.

Adjuvants containing nucleic acid (NA) targeting TLR9 have been shown to boost anti-cancer immune responses. Nucleic acids can also be recognized by cytosolic receptors and trigger cGAS-STING/RIG1-MAVS pathways to produce proinflammatory cytokines. The review of Iurescia et al. described how these NA have been used in recent clinical trials and represented a novel strategy for cancer immunotherapy.

On the other hand, Anchim et al. provide an original research article investigating the role of epitope display on Adenovirus capside. paving the way for the development of Ad vaccines able to trigger specific response against the epitope inserted and not against the transgene.

Another mini review article by Hos et al. focused on "approaches to improve chemically defined synthetic peptide vaccines". The history of peptides vaccines, made of beneficial outcomes in preclinical models and mixed results obtained in clinical trials is summarized. An aim of this mini review was to report the novel options for a rationale design of peptide vaccines. Moreover, strategies for biochemical visualization and tracking of peptide vaccines at the molecular and subcellular level were described and discussed.

The nature and biology of the targeted cells emphasizes the importance of investigating the molecular mechanisms that modulate the successful delivery of immunogenic molecules. Thus, in this topic, Aksoy et al. summarized the role of the different isoforms of phosphoinositide-3 kinases (PI3Ks) proteins generating phosphoinositides

lipids in DC biology, focusing in antigen presentation and PRR stimulation.

In conclusion, this research topic provides a general frame of the most efficient way to mount a sustained immune response by targeting antigen to antigen presenting cells, and a description of the exploitable strategies to trigger both innate and adaptive efficient immune responses.

AUTHOR CONTRIBUTIONS

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

Conflict of Interest Statement: PD is inventor of the patent n PCT/IB2018/050525 WO2018/138696 entitled "Phages conjugates and uses thereof."

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

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CpG Oligodeoxinucleotides and Flagellin Modulate the Immune Response to Antigens Targeted to CD8α$^+$ and CD8α$^-$ Conventional Dendritic Cell Subsets

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Dendritic cells (DCs) are antigen-presenting cells essential for the induction of adaptive immune responses. Their unprecedented ability to present antigens to T cells has made them excellent targets for vaccine development. In the last years, a new technology based on antigen delivery directly to different DC subsets through the use of hybrid monoclonal antibodies (mAbs) to DC surface receptors fused to antigens of interest opened new perspectives for the induction of robust immune responses. Normally, the hybrid mAbs are administered with adjuvants that induce DC maturation. In this work, we targeted an antigen to the CD8α$^+$ or the CD8α$^-$ DC subsets in the presence of CpG oligodeoxinucleotides (ODN) or bacterial flagellin, using hybrid αDEC205 or αDCIR2 mAbs, respectively. We also accessed the role of toll-like receptors (TLRs) 5 and 9 signaling in the induction of specific humoral and cellular immune responses. Wild-type and TLR5 or TLR9 knockout mice were immunized with two doses of the hybrid αDEC205 or αDCIR2 mAbs, as well as with an isotype control, together with CpG ODN 1826 or flagellin. A chimeric antigen containing the *Plasmodium vivax* 19 kDa portion of the merozoite surface protein ($MSP1_{19}$) linked to the Pan-allelic DR epitope was fused to each mAb. Specific CD4$^+$ T cell proliferation, cytokine, and antibody production were analyzed. We found that CpG ODN 1826 or flagellin were able to induce CD4$^+$ T cell proliferation, CD4$^+$ T cells producing pro-inflammatory cytokines, and specific antibodies when the antigen was targeted to the CD8α$^+$ DC subset. On the other hand, antigen targeting to CD8α$^-$ DC subset promoted specific antibody responses and proliferation, but no detectable pro-inflammatory CD4$^+$ T cell responses. Also, specific antibody responses after antigen targeting to CD8α$^+$ or CD8α$^-$ DCs were reduced in the absence of TLR9 or TLR5 signaling, while CD4$^+$ T cell proliferation was mainly affected after antigen targeting to CD8α$^+$ DCs and in the absence of TLR9 signaling. These results extend our understanding of

the modulation of specific immune responses induced by antigen targeting to DCs in the presence of different adjuvants. Such knowledge may be useful for the optimization of DC-based vaccines.

Keywords: dendritic cells, hybrid monoclonal antibodies, CpG oligodeoxinucleotides 1826, flagellin, antigen targeting

INTRODUCTION

Dendritic cells (DCs) are innate immune cells specialized in antigen presentation to naïve T lymphocytes (1). DCs express pattern recognition receptors (PRRs), such as toll-like (TLRs) and nod-like (NLR) receptors, which recognize pathogen- or damage-associated molecular patterns (PAMPs or DAMPs), respectively (2). After pathogen contact, DCs mature, produce cytokines, and upregulate costimulatory molecules that prime $CD4^+$ and $CD8^+$ T cell responses, and stimulate B cells to produce antibodies (3–5). Thus, DCs play a central role in immunity, promoting, and controlling the adaptive immune response during inflammation (6).

Dendritic cells are a heterogeneous lineage of cells that differentiate from bone-marrow precursors and migrate to different regions of the body, such as blood, thymus, liver, lymphoid organs, spleen, and skin (7–9). DCs can be divided in two main subtypes: plasmacytoid DCs that are mainly associated with antiviral response and conventional DCs mainly related with antigen presentation (7). Classically, murine conventional spleen DCs ($CD11c^+MHCII^+$) can be classified according to the expression of the CD8 molecule alpha chain. $CD8\alpha^+$ DCs ($CD11c^+CD8\alpha^+$) are mainly associated with cross-presentation to $CD8^+$ T cells, while $CD8\alpha^-$ DCs ($CD11c^+CD8\alpha^-$) with antigen presentation to $CD4^+$ T cells (10–12). More recently, conventional DCs were classified into two distinct subtypes based on their ontogeny: the conventional type 1 DCs (cDC1s, $CD11c^+CD26^+XCR1^{hi}$ $CD172a^{lo}IRF8^{hi}IRF4^{lo}$) and conventional type 2 DCs (cDC2s, $CD11c^+CD26^+XCR1^{lo}CD172a^{hi}$ $IRF8^{lo}IRF4^{hi}$) (13). Evidences support the notion that the $CD8\alpha^+$ DCs correspond to cDC1s, while $CD8\alpha^-$ DCs correspond to cDC2s (14, 15).

In addition to the markers mentioned above, conventional DCs also express endocytic receptors that belong to the C-type lectin family. While the $CD8\alpha^+$ DCs express the DEC205 receptor (16), the $CD8\alpha^-$ DCs express a receptor known as DCIR2 (17). αDEC205 and αDCIR2 monoclonal antibodies (mAbs) have been successfully used to target antigens to $CD8\alpha^+$ DCs and $CD8\alpha^-$ DCs, respectively (18–20). This is accomplished by fusing the antigen of interest to the carboxyl terminus portion of the αDEC205 or αDCIR2 heavy chains. The result is a hybrid mAb that, once administered to mice, delivers the antigen of interest to the DCs *in vivo* and consequently promotes antigen processing and presentation (21). Nevertheless, the use of this strategy to induce an immune response against proteins expressed by different pathogens requires the administration of an adjuvant to mature the DCs, and avoid the development of tolerance (22, 23).

The αCD40 agonistic mAb was frequently used as an adjuvant in immunizations using αDEC205 and αDCIR2 fusion mAbs to promote DC maturation (24) and robust adaptive immune responses (12, 18, 25, 26). Furthermore, PRR ligands have also been used to mature DCs. Polyinosinic:polycytidylic acid (poly (I:C)) is a TLR3 and MDA-5 (melanoma differentiation-associated gene 5) ligand that has been largely used together with hybrid mAbs in protocols intended to target antigens to DCs, especially through the DEC205 receptor (19, 20, 26–28). In fact, it was shown that poly (I:C) administered together with an αDEC205 fusion mAb was the best adjuvant to induce potent IFN-γ-producing $CD4^+$ T cells (27, 29).

Despite the use of αCD40 agonistic mAb and poly (I:C) as adjuvants, the search for new adjuvants that may be used together with the hybrid mAbs is still relevant, especially when targeting the $CD8\alpha^-$ DCs with the αDCIR2 mAb. Here, we analyzed two other adjuvants in the context of DC targeting. We studied the immune response induced after antigen targeting to $CD8\alpha^+$ and $CD8\alpha^-$ DCs using CpG oligodeoxynucleotides (CpG ODN) or bacterial flagellin as adjuvants. CpG ODN are PAMPs formed by an unmethylated DNA motif present in microbes that are recognized by TLR9, an intracellular receptor anchored in the endosome internal membrane (30, 31). Flagellin is the main component of bacterial flagellum, and it is recognized by extracellular TLR5 (32, 33) and by the intracellular NLR receptors Naip5 (34) and NLRC4 (35). While both TLRs (5 and 9) signal through the same pathway that involves MyD88 activation followed by NF-κB translocation to the nucleus and subsequent pro-inflammatory cytokine production (36), Naip5 and NLRC4 activate the caspase-1 cascade that culminates in the release of inflammatory cytokines such as IL-1β and IL-18 (34, 35). Due to their potent adjuvant effects, both CpG ODN (37) and flagellin (38, 39) have already been used as adjuvants in a number of clinical trials.

Although CpG ODN and flagellin are well-described adjuvants, their use in DC-targeted vaccination protocols may be further explored. In this paper, we hypothesized that the use of different adjuvants together with antigen targeting to the $CD8\alpha^+$ and $CD8\alpha^-$ DC subsets might induce differential immune responses based on the DC subtype biology. We used recombinant flagellin as a TLR5 ligand and synthetic CpG ODN as TLR9 ligands. In addition, we investigated the direct role of TLR5 or TLR9 signaling using knockout mice to analyze the influence of their signaling specifically on antigen targeting to $CD8\alpha^+$ and $CD8\alpha^-$ DCs. Previous studies showed that $CD8\alpha^+$ and $CD8\alpha^-$ DCs promote $CD4^+$ T cell differentiation into diverse Th subsets, indicating that different DC subtypes are diverse in priming naïve T cells suggesting biological differences between them (40–42).

Using a *Plasmodium vivax* protein fused to a well-described $CD4^+$ T cell epitope (43), we tested the influence of the adjuvant on cellular and humoral immune responses after antigen targeting to DCs. The antigen is composed by the C-terminal 19 kDa fragment of the Merozoite Surface Protein 1 ($MSP1_{19}$) of *P. vivax*

fused to a Pan allelic DR epitope (PADRE) (44, 45) in a construct known as $MSP1_{19}$_PADRE. Targeting of $MSP1_{19}$_PADRE to different DC subsets allows us to study the humoral immune response through the evaluation of anti-$MSP1_{19}$ antibody titers, as well as, the specific $CD4^+$ T cell response using the PADRE epitope.

Our results demonstrate that antigen targeting to $CD8\alpha^+$ or $CD8\alpha^-$ DCs in the presence of flagellin or CpG ODN induce different immune responses that may be linked to the differential activation of these DC subtypes promoted by TLR5 or TLR9 engagement and signaling. In summary, humoral immune responses were successfully induced after antigen targeting to both DC subsets in the presence of either CpG ODN or flagellin. CpG ODN was more suitable to induce specific $CD4^+$ T cell proliferation and pro-inflammatory cytokines when the antigen was targeted to $CD8\alpha^+$ DCs. TLR9 signaling was essential for this response. On the other hand, flagellin induced more pronounced $CD4^+$ T cell proliferation when the antigen was targeted to the $CD8\alpha^-$ DC subset. TLR5 signaling did not seem to play a major role in this response. The results presented here contribute to shed more light on the use of different adjuvants associated with DC targeted vaccines.

MATERIALS AND METHODS

Mice

C57BL/6 mice of both sexes, and 5- to 9-week-old, were bred at the Isogenic Mouse Facility of the Parasitology Department, University of São Paulo, Brazil. C57BL/6 background TLR5-deficient (KO) (46) and TLR9 KO (47) were kindly provided by Dr. Michel C. Nussenzweig (The Rockefeller University, USA), and bred and used at the same conditions as the C57BL/6 mice. All experimental procedures and animal handling were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the Brazilian National Law on animal care (11.794/2008). The Institutional Animal Care and Use Committee (CEUA) of the University of São Paulo approved all procedures under the protocol number 082.

Cloning and Expression of the Fusion mAbs and Recombinant Protein Production

The $MSP1_{19}$_PADRE sequence was amplified from the pET14b-$MSP1_{19}$_PADRE plasmid previously described (43) using forward (5′-GGCTCGAGGAGTTCGGTAGGTTCATGAGCTCCGAGCACACATG-3′) and reverse (5′-GGGCGGCCGCTTATTGCTCAGCGGTGGCAG-3′) primers. Underlined sequences indicate *Xho* I and *Not* I restriction sites, respectively. After amplification using Phusion High-Fidelity DNA Polymerase (New England Biolabs), the insert was digested with *Xho* I and *Not* I, and cloned in frame with the mouse anti-DEC205 (NLDC145 clone), anti-DCIR2 (33D1 clone), or isotype control (GL117 clone) heavy chain carboxyl terminus. The original plasmid constructs are described elsewhere (12, 22). Plasmids pDEC-$MSP1_{19}$_PADRE, pDCIR2-$MSP1_{19}$_PADRE and pISO-$MSP1_{19}$_PADRE were then generated. These plasmids and the plasmids encoding their respective light chains were amplified in DH5α bacteria and subsequently purified in large scale using the QIAGEN Maxi Prep Kit (Qiagen). Transient transfection in human embryonic kidney (HEK) 293T (ATCC No CRL-11268) cells was performed exactly as described elsewhere (19). After purification with protein G beads (GE Healthcare), fusion mAbs were dialyzed in PBS, filtered, and had their concentrations estimated by Bradford assay (Pierce). Samples were stored at −20°C until use.

To analyze the cellular and humoral immune responses after immunization with the fusion mAbs, we produced recombinant $MSP1_{19}$ and $MSP1_{19}$_PADRE proteins exactly as described by Cunha et al. (48) and Rosa et al. (43), respectively.

Fusion mAbs Integrity Evaluation and Binding Assay

The integrity of the purified fusion mAbs was assessed in 12% SDS-PAGE gels under reducing conditions as previously described (28).

The binding assay was performed using Chinese hamster ovary (CHO) cells expressing the mouse DEC205 or DCIR2 receptors. These cells were kindly provided by Dr. Michel C. Nussenzweig (The Rockefeller University, USA). Before use, cells were detached with 1× PBS containing 1 mM of EDTA for 10 min at 37°C. EDTA was neutralized with 500 µL of fetal bovine serum, and cells were washed three times with PBS 1×. One hundred thousand CHO cells expressing each receptor were incubated with 5, 0.5, or 0.05 µg/mL of each fusion mAb on ice for 40 min. Cells were then washed twice with PBS plus 2% fetal bovine serum (Life Technologies) and incubated with anti-mouse IgG-Alexa Fluor 488 (Thermo Scientific) for 40 min on ice. After two additional washes, 20,000 events were acquired using BD LSRFortessa flow cytometer (BD Biosciences).

This assay was also performed on splenocytes isolated from C57BL/6 naïve mice. Five million splenocytes were initially incubated with anti-CD16/32 (BD Fc Block) for 15 min and then incubated with 5, 0.5, or 0.05 µg/mL of each fusion mAb on ice for 40 min. After two washes, biotinylated anti-CD3 (clone 145.2C11), anti-CD49b (clone DX5) and anti-CD19 (clone 1D3) were incubated on ice for 40 min. Splenocytes were then washed twice and incubated with anti-IgG1-PE (clone A85-1), anti-CD11c-BV421 (clone N418), anti-MHCII (I-A/I-E)-FITC (clone 2G9), anti-CD8α-APC (clone 53–67), streptavidin-PerCP, and Live and Dead Aqua (Thermo Fisher Scientific) for 40 min on ice. All antibodies were purchased from BD Biosciences. One million events were acquired using BD LSRFortessa flow cytometer (BD biosciences). Analyses were performed using FlowJo software (version 9.3, Tree Star, San Carlo, CA, USA).

Flagellin Production and Purification

The *Salmonella* flagellin FliC*d*, originally produced by the *S.* Muenchen patovar, was produced from a recombinant *S.* Dublin strain exactly as described previously (49) and its concentration was determined by the BCA assay (Pierce). Purity was monitored by 12% polyacrylamide gels stained with Coomassie Blue (Amresco). LPS was removed using detoxi-gel columns (Pierce) according to the manufacturer's protocol. Residual LPS

contamination was monitored using the Limulus Amebocyte Lysate assay (Lonza) and shown to be below 3 EU/μg of protein.

Immunizations

Groups of five animals were immunized with 5 μg of each mAb administered intraperitoneally (i.p.) combined with either 25 μg/animal of CpG ODN 1826 (Invivogen) or 5 μg/animal of *Salmonella* flagellin. Two doses were administered with a 30-day interval between each one. Five days before and 14 days after the administration of the booster dose, sera were collected. The cellular immune response was analyzed 20 days after the administration of the booster dose, when mice were euthanized and had their spleens removed.

Analysis of $MSP1_{19}$-Specific Antibodies

The presence of anti-$MSP1_{19}$ specific total IgGs, or IgG1, IgG2b, IgG2c, and IgG3 subclasses was detected by ELISA exactly as previously described (28). Antibody titers were normalized in a log10 scale considering the highest serum dilution showing an $OD_{490} > 0.1$. The IgG1/IgG2c ratio was calculated by dividing the mean values of the highest serum dilution obtained for IgG1 by the mean value of the highest serum dilution obtained for IgG2c without normalization.

CFSE-Based Proliferation Assay and Detection of Cytokine-Producing Cells by Intracellular Staining

Splenocytes were isolated and processed as previously described (19, 28). For the proliferation assay, fifty million splenocytes obtained from each group of immunized mice were resuspended in 1 mL of PBS previously heated at 37°C containing 1.25 μM CFDA dye (Vybrant CFDA SE—Cell Tracer Kit, Molecular Probes). The cells were then incubated for 10 min at 37°C, centrifuged at 600 × *g* for 5 min, washed three times, and resuspended in 1 mL of R10 [RPMI supplemented with 10% of fetal bovine serum, 2 mM L-glutamine, 10 mM Hepes, 1 mM sodium pyruvate, 1% vol/vol non-essential aminoacid solution, 1% vol/vol vitamin solution, 5×10^{-5} M 2-mercaptoetanol (all from Life Technologies), and 20 μg/mL of ciprofloxacin (Isofarma, Brazil)]. In *U*-shaped 96-well plates (Costar), 3×10^5 cells were stimulated with 1 μg/mL of either $MSP1_{19}$_PADRE or $MSP1_{19}$ recombinant proteins in each well and incubated for 5 days at 37°C and 5% CO_2. After this period, the plates were centrifuged, washed, and the triplicates were combined in a single well for labeling with anti-CD4-PerCP (clone RM 4–5) and anti-CD3-APC.Cy7 (clone 145.2C11) for 40 min on ice. Cells were then washed three times with PBS-FBS (PBS plus 2% fetal bovine serum). One hundred thousand events were acquired using FACS Canto II flow cytometer (BD biosciences). The percent of CFSE low cells was calculated after subtraction of the percent of CFSE low cells in the non-pulsed wells.

Detection of cytokine-producing cells by intracellular staining was performed as described elsewhere (28). Briefly, 1×10^6 splenocytes/well were plated in triplicates in *U*-shaped 96-well plates and pulsed with 5 μg/mL of the recombinant $MSP1_{19}$_PADRE protein. As negative control, splenocytes were not pulsed. Incubation was performed in R10 medium containing 2 μg/mL of αCD28 agonist antibody. After incubation for an hour at 37°C and 5% CO_2, Golgi Plug (Brefeldin A, BD Biosciences) was added to each well (0.5 μg/well). Splenocytes were then incubated in the same conditions for 12 additional hours. Plates were centrifuged for 5 min at 1,000 × *g* and washed twice with PBS-FBS. Cells were stained on ice for 45 min with αCD4-PerCP-Cy5.5 mAb (clone RM 4–5). After three washes with PBS-FBS, cells were fixed and permeabilized for 15 min using the Cytofix/Cytoperm kit (BD Biosciences). After three washes with PermWash buffer (BD Biosciences), the intracellular staining was performed on ice for 45 min using the following mAbs: αCD3-APC-Cy7 (clone 145-2C11), αIFNγ-APC (clone XMG1.2), αIL2-PE (clone JES6-5H4), and αTNFα-PE-Cy7 (clone MP6-XT22). Cells were washed three times with PermWash buffer (BD Biosciences) and resuspended in PBS-FBS. One million events were acquired in a FACS Canto II flow cytometer (BD biosciences). The percent of cytokine producing cells was calculated after subtraction of the percent of cytokine producing cells in the non-pulsed wells. All data were analyzed using FlowJo software (version 9.3, Tree Star, San Carlo, CA, USA).

Expression of Co-stimulatory Molecules on DC Subsets

Mice were immunized i.p. with 25 μg/animal of CpG ODN 1826 (InvivoGen) or with 5 μg/animal of *Salmonella* flagellin (FliC). 6 h after immunization, mice were euthanized and splenocytes were labeled. Fc receptors were blocked with Fc Block (BD Biosciences) and subsequently stained first with anti-CD19-Biotin (clone 1D3), anti-CD3-Biotin (clone 145.2C11), and anti-CD49b-Biotin (clone DX5) for 40 min on ice. After two washes with PBS-2% FBS, cells were then incubated anti-MHCII (I-A/I-E)-Alexa Fluor 700 (clone M5/114.15.2), anti-CD11c-BV421 (clone N418), anti-CD11b-PE.Cy7 (clone M1/70), anti-CD8α-BV786 (clone 52–67), anti-CD80-FITC (clone 16-10A1), anti-CD86-APC (clone GL1), anti-CD40-PE (clone 1C10), Streptavidin APC.Cy7 (all antibodies and the streptavidin were purchased from BD Biosciences) and Live and Dead Aqua (Life Technologies). Flow cytometry was performed using LSRFortessa (BD Biosciences) and results were analyzed in FlowJo software (version 9.3, Tree Star, San Carlo, CA, USA).

Statistical Analysis

We used Prism 5.0 (GraphPad, CA, USA) for all the analyses. Regular two-way ANOVA and two-way ANOVA for repeated measures were used for multiple comparisons, followed by Bonferroni's multiple comparison posttest for comparison of specific groups. $p < 0.05$ was considered significant.

RESULTS

αDEC205-$MSP1_{19}$_PADRE and αDCIR2-$MSP1_{19}$_PADRE mAbs Were Successfully Produced and Bound to Their Respective Receptors

Transfection of HEK293T cells with plasmids encoding the heavy and light chains of the fusion mAbs allowed us to successfully

produce and purify αDEC205-MSP1$_{19}$_PADRE, αDCIR2-MSP1$_{19}$_PADRE and ISO-MSP1$_{19}$_PADRE. A schematic representation of the fusion mAbs is depicted in Figure S1 in Supplementary Material. **Figure 1A** shows a reduced gel in which we observe the heavy (~69 kDa) and light (~25 kDa) chains of all mAbs. To test whether the fusion mAbs maintained their binding capacities to the respective receptors, we performed binding assays using CHO cells constitutively expressing mouse DEC205 or mouse DCIR2 (**Figure 1B**). We observed that the αDEC205-MSP1$_{19}$_PADRE mAb bound specifically, and in a dose dependent manner, to CHO cells expressing exclusively the mouse DEC205 receptor. On the other hand, αDCIR2-MSP1$_{19}$_PADRE mAb was able to bind to CHO cells expressing the DCIR2 receptor. As expected, the ISO-MSP1$_{19}$_PADRE mAb did not bind to any receptor. To further characterize the binding capacity of the fusion mAbs, we performed a binding assay using splenocytes (**Figure 1C**). Different concentrations of the fusion mAbs were incubated with C57BL/6 splenocytes. After exclusion of T, B, and NK cells, DC subsets were divided into CD11c$^+$MHCII$^+$CD8α$^+$ or CD11c$^+$MHCII$^+$CD8α$^-$. We observed a dose dependent binding of the αDEC205-MSP1$_{19}$_PADRE mAb to the CD8α$^+$ DC subset, while the αDCIR2-MSP1$_{19}$_PADRE mAb was shown to bind specifically to the CD8α$^-$ DC subset. Once more, the ISO-MSP1$_{19}$_PADRE mAb did not bind specifically to any DC subset. To verify if the fusion of MSP1$_{19}$_PADRE protein to the C-terminal portion of the αDEC205 and αDCIR2 mAbs would affect their binding capacity, we performed an experiment comparing fused and non-fused αDEC205 and αDCIR2 mAbs (Figure S2 in Supplementary Material). We observed that the fusion of the MSP1$_{19}$_PADRE protein to αDEC205 and αDCIR2 mAbs did not affect their binding capacity. Taken together, these results led us to conclude that all fusion mAbs were produced successfully and maintained the binding capacity to their respective receptors.

CpG ODN Promotes Robust Antibody Responses Partially Dependent on TLR9 Signaling after Antigen Targeting to CD8α$^+$ or CD8α$^-$ DC Subsets

To study the role of CpG ODN signaling in the induction of humoral immune response after antigen targeting to CD8α$^+$ and CD8α$^-$ DC subsets, we immunized wild type (WT) and TLR9 knockout (TLR9 KO) mice with αDEC205-MSP1$_{19}$_PADRE, αDCIR2-MSP1$_{19}$_PADRE, or with ISO-MSP1$_{19}$_PADRE as a non-targeted control. To demonstrate that DCs derived from WT and TLR9KO mice expressed similar amounts of DEC205 or DCIR2 receptors, we stained splenocytes with commercially available αDEC205 (NLDC-145 clone) and αDCIR2 (33D1 clone) mAbs. Figure S3 in Supplementary Material confirms that WT and TLR9KO DCs express similar amounts of DEC205 or DCIR2 receptors. Mice then received two doses of the fusion mAbs in the presence of CpG ODN 1826 and were bled 5 days before (pre-boost) and 14 days after (post-boost) the administration of the second dose (**Figure 2**). When groups were compared before boost, CD8α$^-$ DC targeting through DCIR2 in WT mice induced higher anti-MSP1$_{19}$ antibody titers when compared to targeting through DEC205 or no targeting, indicating that antigen delivery to the CD8α$^-$ DC subset induces a more robust primary response. The absence of TLR9 signaling reduced the response in all groups. After the administration of the booster dose, anti-MSP1$_{19}$ antibody titers increased in all groups. Besides, titers were higher ($p < 0.05$) in WT when compared to the TLR9 KO mice, suggesting that CpG ODN 1826 signaling through TLR9 contributes to increase antibody titers after MSP1$_{19}$_PADRE targeting to CD8α$^+$ or CD8α$^-$ DC *via* DEC205 or DCIR2, respectively (**Figure 2A**). A decrease in anti-MSP1$_{19}$ antibody titers was also observed in mice immunized with the isotype control, indicating that TLR9 signaling also plays a role in the absence of antigen targeting to DCs. Interestingly, after boost, anti-MSP1$_{19}$ antibody titers were not different in mice immunized with αDEC205-MSP1$_{19}$_PADRE when compared to animals immunized with αDCIR2-MSP1$_{19}$_PADRE, despite the difference observed before the boost. In the absence of antigen targeting (i.e., in animals immunized with the isotype control), anti-MSP1$_{19}$ titers were significantly lower. The same was observed in TLR9 KO mice (**Figure 2A**).

To study the humoral response in more detail, we also analyzed the anti-MSP1$_{19}$ IgG subclasses elicited after the boost. We observed that WT mice immunized with αDEC205-MSP1$_{19}$_PADRE or αDCIR2-MSP1$_{19}$_PADRE presented all IgG subclasses tested (IgG1, IgG2b, IgG2c, and IgG3), while ISO-MSP1$_{19}$_PADRE immunized WT mice did not present IgG1 antibodies (**Figure 2B**). Interestingly, we detected differences in the IgG1/IgG2c ratio when WT mice were immunized with αDEC205-MSP1$_{19}$_PADRE or with αDCIR2-MSP1$_{19}$_PADRE. These differences indicate that antigen targeting, in the presence of CpG ODN 1826, to the CD8α$^+$ DCs induced a Th1 prone type of response (IgG1/IgG2c ratio = 0.70), while a more Th2 type of response was induced after antigen targeting to CD8α$^-$ DCs (IgG1/IgG2c ratio = 4.36). TLR9 signaling played a role in antibody class switch as we observed a pronounced decrease of IgG2b and 2c in TLR9 KO mice immunized with either αDEC205-MSP1$_{19}$_PADRE (IgG1/IgG2c ratio = 24.09) or with αDCIR2-MSP1$_{19}$_PADRE (IgG1/IgG2c ratio = 25.20). We did not detect antibody titers after immunization with ISO-MSP1$_{19}$_PADRE in the TLR9 KO mice (**Figure 2B**). Taken together, these results indicate that CpG ODN 1826 increases the humoral immune response when the antigen is targeted to both DC subtypes and that antibody class switch is influenced by TLR9 signaling.

Antigen Targeting to the CD8α$^+$ DC Subset in the Presence of CpG ODN 1826 Elicits Strong CD4$^+$ T Cell Response That Is Greatly Diminished in the Absence of TLR9 Signaling

Next, we analyzed the PADRE specific CD4$^+$ T cell response in WT and TLR9 KO mice when MSP1$_{19}$_PADRE was targeted to either CD8α$^+$ or CD8α$^-$ DCs (**Figure 3**). CFSE-labeled splenocytes derived from immunized mice were pulsed *in vitro* with MSP1$_{19}$_PADRE or MSP1$_{19}$ recombinant proteins, and after 5 days of culture, the frequency of CD3$^+$CD4$^+$CFSE^{low} T cells was analyzed by flow cytometry (**Figure 3A**). A representative gating strategy is depicted in Figure S4 in Supplementary Material. We observed robust CD4$^+$ T cell proliferation in WT

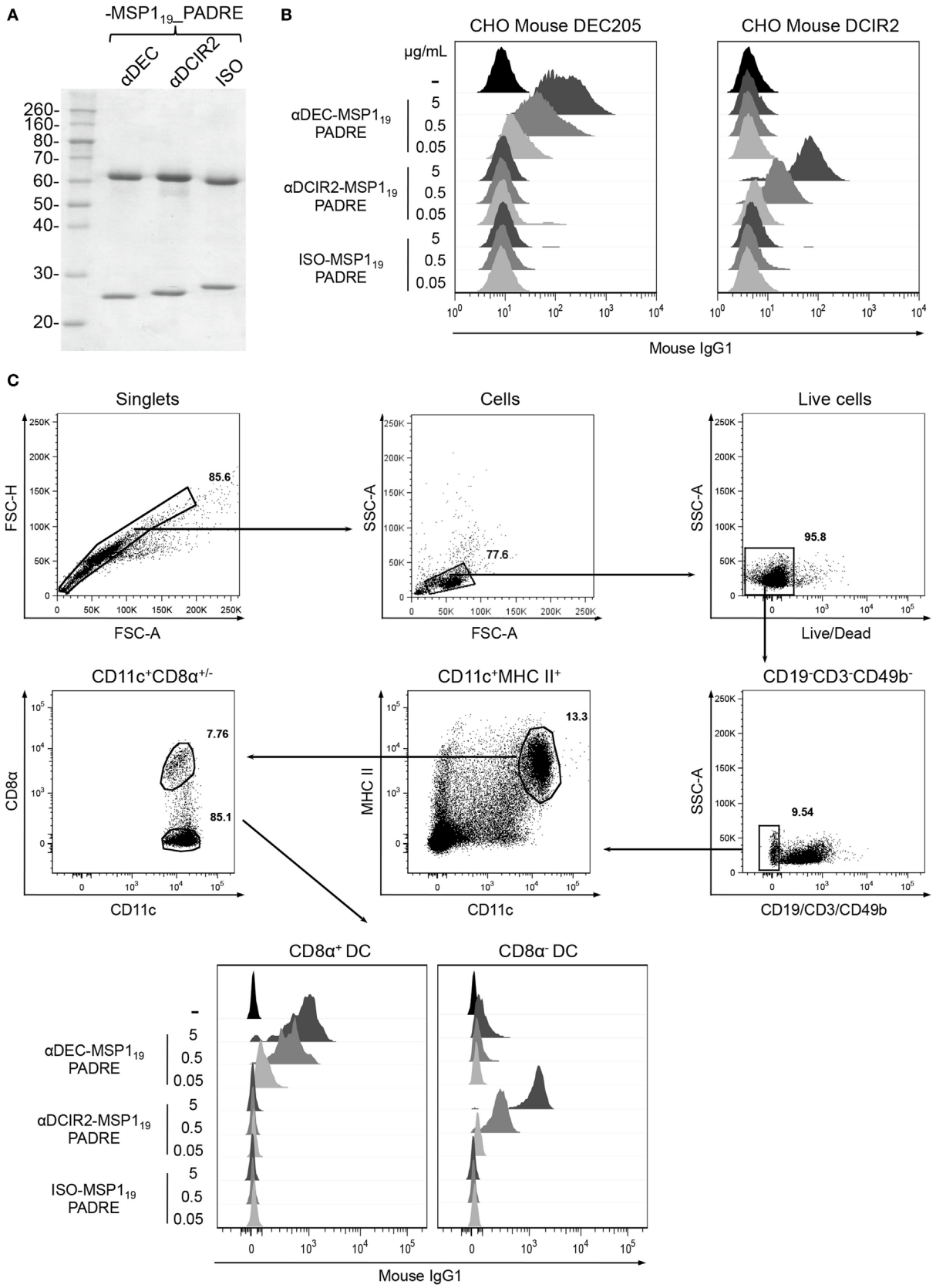

FIGURE 1 | Continued

FIGURE 1 | Continued
Production and binding of the hybrid monoclonal antibodies (mAbs) αDEC205-MSP1$_{19}$_PADRE and αDCIR2-MSP1$_{19}$_PADRE to their respective receptors. **(A)** SDS-PAGE under reducing conditions of each hybrid mAb (~1 μg) stained with Coomassie blue dye. Numbers on the left indicate molecular weights in kDa. **(B)** Chinese hamster ovary (CHO) cells expressing the murine DEC205 (left) or DCIR2 (right) receptors were incubated with 0.05, 0.5, or 5 μg of αDEC205-MSP1$_{19}$_PADRE, αDCIR2-MSP1$_{19}$_PADRE or ISO-MSP1$_{19}$_PADRE and then labeled with anti-IgG-Alexa fluor 488. **(C)** Naïve C57BL/6 splenocytes were incubated with 0.05, 0.5, or 5 μg/mL of each hybrid mAb and stained with fluorescent antibodies. The gating strategy involved the selection of singlets, size versus granulosity and viable cells. Then, CD19$^-$CD3$^-$CD49b$^-$ cells were excluded and CD11c$^+$MHCII$^+$ dendritic cells (DCs) were gated and subsequently divided in CD8α$^+$ and CD8α$^-$ DCs. Binding was detected using anti-IgG1-PE antibody. **(B,C)** Analysis was performed using FlowJo software. One experiment representative of three is depicted.

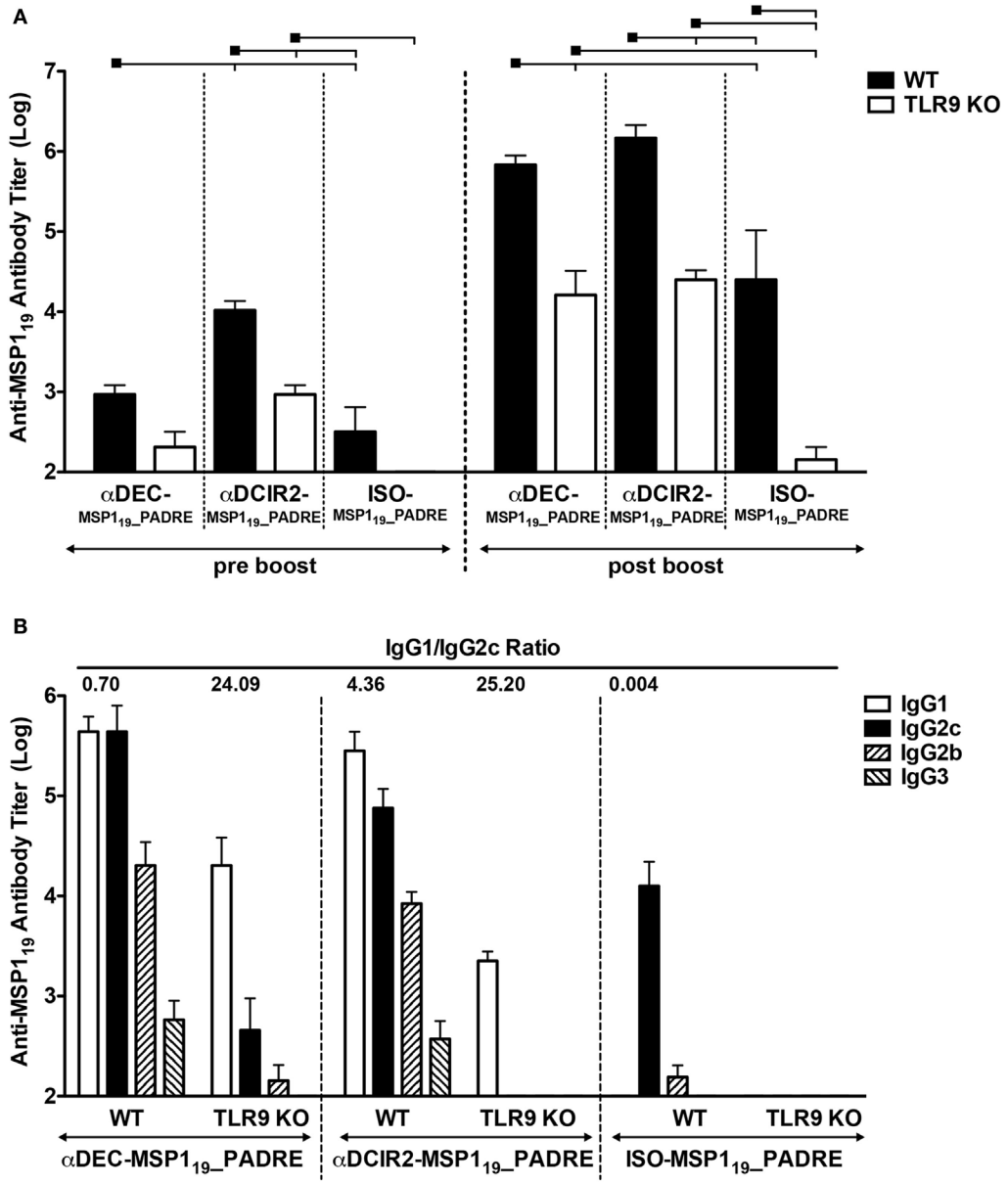

FIGURE 2 | CpG oligodeoxynucleotides (ODN) 1826 as adjuvant induces robust humoral immune response after antigen targeting to CD8α$^+$ or CD8α$^-$ DC subsets that is partially dependent on TLR9 signaling. WT and TLR9 KO mice were immunized with 5 μg of αDEC205-MSP1$_{19}$_PADRE, αDCIR2-MSP1$_{19}$_PADRE or ISO-MSP1$_{19}$_PADRE together with 25 μg of CpG ODN 1826 as adjuvant. **(A)** Five days before (pre-boost) and 14 days after (post-boost) the administration of the booster dose, blood was collected and serum obtained. Total anti-MSP1$_{19}$ IgG antibodies were detected by ELISA. Graphs show the mean ± SEM of anti-MSP1$_{19}$ titers in different groups normalized in log10 scale ($n = 5$ animals/group). Experiments were analyzed by two-way ANOVA for repeated measures followed by Bonferroni posttest. Black squares indicate the reference group against which comparisons are being made. Horizontal capped lines only depict significant differences ($p < 0.05$). **(B)** Anti-MSP1$_{19}$ IgG1, IgG2b, IgG2c, and IgG3 subclasses were also determined by ELISA 14 days after the boost. The numbers above the bars indicate the IgG1/IgG2c ratio calculated for each group.

mice immunized with αDEC205-MSP1$_{19}$_PADRE using CpG ODN 1826 as an adjuvant only when cells were pulsed with the recombinant MSP1$_{19}$_PADRE. This result was expected since PADRE is an immunodominant peptide and no other peptides, recognized by the C57BL/6 haplotype, have been described in the *P. vivax* MSP1$_{19}$ sequence. In this way, we used the recombinant MSP1$_{19}$ protein pulse as an internal negative control. On the other hand, spleen cells derived from αDEC205-MSP1$_{19}$_PADRE immunized TLR9 KO mice showed a very pronounced reduction in proliferation, not different from the one obtained in animals immunized with CpG ODN 1826 only. This result indicates that TLR9 signaling after CpG ODN 1826 stimulation plays a crucial role in the promotion of a CD4$^+$ T cell proliferative response after antigen targeting to CD8α$^+$ DCs *via* DEC205. In contrast, antigen targeting to CD8α$^-$ DCs *via* DCIR2 in the presence of CpG ODN 1826 did not elicit strong specific CD4$^+$ T cell proliferation in WT mice. This result was surprising and led us to conclude that, under our experimental conditions, CpG ODN 1826 does not seem to be a good adjuvant to induce CD4$^+$ T cell proliferation when the antigen is targeted to the CD8α$^-$ DCs *via* DCIR2. We also did not observe proliferation when the ISO-MSP1$_{19}$_PADRE mAb was used to immunize WT or TLR9 KO mice.

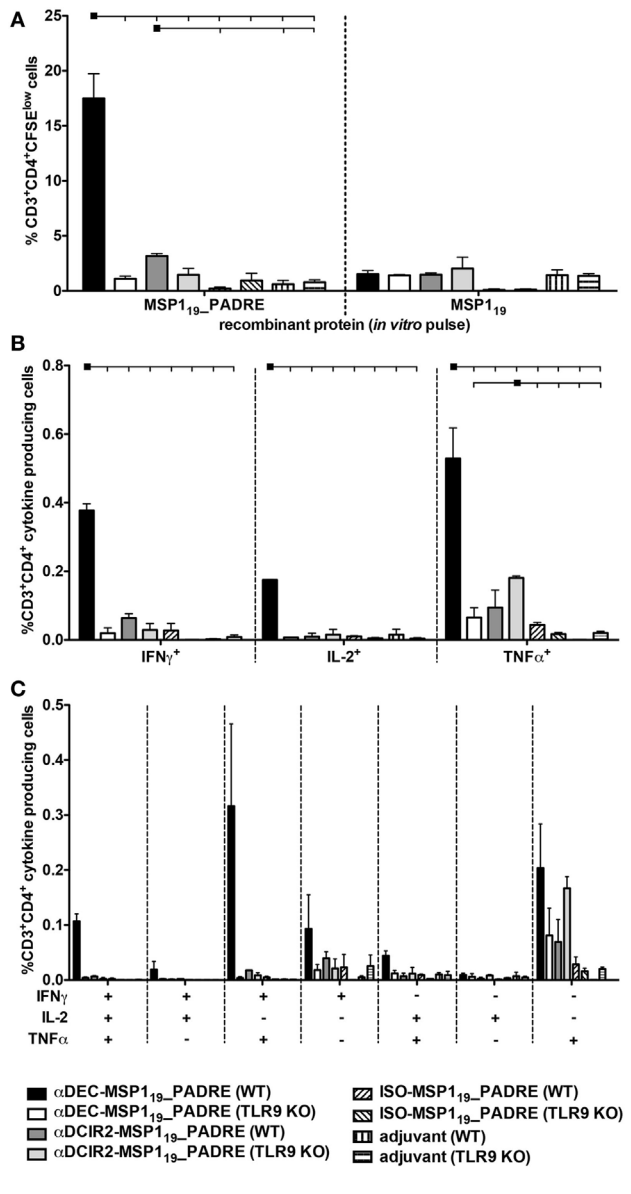

FIGURE 3 | Continued

FIGURE 3 | Continued
Antigen targeting to the CD8α$^+$ dendritic cells (DCs) *via* DEC205 receptor in the presence of CpG oligodeoxynucleotides (ODN) 1826 induces strong CD4$^+$ T cell response that is practically abolished in the absence of TLR9 signaling. WT and TLR9 KO mice were immunized with the different hybrid mAbs as described in **Figure 2**. Twenty days after the administration of the booster dose, mice were euthanized and **(A)** Splenocytes from pooled WT or TLR9 KO mice (n = 5) were labeled with CFSE and cultured with 5 μg/mL of either MSP1$_{19}$_PADRE or MSP1$_{19}$ recombinant proteins for 96 h. Cells were then stained with fluorescent antibodies, and CD4$^+$ T cell proliferation by CFSE dilution was analyzed. The graph shows the percentage of CD3$^+$CD4$^+$CFSE^{low} T cells after the subtraction of values obtained in the absence of any stimulus. Bars indicate mean ± SEM, and the experiment was analyzed by two-way ANOVA followed by the Bonferroni posttest. **(B)** Splenocytes from pooled mice (n = 5 animals/group) were pulsed *ex vivo* with 5 μg/mL of MSP1$_{19}$_PADRE recombinant protein and incubated in the presence of brefeldin for 12–16 h. Graphs show the percentage of cells producing IFN-γ, IL-2, or TNFα in the CD3$^+$CD4$^+$ gate after subtraction of values obtained in the absence of any stimulus. Bars indicate mean ± SEM, and the experiment was analyzed by two-way ANOVA followed by the Bonferroni posttest. Black squares indicate the reference group against which comparisons are being made. Horizontal capped lines only depict significant differences ($p < 0.05$). **(C)** Boolean combinations were created using FlowJo software to determine the frequency of each cytokine production based on all possible combinations. The experiment was performed in duplicates using samples from pooled mice. One representative experiment of two is depicted.

To further evaluate the PADRE-specific CD4$^+$ T cell response, we tested, by intracellular staining, the production of inflammatory cytokines IFN-γ, IL-2, and TNF-α (**Figure 3B**). Splenocytes from mice immunized with the different fusion mAbs were pulsed with the recombinant MSP1$_{19}$_PADRE protein, and intracellular cytokines were labeled after overnight stimulation (representative gating strategy shown in Figure S5 in Supplementary Material). Similarly to what was observed when the CD4$^+$ T cell proliferation was analyzed, we detected specific CD4$^+$ T cells positive for IFN-γ, IL-2, or TNF-α mainly in αDEC205-MSP1$_{19}$_PADRE immunized WT mice. Once more, when TLR9 KO mice were immunized with αDEC205-MSP1$_{19}$_PADRE, the frequencies of cytokine-producing cells were negligible. Antigen targeting to CD8α$^-$ DCs *via* DCIR2 did not induce specific cells that produced IFN-γ or IL-2. However, we observed a small percentage of TNF-α producing cells in WT or TLR9 KO mice immunized with αDCIR2-MSP1$_{19}$_PADRE. In the absence of antigen targeting (when ISO-MSP1$_{19}$_PADRE was used), only negligible frequencies of cytokine-producing cells were detected (**Figure 3B**).

To analyze the cytokine response in more detail, we performed Boolean analysis in order to study cell polyfunctionality. We observed that the specific CD4$^+$ T cells produced different

combinations of the three cytokines in WT mice immunized with αDEC205-MSP1$_{19}$_PADRE, namely IFN-γ$^+$IL-2$^+$TNF-α$^+$, IFN-γ$^+$TNF-α$^+$, and TNF-α$^+$. As expected, immunization of TLR9 KO mice in the same conditions failed to promote an inflammatory response (**Figure 3C**). Based on these results, we conclude that CpG ODN 1826 stimulation is critical for proliferation and induction of polyfunctional CD4$^+$ T cells when the antigen is targeted to CD8α$^+$ DCs *via* DEC205. Also, this response is strongly dependent on TLR9 signaling.

TLR5 Signaling Contributes to Improve the Antibody Response after Priming When the Antigen Is Targeted to CD8α$^-$ DCs and after Boosting When the Antigen Is Targeted to CD8α$^+$ DCs

To study the contribution of flagellin and TLR5 signaling in the development of a humoral immune response elicited by antigen targeting to CD8α$^+$ or CD8α$^-$ DCs, groups of WT and TLR5 KO mice were immunized with αDEC205-MSP1$_{19}$_PADRE, αDCIR2-MSP1$_{19}$_PADRE, or ISO-MSP1$_{19}$_PADRE in the presence of recombinant flagellin as adjuvant. It is important to highlight that DCs derived from the TLR5KO mice also expressed similar amounts of DEC205 and DCIR2 receptors when compared to WT (Figure S3 in Supplementary Material). Anti-MSP1$_{19}$ antibody titers were determined before and after the boost. **Figure 4A** shows that TLR5 signaling is dispensable for antigen targeting to CD8α$^+$ DCs before the boost, but it is important if the antigen is directed to CD8α$^-$ DCs. In the absence of targeting (i.e., mice immunized with the isotype control), we observed an increase in antibody titers in the absence of TLR5 signaling. After the administration of the booster dose, antibody titers increased in WT mice immunized with all the different fusion mAbs. In TLR5 KO mice, no differences were observed before or after the boost following immunization with αDEC205-MSP1$_{19}$_PADRE or ISO-MSP1$_{19}$_PADRE. However, in TLR5 KO mice immunized with αDCIR2-MSP1$_{19}$_PADRE mAb, the anti-MSP1$_{19}$ titers were increased. When all groups were compared after the boost, we noticed that TLR5 signaling seems to play a role only when CD8α$^+$ DCs are targeted *via* DEC205, as we did not observe statistical differences between the WT and TLR5 KO groups immunized with either αDCIR2-MSP1$_{19}$_PADRE or ISO-MSP1$_{19}$_PADRE. In summary, in the presence of flagellin and after the second dose, DC targeting to both DC subsets leads to an increased humoral response in comparison with the absence of targeting.

Moreover, anti-MSP1$_{19}$ IgG1, IgG2b, IgG2c, and IgG3 subclasses were determined by ELISA after boost. All groups, except the TLR5 KO mice immunized with αDEC205-MSP1$_{19}$_PADRE, presented detectable titers of IgG1, IgG2b, and IgG2c. Very low (or undetectable) levels of IgG3 titers were also detected. Contrary to what was observed in the WT animals immunized with CpG ODN 1826, mice immunized with flagellin did not promote vigorous class switch as the IgG1/IgG2c ratio was higher than 1 in all groups, except in the TLR5 KO mice immunized with ISO-MSP1$_{19}$_PADRE (**Figure 4B**). Interestingly, WT and TLR5 KO mice immunized with αDCIR2-MSP1$_{19}$_PADRE presented high IgG1/IgG2c ratios (56.45 and 70.20, respectively), while in mice immunized with αDEC205-MSP1$_{19}$_PADRE these ratios were much lower (12.67 and 1.77, respectively). Of note, IgG2b titers were drastically reduced in the absence of TLR5 signaling when the antigen was targeted to both DC subsets. This result indicates that TLR5 signaling influences class switch.

Flagellin Is Important for the Induction of CD4$^+$ T Cell Proliferation but Not for the Production of Inflammatory Cytokines When CD8α$^-$ DCs Are Targeted *via* DCIR2

We next analyzed the CD4$^+$ T cell proliferation elicited when MSP1$_{19}$_PADRE was targeted to both DC subsets in the presence of flagellin (representative gating strategy depicted in Figure S4 in Supplementary Material). We observed higher CD4$^+$ T cell proliferation in WT mice immunized with αDCIR2-MSP1$_{19}$_PADRE when compared to the groups immunized with αDEC205-MSP1$_{19}$_PADRE or ISO-MSP1$_{19}$_PADRE. Once more, MSP1$_{19}$ recombinant protein was used as a negative control, and we did not observe significant proliferation among all the groups. Interestingly, for αDEC205-MSP1$_{19}$_PADRE or αDCIR2-MSP1$_{19}$_PADRE mAbs, despite a reduction, proliferation does not seem to depend on TLR5 signaling, as we did not observe statistically significant differences when we compared WT with TLR5 KO mice. On the other hand, TLR5 signaling seems important in the absence of targeting (i.e., in mice immunized with ISO-MSP1$_{19}$_PADRE, **Figure 5A**). These results indicate that flagellin is important for the induction of CD4$^+$ T cell proliferation when CD8α$^-$ DCs are targeted *via* DCIR2. However, TLR5 signaling does not seem to play a major role in the CD4$^+$ T cell proliferation when the antigen is delivered to either CD8α$^+$ or CD8α$^-$ DCs *via* DEC205 or DCIR2, respectively.

Surprisingly, when the frequency of CD4$^+$ T cells producing inflammatory cytokines (IFN-γ, IL-2, and TNF-α) was analyzed (a representative gating strategy is depicted in Figure S5 in Supplementary Material), we did not detect many cells producing any of these cytokines in the WT or TLR5 KO groups immunized with αDCIR2-MSP1$_{19}$_PADRE. On the other hand, specific CD4$^+$ T cells producing IFN-γ, IL-2, or TNF-α were detected in the WT group immunized with αDEC205-MSP1$_{19}$_PADRE. This response was reduced in TLR5 KO mice (**Figure 5B**). Similar results were obtained when polyfunctional CD4$^+$ T cells were analyzed (**Figure 5C**). We conclude that antigen targeting to CD8α$^-$ DCs *via* DCIR2 in the presence of flagellin induces CD4$^+$ T cell proliferation. However, induction of inflammatory polyfunctional CD4$^+$ T cells is only observed when the antigen is targeted specifically to the CD8α$^+$ DCs *via* DEC205 and is partially dependent on TLR5 signaling.

In an attempt to verify if other cytokines were being produced, we analyzed the production of IL-4, IL-6, IL-17A, and IL-10 in the supernatant of cell cultures, 96 h after pulse, using recombinant MSP1$_{19}$_PADRE or MSP1$_{19}$ proteins in WT mice immunized with the different fusion mAbs (Figure S6 in Supplementary Material). We detected higher levels of IL-4 and IL-10 in WT mice immunized with αDCIR2-MSP1$_{19}$_PADRE when compared to mice

immunized with αDEC205-MSP1$_{19}$_PADRE together with CpG ODN 1826 (Figures S6A,B in Supplementary Material, respectively) or flagellin (Figures S6C,D in Supplementary Material, respectively). The production of IL-6 and IL-17A was below the kit detection threshold (data not shown). Taken together, these results suggest that antigen targeting to CD8α$^-$ DCs *via* DCIR2 in the presence of CpG ODN 1826 or flagellin induces more Th2/regulatory response.

Differential Expression of Co-stimulatory Molecules in CD8α$^+$ and CD8α$^-$ DCs Induced by CpG ODN 1826 or Flagellin

Due to differences in CD4$^+$ T cell proliferation induced by antigen targeting to CD8α$^+$ and CD8α$^-$ DCs using CpG ODN 1826 or flagellin as adjuvants, we hypothesized that CD8α$^+$ and CD8α$^-$ DCs may differently respond to these activation stimuli. CD8α$^+$

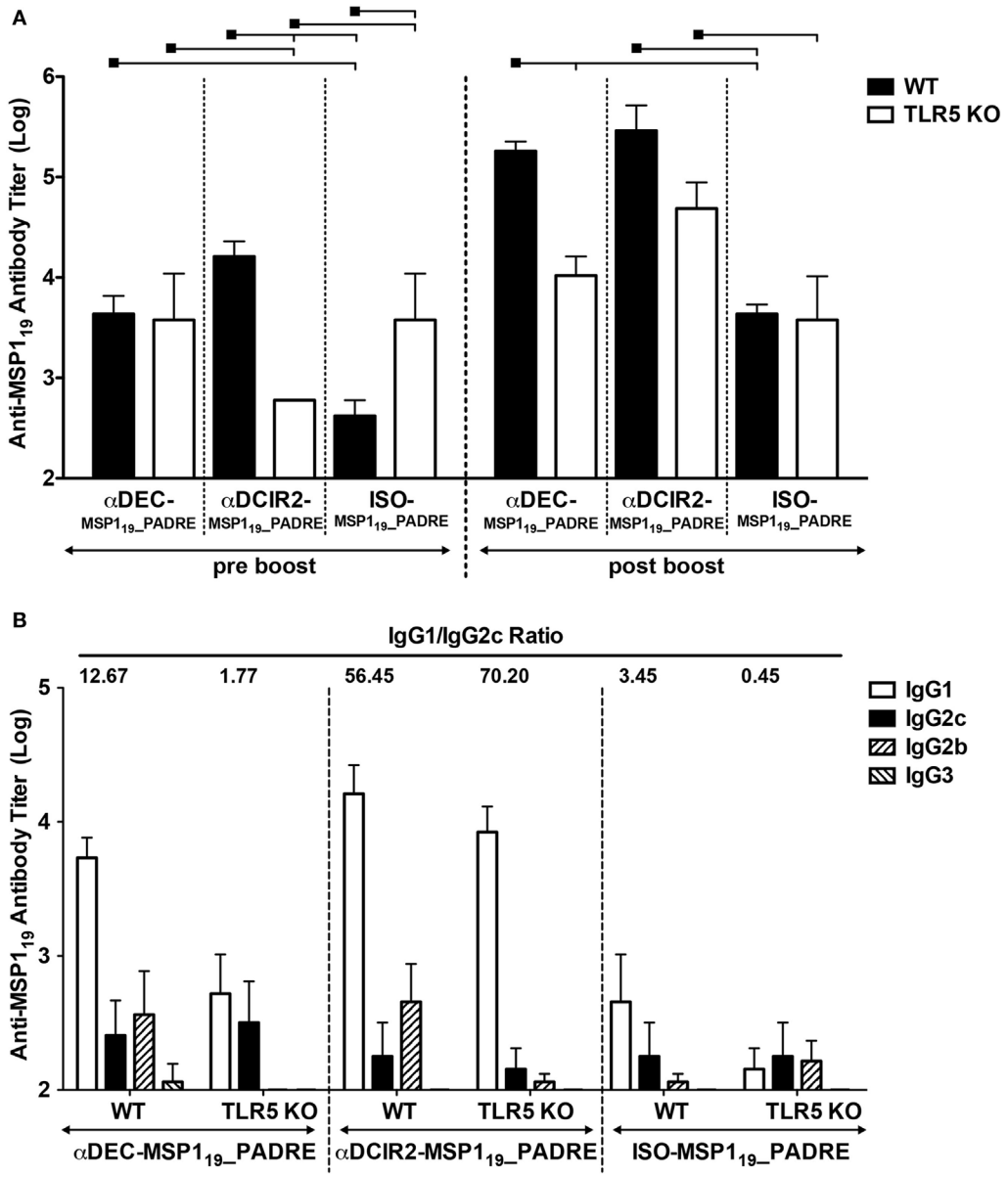

FIGURE 4 | TLR5 signaling contributes after prime or boost, depending on the targeted dendritic cell (DC) subset. WT and TLR5 KO mice were immunized with 5 μg of αDEC205-MSP1$_{19}$_PADRE, αDCIR2-MSP1$_{19}$_PADRE or ISO-MSP1$_{19}$_PADRE together with 5 μg of flagellin as adjuvant. **(A)** Five days before (pre-boost) and 14 days after (post-boost) the administration of the booster dose, blood was collected and serum obtained. Total anti-MSP1$_{19}$ IgG antibodies were detected by ELISA. Graphs show the mean ± SEM of anti-MSP1$_{19}$ titers in different groups normalized in log10 scale (n = 5 animals/group). Experiments were analyzed by two-way ANOVA for repeated measures followed by Bonferroni posttest. Black squares indicate the reference group against which comparisons are being made. Horizontal capped lines only depict significant differences ($p < 0.05$). **(B)** Anti-MSP1$_{19}$ IgG1, IgG2b, IgG2c, and IgG3 subclasses were also determined by ELISA 14 days after the boost. The numbers above the bars indicate the IgG1/IgG2c ratio calculated for each group.

DC targeting *via* DEC205 induced Th1 CD4$^+$ T cell polarization when CpG ODN 1826 or flagellin were used. On the other hand, CD8α$^-$ DC targeting using the same adjuvants induced more IL-4 and IL-10 in culture supernatants, and robust CD4$^+$ T cell proliferation when flagellin was used.

To further gain insight into those differences, we sorted spleen CD8α$^+$ and CD8α$^-$ DCs. After isolation, both DC subsets were stimulated with CpG ODN 1826 or flagellin for 48 h. Negative controls were left untreated. Then, TNFα and IL-6 in culture supernatants were measured (Figure S7 in Supplementary Material). DC stimulation with CpG ODN 1826 induced TNFα and IL-6 production in both DC subsets. However, CD8α$^-$ DCs were much more responsive and produced approximately 7 times more TNFα or 13 times more IL-6. When flagellin was used as adjuvant, CD8α$^+$ DCs did not secrete TNFα or IL-6. On the other hand, CD8α$^-$ DCs secreted more TNFα or IL-6, although the latter difference was not statistically significant (when compared to non-stimulated DCs). These results indicate that CpG ODN 1826 is able to directly activate CD8α$^-$ DCs to produce more TNFα or IL-6 when compared to CD8α$^+$ DCs, while flagellin only directly activates CD8α$^-$ DCs.

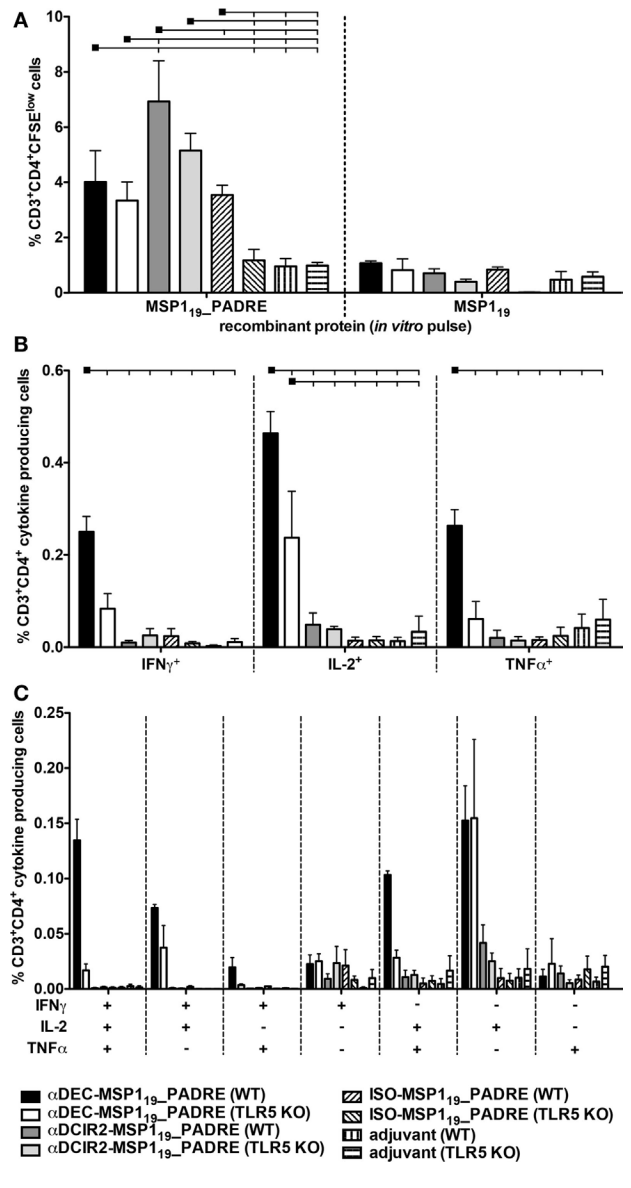

FIGURE 5 | Continued

FIGURE 5 | Continued
Antigen targeting to CD8α$^-$ dendritic cells (DCs) *via* DCIR2 in the presence of flagellin induces CD4$^+$ T cell proliferation but no production of pro-inflammatory polyfunctional CD4$^+$ T cells. WT and TLR5 KO mice were immunized with the different hybrid monoclonal antibodies (mAbs) as described in **Figure 4**. Twenty days after the administration of the booster dose, mice were euthanized and **(A)** Splenocytes from pooled WT or TLR5 KO mice (n = 5) were labeled with CFSE and cultured with 5 μg/mL of either MSP1$_{19}$_PADRE or MSP1$_{19}$ recombinant proteins for 96 h. Cells were then stained with fluorescent antibodies, and CD4$^+$ T cell proliferation was analyzed by CFSE dilution. The graph shows the percentage of CD3$^+$CD4$^+$CFSE^{low} T cells after the subtraction of values obtained in the absence of any stimulus. Bars indicate mean ± SEM, and the experiment was analyzed by two-way ANOVA followed by the Bonferroni posttest. **(B)** Splenocytes from pooled mice (n = 5 animals/group) were pulsed *ex vivo* with 5 μg/mL of MSP1$_{19}$_PADRE recombinant protein and incubated in the presence of brefeldin for 12–16 h. Graphs show the percentage of cells producing IFN-γ, IL-2, or TNFα in the CD3$^+$CD4$^+$ gate after subtraction of values obtained in the absence of any stimulus. Bars indicate mean ± SEM, and the experiment was analyzed by two-way ANOVA followed by the Bonferroni posttest. Black squares indicate the reference group against which comparisons are being made. Horizontal capped lines only depict significant differences ($p < 0.05$). **(C)** Boolean combinations were created using FlowJo software to determine the frequency of each cytokine production based on all possible combinations. The experiment was performed in duplicates using samples from pooled mice. One representative experiment of two is depicted.

We then decided to investigate DC subset expression of co-stimulatory molecules after *in vivo* administration of CpG ODN 1826 or flagellin to WT and KO mice. As negative controls, we used WT mice immunized with saline. We analyzed the upregulation of CD80, CD86, and CD40 on CD8α$^+$CD11b$^-$ (DEC205$^+$) and CD8α$^-$CD11b$^+$ (DCIR2$^+$) DC subtypes according to the gating strategy depicted in Figure S8 in Supplementary Material. 6 h after injection, we observed a significant increase in the median fluorescence intensity (MFI) of CD86 and CD40 in both DC subsets in WT mice immunized with CpG ODN 1826 when compared to saline. This increase was reverted in TLR9 KO mice (**Figures 6A–B**, middle and lower panels). Although we observed a slight increase in CD80 expression, especially in CD8α$^+$CD11b$^-$ DCs when compared to saline or TLR9 KO mice, the difference was not statistically significant (**Figures 6A–B**, upper panels). When we analyzed DCs derived from mice immunized with flagellin, we observed an increase in CD80, CD86, and CD40 MFIs in both DC subtypes when compared to saline. The absence of TLR5 signaling also impaired MFI upregulation (**Figure 6**, all panels). We conclude that either CpG ODN 1826 or flagellin administration induces significant upregulation of co-stimulatory molecules in both DC subsets *in vivo* after 6 h of inoculation.

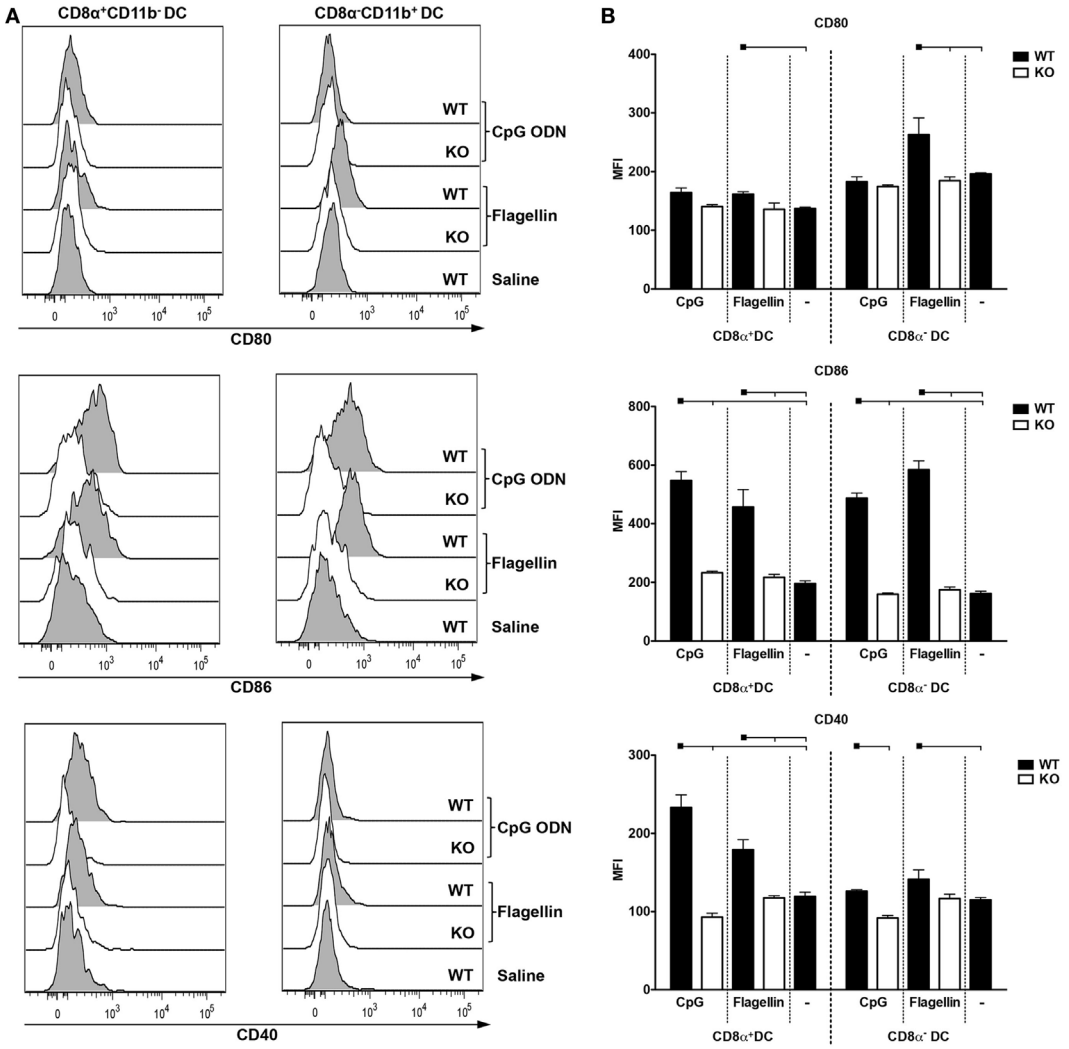

FIGURE 6 | CpG oligodeoxynucleotides (ODN) 1826 or flagellin induce differential expression of co-stimulatory molecules in CD8α$^+$ and CD8α$^-$ dendritic cells (DCs). C57BL/6 naive mice were injected i.p. with 25 μg of CpG ODN 1826 or with 5 μg of flagellin. 6 h later, mice were euthanized and splenocytes were stained with different fluorescent antibodies. The gating strategy is depicted in Figure S8 in Supplementary Material. **(A)** Representative histograms showing the expression of the co-stimulatory molecules CD80, CD86, and CD40 on CD8α$^+$ and CD8α$^-$ DCs. **(B)** Graphs show the mean ± SEM of the median fluorescence intensity for CD80, CD86, and CD40 obtained on CD8α$^+$CD11b$^-$ and CD8α$^-$CD11b$^+$ DCs from three mice per group. The experiment was analyzed by two-way ANOVA followed by the Bonferroni posttest. Black squares indicate the reference group against which comparisons are being made. Horizontal capped lines only depict significant differences ($p < 0.05$). One representative experiment of three is depicted.

DISCUSSION

Antigen targeting to DCs through DEC205 and DCIR2 receptors is a largely used strategy to induce specific immune responses to antigens. As previously described, the use of an adjuvant is required to promote a non-tolerogenic immune response (12, 25). Here, we studied the immune responses induced by MSP1$_{19}$_PADRE antigen targeting to CD8α$^+$ and CD8α$^-$ DCs *via* DEC205 and DCIR2 receptors using CpG ODN 1826 or flagellin as adjuvants. First, we successfully produced the fusion mAbs αDEC205- MSP1$_{19}$_PADRE, αDCIR2- MSP1$_{19}$_PADRE, and the isotype control (ISO-MSP1$_{19}$_PADRE). MSP1$_{19}$_PADRE is a chimeric antigen designed to increase MSP1$_{19}$ antigenicity. Our immunization results confirmed that PADRE epitope elicited robust cellular immune responses while MSP1$_{19}$ induced high antibody titers as previously described (43). We showed that CpG ODN 1826 and flagellin were efficient to induce antibody production, proliferation, and pro-inflammatory CD4$^+$ T cell responses when MSP1$_{19}$_PADRE was targeted to CD8α$^+$ DCs *via* DEC205. However, when the CD8α$^-$ DCs were targeted, different outcomes were observed. In CpG ODN 1826 immunized mice, we observed an increase in antibody responses, and the development of a more Th2 type of response, corroborated by the increase in IL-4 production. On the other hand, when we analyzed CD4$^+$ T cell proliferation or pro-inflammatory cytokine production, the response was negligible. An interesting

observation was made when flagellin was used as adjuvant. In this case, we observed $CD4^+$ T cell proliferation but no induction of pro-inflammatory cytokines. Again, we detected an increase in IL-4 production. These results led us to conclude that each adjuvant seemed to differentially influence the promotion of adaptive immune responses when the antigen was targeted to $CD8\alpha^+$ and $CD8\alpha^-$ DCs.

CpG oligodeoxynucleotides (ODN) 1826 is a TLR9 ligand expressed in antigen-presenting cells, including DCs and B cells (31). It can stimulate activated B cells by direct TLR9 signaling and promote their differentiation into plasma cells. Also, antigen-experienced B cells upregulate TLR9 and can be activated by CpG ODN 1826, increasing antibody production (50–52). In our system, the use of CpG ODN 1826 as an adjuvant induced high antibody titers when mice were immunized with either αDEC205-$MSP1_{19}$_PADRE or αDCIR2-$MSP1_{19}$_PADRE. These titers were significantly reduced in the absence of TLR9 signaling. Our results also indicated that TLR9 signaling *via* CpG ODN 1826 influences antibody class switch, promoting IgG2b and mainly IgG2c subclasses when mice are immunized with αDEC205-$MSP1_{19}$_PADRE. Immunization with αDCIR2-$MSP1_{19}$_PADRE showed an even more pronounced effect as class switch to IgG2b and IgG2c was completely abolished in TLR9 KO mice. A possible explanation for this effect may be related to the fact that CpG ODN 1826 increases germinal center reaction induced by helper T cells primed by matured DC, supporting class switch to IgG2b and IgG2c subclasses (53). In fact, it was previously shown that $CD8\alpha^-$ DCs are known to induce functional antigen-specific Tfh cells that play a central role in antibody production (41, 54). It is interesting to mention that in the absence of antigen targeting, CpG ODN 1826 signaling also played a crucial role in antibody production and class switch.

A different set of results was obtained when flagellin was used as adjuvant. First, TLR5 signaling was only partially important for the induction of antibodies when $CD8\alpha^+$ DCs were targeted. Antigen targeting to $CD8\alpha^-$ DCs, or absence of targeting, were not influenced by flagellin signaling through TLR5. Class switching was mainly restricted to IgG1 and not influenced by the absence of TLR5 signaling, mainly when the antigen was delivered through αDCIR2-$MSP1_{19}$_PADRE. The effect of flagellin in inducing a Th2 type of response with the production of high levels of specific IgG1 was previously reported (55, 56). The partial effect observed in TLR5 KO mice may also be explained by the fact that, once intracellular, flagellin is able to signalize through Naip5/NLRC4 inflammasome (32, 34, 57), and induce DC activation. Furthermore, there are data indicating that flagellin can stimulate antibody production in a TLR5 and NAIP5 independent fashion (56).

Interesting results were also obtained when we analyzed the proliferation of specific $CD4^+$ T cells when the antigen was targeted to $CD8\alpha^+$ and $CD8\alpha^-$ DCs in the presence of CpG ODN 1826 or flagellin. When CpG ODN 1826 was used as adjuvant, a very pronounced T cell proliferation was only observed in WT mice immunized with αDEC205-$MSP1_{19}$_PADRE. This response was almost completely abolished in the absence of TLR9 signaling. More interesting was the result obtained when the $CD8\alpha^-$ DCs were targeted *via* DCIR2. In this case, we were unable to detect specific proliferation in WT or TLR9 KO mice, indicating that antigen delivery to this particular DC subset in the presence of CpG ODN 1826 is not an efficient way to induce $CD4^+$ T cell proliferation under our experimental conditions. This result contrasts with reports that observed vigorous $CD4^+$ T cell proliferation after antigen targeting to the $CD8\alpha^-$ DCs (12, 58). This difference may be explained by differences in the immunization protocols and/or in the adjuvants used. While both reports used transgenic T cell transference and analyzed proliferation 3 or 9 days after the administration of one dose of the chimeric mAbs, or *in vitro*, we administered two doses of each mAb and analyzed the $CD4^+$ T cell immune response 20 days after the boost. Also, both authors used either the agonist αCD40 mAb or a combination of αCD40 mAb plus poly (I:C). Another important point is that, as mentioned before, the $CD8\alpha^-$ DC subset is very efficient to induce Tfh cells (41, 54) that may not necessarily present strong proliferation capacity. On the other hand, when flagellin was used, we detected specific T cell proliferation in response to antigen targeting especially to $CD8\alpha^-$ DCs, result that agrees with previous reports (12, 58). Despite a reduction, the response obtained in the absence of TLR5 signaling was not significantly different from that obtained in its presence. Furthermore, antigen targeting to the $CD8\alpha^+$ DCs induced a lower level of $CD4^+$ T cell proliferation in the presence or absence of TLR5 signaling. In summary, TLR5 direct signaling seems dispensable for the induction of antigen-specific $CD4^+$ T cell proliferation after antigen targeting to $CD8\alpha^+$ or $CD8\alpha^-$ DC subsets.

When we analyzed the induction of specific $CD4^+$ T cells that produced pro-inflammatory cytokines, we noticed that the response was mainly dependent on the targeted DC subset. The $CD4^+$ T cells response was similar when the antigen was targeted to $CD8\alpha^+$ DCs *via* DEC205 using either CpG ODN 1826 or flagellin as adjuvants. Immunizations with αDEC205-$MSP1_{19}$_PADRE in the presence of CpG ODN 1826 or flagellin induced polyfunctional IFN-γ^+IL-2^+TNFα^+ $CD4^+$ T cells. Antigen targeting to $CD8\alpha^+$ DCs *via* DEC205 also induced inflammatory cytokines in the presence of poly (I:C), a TLR3/MDA5 ligand (27–29, 59). Taken together, these results confirm that antigen targeting to $CD8\alpha^+$ DCs is independent of the adjuvant but dependent of DC subtype. On the contrary, very low percentages of pro-inflammatory cytokine-producing cells were obtained when the antigen was targeted to $CD8\alpha^-$ DCs using either CpG ODN 1826 or flagellin, while higher levels of IL-4 and IL-10 were detected in culture supernatants. Detection of IL-4 in culture supernatants was previously reported when $CD8\alpha^-$ DCs were targeted *via* DCIR2 (58). This lack of pro-inflammatory cytokine production when the antigen is delivered through DCIR2 may also be explained by the fact that $CD8\alpha^-$ DCs are specialized in antigen presentation and induction of Tfh cells (41, 54). In this way, it is plausible to speculate that they may not induce the activation of Th1 cells capable of producing IFN-γ, IL-2, and TNFα.

Up to this point, our results suggested that the adjuvants might help in the development of humoral immune responses, while it is the DC subset that essentially dictates the fate of the $CD4^+$ T cell response. To explore in more detail DC subset activation

by the two adjuvants, we performed experiments *in vitro* and *in vivo*. Purified splenic WT DCs were incubated with either CpG ODN 1826 or flagellin, and TNFα or IL-6 secretion was detected 48 h later. We observed that CpG ODN 1826 was able to induce cytokine production by both DC subsets while flagellin only activated the CD8α$^-$ DCs. In *in vivo* experiments, we administered CpG ODN 1826 or flagellin to WT or KO mice, and 6 h later analyzed the upregulation of co-stimulatory molecules. CpG ODN 1826 induced mainly upregulation of CD86 and CD40 in both DC subsets. Previous reports showed that both DCs subsets are in fact able to respond to CpG ODN as they express similar levels of TLR9 transcripts (60), and also upregulate co-stimulatory molecules (61). Interesting results were obtained when flagellin was used *in vitro* and *in vivo*. In this case, flagellin was not able to directly activate CD8α$^+$ DCs. This can be explained by the fact that this particular subset does not express TLR5 (60). However, an upregulation in co-stimulatory molecules was observed *in vivo*. Previous reports also show conflicting results when flagellin was used. Some investigators showed direct activation of murine bone marrow-derived DCs (55, 62, 63), while others reported an effect on human, but not murine, DCs (64). Salazar-Gonzalez et al. obtained similar results to ours when flagellin was administered to mice, but no effect when flagellin was added directly to purified DCs. In this way, they suggested that the stimulatory effect of flagellin on splenic DCs is indirect (65).

In summary, our results indicate that the combination of CpG ODN 1826 and flagellin with antigen delivery to the two major conventional DC subsets induces different effects on the humoral and cellular immune responses. While both adjuvants are efficient to induce Th1 responses when the antigen is directed to CD8α$^+$ DCs, a more Th2/Treg type of response is obtained when the antigen is directed to the CD8α$^-$ DCs. This knowledge may be explored for the design of DC-targeted vaccines aiming to use CpG ODN 1826 or flagellin as adjuvants. The best combination of antigen targeting/adjuvant will depend mainly on the correlates of protection for a given disease.

ETHICS STATEMENT

All experimental procedures and animal handling were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the Brazilian National Law on animal care (11.794/2008). The Institutional Animal Care and Use Committee (CEUA) of the University of São Paulo approved all procedures under the protocol number 082.

AUTHOR CONTRIBUTIONS

RA, FS, and SB designed the experiments. RA, FS, KA, BA, NF, and MY conducted most of the experiments. RA, FS, and SB analyzed the data. FS and SB prepared the figures and wrote the manuscript. IS, LF, and DR contributed reagents. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01727/full#supplementary-material.

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Vaccination With Recombinant Filamentous *fd* Phages Against Parasite Infection Requires TLR9 Expression

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Recombinant filamentous *fd* bacteriophages (*rfd*) expressing antigenic peptides were shown to induce cell-mediated immune responses in the absence of added adjuvant, being a promising delivery system for vaccination. Here, we tested the capacity of *rfd* phages to protect against infection with the human protozoan *Trypanosoma cruzi,* the etiologic agent of Chagas Disease. For this, C57BL/6 (B6) and *Tlr9*$^{-/-}$ mice were vaccinated with *rfd* phages expressing the $OVA_{257–264}$ peptide or the *T. cruzi*-immunodominant peptides PA8 and TSKB20 and challenged with either the *T. cruzi* Y-OVA or Y-strain, respectively. We found that vaccination with *rfd* phages induces anti-PA8 and anti-TSKB20 IgG production, expansion of Ag-specific IFN-γ, TNF-α, and Granzyme B-producing $CD8^+$ T cells, as well as *in vivo* Ag-specific cytotoxic responses. Moreover, the *fd*-TSKB20 vaccine was able to protect against mortality induced by a high-dose inoculum of the parasite. Although vaccination with *rfd* phages successfully reduced both parasitemia and parasite load in the myocardium of WT B6 mice, *Tlr9*$^{-/-}$ animals were not protected against infection. Thus, our data extend previous studies, demonstrating that *rfd* phages induce Ag-specific IgG and $CD8^+$ T cell-mediated responses and confer protection against an important human parasite infection, through a TLR9-dependent mechanism.

Keywords: *fd* phages, *Tlr9*, *Trypanosoma cruzi*, cytotoxic T cell, delivery system, vaccine, CD8 T cells

INTRODUCTION

Filamentous *fd* bacteriophages are non-lytic viruses that infect and replicate only in host bacteria and, therefore, have been considered safe for the vertebrate host so far. Both the minor coat protein involved in host cell recognition, pIII, and the major coat protein pVIII of *fd* phages can be fused to antigenic peptides (1). Previous studies have shown that antigen peptides expressed on the phage capsid can be displayed through MHC class I and class II pathways on antigen-presenting cells (APCs), resulting in the capacity of inducing immune responses mediated by specific antibodies

and by helper and cytotoxic T lymphocytes (2, 3). Importantly, a single-chain antibody fragment (scFv), able to target dendritic cells (DCs) through the endocytic receptor DEC-205 (also known as CD205), can be introduced at the N-terminus of the pIII protein, further improving phage-uptake by DCs and their maturation. These *fd*sc-αDEC particles were shown to induced a strong and sustained $CD8^+$ T cell-mediated anti-tumor response (1). Moreover, *fd*sc-αDEC virions were shown to activate the TLR9 pathway, which induces the maturation of DCs (4, 5).

The intracellular protozoan *Trypanosoma cruzi* is the etiologic agent of Chagas' disease (American trypanosomiasis), which is the leading cause of myocardial disease and endemic in Latin America. Eight million people are estimated to be infected worldwide, of which 300,000 in the US, where enzootic cycles of *T. cruzi* have been recently established (6, 7). Since no mandatory screening exists for blood and tissue donors in non-endemic countries, it is expected that an altered epidemiology of Chagas disease will evolve considerably in a near future. No human vaccine is available to date and the approved drugs, nitrofuran and nitroimidazole, have limited efficacy in the chronic stage and important adverse side effects (8). Experimental *T. cruzi* infection in murine models has provided the means for the identification of protective immune responses, which need to be fully elucidated in order to allow the development of appropriated and safe human vaccines [reviewed in Ref. (9)]. Eliminating the parasite at the acute phase prevents parasite survival and may avoid chronic phase immunopathology. Therefore, prophylactic vaccination, by reducing or completely eliminating the parasite burden, represents a desirable method to restrict the development of the chronic symptoms of the disease. *T. cruzi* antigens recognized by immune sera from infected humans or animals served as the basis for studies employing recombinant proteins. These recombinant proteins included members of the large trans-sialidase (Ts) surface protein family, which are expressed mainly in the infective trypomastigote and amastigote forms of the parasite. Proteins belonged to the family of cysteine-proteases (cruzipain) and other antigens, such as the flagellar calcium-binding protein, paraflagellar rod protein-2, LYT-1 antigen, ribosomal protein L7a-like protein, and KMP11, among others, have also been used in different formulations and delivery systems, including recombinant proteins mixed with distinct adjuvants and platforms using DNA delivery or recombinant viruses [reviewed in Ref. (10)].

Here, we investigated the capacity of recombinant filamentous fd bacteriophages (r*fd*) phages to act as a delivery system for vaccination against the experimental infection with *T. cruzi* in mice, in the absence of any added adjuvant. For this, we genetically modified *fd* phages, by introducing the $OVA_{257–264}$ peptide or the *T. cruzi* immunodominant peptides TSKB20 and PA8, derived from *trans*-sialidase (Ts) and amastigote surface protein-2 (ASP-2), respectively (11, 12), as an N-terminal fusion with the pVIII coat protein. Our results demonstrated that vaccination with r*fd* phages induces specific IgG and a strong $CD8^+$ T cell-mediated response, enhancing the percentages of Ag-specific IFN-γ and TNF-secreting $CD8^+$ T cells in the spleen, as well as the level of specific cytotoxicity *in vivo*. Accordingly, vaccinated mice displayed significant lower levels of parasitemia and parasite load in the myocardium and, moreover, exhibited an increased survival rate. Furthermore, we also demonstrated here that r*fd* phages devoid of the scFv anti-DEC-205 on pIII also require the expression of TLR9 by the host in order to confer protection against infection. Therefore, the present work extends previous studies on the immunogenicity mechanisms mediated by *fd* phages, reinforcing the potential use of these particles as a valuable delivery system for immunization without the need of exogenous adjuvant administration and shows, for the first time, its promising use in vaccination against intracellular parasites.

MATERIALS AND METHODS

Construction of *fd*-PA8 and *fd*-TSKB20 Filamentous Bacteriophages

Oligonucleotide sequences encoding PA8 peptide (VNHRFTLV) from ASP-2 or TSKB20 peptide (ANYKFTLV) derived from *trans*-sialidase, flanked by the sequences for the +2 to +3 and +4 to +10 residues of the pVIII protein and by the 5′-protruding ends of SacII-StyI restriction sites were designed and purchased from Eurofins Genomics, Germany (PA8-up: 5′-GGAGGGTgt taaccaccgtttcaccctggttGACGATCCCGC-3′;PA8-dw:5′-CTTGG CGGGATCGTCaaccagggtgaaacggtggttaacACCCTCCGC-3′; TSKb20-up: 5′-GGAGGGTgcgaactataaattcaccctggtgGAC GATCCCGC-3′; TSKb20-dw: 5′- CTTGGCGGGATCGTCcacc agggtgaatttatagttcgcACCCTCCGC-3′). Each pair of sequence was annealed and ligated into bacteriophage *fd*AMPLAY388-HA DNA previously digested with SacII and StyI restriction enzymes (1). The DNA were transformed into *Escherichia coli* TG1 recO cells, and their identities were confirmed by DNA sequencing.

Purification of Bacteriophages Particles

Wild-type and hybrid *fd*-PA8, *fd*-TSKB20, and *fd*-OVA (expressing the recombinant $OVA_{257–264}$-pVIII proteins) and *fd*-PA8αDEC (expressing also the recombinant scFv anti-DEC-205-pIII proteins) filamentous bacteriophages were purified from the supernatant of *Escherichia coli* TG1recO cells (1). Briefly, bacterial cells were grown in TY2X medium and the expression of the recombinant proteins was induced adding 0.1 mM isopropyl-beta–D-thiogalactopyranoside (Sigma-Aldrich) to the cultures. The bacteriophage virions were precipitated from *E. coli* supernatant using Polyethylene glycol 6000 (Sigma-Aldrich), purificated using cesium chloride (Sigma-Aldrich) gradient, and dialyzed against phosphate buffered saline (PBS) 1×. Elimination of LPS from phage particles was performed using Triton X-114 (Sigma-Aldrich) as previously described (13). Residual LPS contamination was assayed using the Limulus Amebocyte Lysate assay (Limulus Amebocyte Lysate QCL-1000 chromogenic modification, Lonza), according to the manufacturer's instructions. The number of copies of pVIII displaying the exogenous peptides was estimated by N-terminal sequence analysis of the purified virions and resulted in 15–20% for *fd*-OVA and 40% for *fd*-PA8 and *fd*-TSKB20. The expression of the scFv anti-DEC-205 in the pIII protein of the purified virions was assessed by western blot analysis using a mouse anti-HA tag mAb (Roche-Boehringer, Basel, Switzerland).

Mice and Ethics Statement

All animal experiments were conducted in accordance with guidelines of the Animal Care and Use Committee of the Federal University of Rio de Janeiro (Comitê de Ética do Centro de Ciências da Saúde CEUA-CCS/UFRJ). *Tlr9*$^{-/-}$ mice were generated by and obtained from Dr. S. Akira (Osaka University, Japan). Procedures and animal protocols were approved by CEUA-CCS/UFRJ license no.: IMPPG022.

Parasite and Experimental Infection

Mice used for experiments were sex- and aged-matched, and housed with a 12-h light–dark cycle. Bloodstream trypomastigotes of the Y-strain of *T. cruzi* were obtained from Swiss mice infected 7 days earlier. The concentration of parasites was estimated and at least four mice per group were inoculated intraperitoneally (i.p.) with 2×10^3 trypomastigotes (in 0.2 ml PBS), unless otherwise stated. The Y-OVA strain was obtained as follows: the *Ova* gene fragment was digested using XbaI and XhoI and then inserted into the pROCK-HYGRO vector, previously digested with the same restriction enzymes, to produce pROCK-Ova (14). *T. cruzi* Y-strain epimastigote forms were grown in liver infusion tryptose medium (pH 7.3) supplemented with 10% fetal bovine serum, streptomycin sulfate (0.2 g/l), and penicillin (200,000 units/l) at 28°C (all from Gibco Thermo Scientific). The parasite transfection was performed using electroporation following a previously described protocol (14). The transfected parasites were cultured for 6 weeks in the presence of hygromycin (200 µg/ml) (Sigma-Aldrich) for selection of parasites containing stably incorporated pROCK-Ova. For infection with Y-OVA strain, trypomastigotes were obtained from LLC-MK2-infected cultures and 2×10^6 trypomastigotes (in 0.2 ml PBS) were injected i.p./mouse. Parasitemia was monitored by counting the number of bloodstream trypomastigotes in 5 µl of fresh blood collected from the tail vein. Mouse survival was followed daily. For tissue parasite load quantification, hearts of infected mice were excised after perfusion, minced and the cardiac tissue immediately homogenized in 1.0 ml of 4.0 M Guanidine thiocyanate (Sigma-Aldrich) containing 8.0 µl/ml of β-mercaptoethanol (Sigma-Aldrich) and processed for DNA extraction. Generation of PCR standards and detection of parasite tissue load by real-time PCR was carried out as described (15); briefly, primers amplify a repeated satellite sequence of *T. cruzi* DNA of 195 base-pairs: TCZ-Fwd: (5′-GCTCTTGCCCACAAGGGTGC-3′) and TCZ-Rev: (5′-CCAAGCAGCGGATAGTTCAGG-3′). Reactions with TNF-α-Fwd: (5′-CCTGGAGGAGAAGAGGAAAGAGA-3′) and TNF-α-Rev: (5′-TTGAGGACCTCTGTGTATTTGTCAA-3′) primers for *Mus musculus* TNF-α gene were used as loading controls. PCR amplifications were analyzed using primers in combination with SYBR Green® on a StepOne Real-Time PCR System (Applied Biosystems, Life Technologies).

Vaccination

Mice were injected i.p. with 100 µg of *fd* phages in 200 µl of PBS at day −17 and −7 and infected with *T. cruzi* at day 0, as illustrated on Figure S1A in Supplementary Material.

Intracellular Cytokine Staining

Splenocytes isolated from infected mice were cultured in the presence of PA8 (VNHRFTLV) peptide at 3.0 µM, or left unstimulated, for 10 h at 37°C in the presence of 5.0 µM monensin (Sigma-Aldrich). Cells were surface stained with anti-CD8-PerCP, CD3-FITC, and H-2K^b-PA8-biotinylated pentamers (Proimmune), followed by 20 min staining with SAv-BV605 and fixed for 10 min with a solution containing PBS, 4% paraformaldehyde at RT. Then, cells were permeabilized for 15 min with PBS, 0.1% bovine serum albumine, 0.1% saponin (Sigma-Aldrich). Intracellular cytokine staining was performed with anti-IFN-γ-PE-Cy7, anti-granzyme B (GzB)-APC, and anti-TNF-PE (all mAbs and SAv from Biolegend). At least 10,000 gated CD8$^+$ lymphocyte events were acquired. Analytical flow cytometry was conducted with a FACSCantoII and the data were processed with FACSDiva™ software (BD Biosciences).

In Vivo Cytotoxicity Assay

For the *in vivo* cytotoxicity assays, splenocytes of naive B6 mice were divided into three populations, each loaded with 2.5 µM of either H-2K^b -restricted $OVA_{257–264}$ (SIINFEKL), or PA8 (VNHRFTLV), or TSKB20 (ANYKFTLV) peptides, or left untreated for 40 min at 37°C. During the last 10 min of incubation, each cell population was labeled with a different concentration of the fluorogenic dye CFSE (Molecular Probes Thermo Scientific) at final concentrations of either 8.6 µM (CFSE^{high}), 2.45 µM (CFSE^{int}), or 0.7 µM (CFSE^{low}). Subsequently, each cell population was extensively washed and mixed with equal numbers of the other two cell populations, before being injected i.v. $15–20 \times 10^6$ total cells per mouse. In each experiment, the same B6 (WT) CFSE-loaded target populations were injected in B6 and *Tlr9*$^{-/-}$ mice. Recipient animals were mice that had been vaccinated and infected (or not) with *T. cruzi* and naive controls. Spleen cells of recipient mice were collected 20 h after transfer, fixed with 1.0% paraformaldehyde and analyzed by cytometry, using a FACSCalibur Cytometer (BD Biosciences). Cells were gated on dot plot FSC × CFSE; percentages of CFSE^{low}, CFSE^{int}, and CFSE^{high} cells were obtained using CellQuest software (BD Biosciences). In all experiments, we refer to M1 as the control cell population without any exogenous peptide-loaded, and M2 and M3 are the cell populations loaded with different exogenous peptides. For calculating the percentage of specific lysis of each peptide-loaded population, M2 (or M3) was gated together M1, giving 100% of the events. Then, the following formula was applied: [1 − ($M2_{peptide\text{-}loaded}$ or $M3_{peptide\text{-}loaded}$ in Experimental group/$M1_{without\ Ag\ peptide}$ in Experimental group)/($M2_{peptide\text{-}loaded}$ or $M3_{peptide\text{-}loaded}$ in CTR group/$M1_{without\ Ag\ peptide}$ in CTR group)] × 100%; Experimental group are either vaccinated mice, infected mice, or both vaccinated and infected mice; CTR group are naive mice.

Anti-Mouse IgG ELISA

Sera from naive B6 mice, *fd*-WT (ctr) and *fd*-PA8 or *fd*-TSKB20 vaccinated and infected mice were diluted 1:5,000 and adsorbed overnight (ON) at 4°C, on treated microplates (Corning-Costar) previously coated with *fd*-WT bacteriophages immobilized by polyclonal rabbit anti-fd phage IgG (Sigma-Aldrich). After

absorption against *fd*-WT antigens, the sera were harvested and tested for reactivity against *fd*-WT and *fd*-PA8 or *fd*-TSKB20 antigens. Briefly, the absorbed sera were incubated at different dilutions overnight at 4°C, on microplates previously coated with *fd*-WT or *fd*-PA8 or *fd*-TSKB20 particles; secondary goat anti-mouse IgG antisera (1:2,500), labeled with peroxidase (HRP) (SouthernBiotech), were added for 4 h at room temperature and the reaction revealed with TMB (Thermo Scientific).

Bone Marrow-Derived Dendritic Cell (BMDC) Culture

Bone marrow-derived dendritic cells were obtained as previously described (16). Briefly, BM cells were cultured in tissue-culture-treated flasks at 1×10^6/ml in complete RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum (all from GIBGO, Thermo Scientific), and 20 ng/ml rmGM-CSF (R&D Systems), at 37°C, 5% CO_2 humid atmosphere. On day 2 of culture, 3 ml of the medium was removed and fresh warmed medium supplemented with GM-CSF (2×, 40 ng/ml) added. At day 3, medium was entirely replaced by fresh warmed medium with GM-CSF (20 ng/ml). On day 6, loosely adherent cells were harvested by gentle washing with PBS and cultured for 20 h in 96-well microplates in the presence of the indicated reagents (LPS, CpG, and *fd*-WT phages at 100 ng/ml, 1.0 μg/ml, and 250 μg/ml, respectively) and subsequently stained for flow cytometry analysis. LPS (*E. coli* 055:B5 strain) and CpG (ODN D-SL03) were from InvivoGen (San Diego, CA, USA).

Confocal Microscopy

LLC-MK2 cells were infected with cell-culture derived Y-strain trypomastigotes at 10:1 ratio overnight at 37°C, 5% CO_2. After 2 days, cells were fixed in 4% paraformaldehyde/PBS for 15 min and permeabilized with three washes with PBS-0,1% Triton X-100 (Bio-Rad). Sera of immunized and/or infected mice, as well as of naïve animals were added and incubated overnight at 4°C. After washing three times with PBS-0, 1% Triton X-100, donkey Cy3-labeled anti-mouse IgG was added for 2 h at RT. After washing, DAPI was added at 1 μg/ml at RT for 5 min. The coverslips were assembled with vectashield and fixed with enamel. Confocal microscopy was performed with a Zeiss Axio Observer.Z1 inverted microscope equipped with a CSU-X1A 5000 Yokogawa Spinning Disk confocal unit using a 100 × NA 1.4, oil-immersion plan-apochromatic objective. Images were captured with QImaging Rolera EM-C2 camera using Zen 2.3 system (Zeiss), and processed off line with Photoshop.

Database Search

The PA8 peptide (VNHRFTLV) sequence was queried at 100% coverage in the NCBI Protein Reference Sequence Database using Basic Local Alignment Search Tool (BLAST)[1] in order to identify proteins expressed by *T. cruzi* that could contain this epitope, other than the amastigote ASP-2 protein.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA[2]). Data were compared using a two-tailed Student's *t* test and are expressed as mean ±SEM. Data were considered statistically significant if p values were <0.05. The Gehan–Breslow–Wilcoxon test was used to compare the mouse survival rate. The differences were considered significant when the p value was <0.05.

RESULTS

fd-$OVA_{257–264}$ Phage Protects B6 but Not *Tlr9*$^{-/-}$ Mice Against Infection With *T. cruzi* Y-OVA Strain

To investigate whether vaccination with phage particles would protect against infection with *T. cruzi*, we first immunized B6 (WT) mice with the model antigen ovalbumin ($OVA_{257–264}$) SIINFEKL peptide, a known H-2K^b-restricted epitope, as an N-terminal fusion with the pVIII phage protein (*fd*-OVA). After vaccination, mice were infected with the *T. cruzi* Y-OVA transgenic strain, following the immunization and challenge scheme shown in Figure S1A in Supplementary Material; injection of *fd*-WT phages, which do not express any *T. cruzi* antigen, was employed as control treatment. As shown in **Figure 1A**, the level of blood parasitemia is significantly lower in B6 mice immunized with *fd*-OVA, when compared to B6 mice immunized with *fd*-WT phage. On the other hand, immunization was not able to reduce parasitemia levels in *Tlr9*$^{-/-}$ mice (**Figures 1B,C**). The same was true when parasite loads in the myocardium were measured at day 20 post-infection (pi) by qPCR (**Figure 1D**). We then investigated the levels of specific *in vivo* cytotoxicity against target cells loaded with the $OVA_{257–264}$ SIINFEKL peptide at day 20 pi in mice vaccinated with *fd*-OVA or *fd*-WT phages. As shown in **Figure 1E**, while *fd*-OVA-vaccinated B6 (WT) mice displayed around 30% of specific lysis against SIINFEKL-loaded targets, no significant increase in Ag-specific cytotoxicity was found in immunized *Tlr9*$^{-/-}$ mice (representative dot blots are shown on Figure S1B in Supplementary Material). However, no difference in the levels of specific cytotoxicity against the endogenous immunodominant *T. cruzi*-derived PA8 (VNHRFTLV) peptide was observed between WT and *Tlr9*$^{-/-}$ mice, immunized with *fd*-OVA or not (**Figure 1E**), in accordance to our previous data showing that the cytotoxic response is preserved in infected *Tlr9*$^{-/-}$ mice (17). Therefore, these results demonstrate that while effective in protecting WT B6 mice, the vaccination with *fd*-OVA did not confer any protection to *Tlr9*$^{-/-}$ mice against infection with *T. cruzi* Y-OVA strain.

fd Phage Particles Induce the Maturation of DCs Through a TLR9-Dependent Pathway

We next tested the *fd* virion property of inducing BMDC maturation *in vitro*. For this, WT (B6) and *Tlr9*$^{-/-}$ BMDC were cultured

[1] www.ncbi.nlm.nih.gov/BLAST/ (Accessed: January 10, 2018).

[2] www.graphpad.com (Accessed: January 10, 2018).

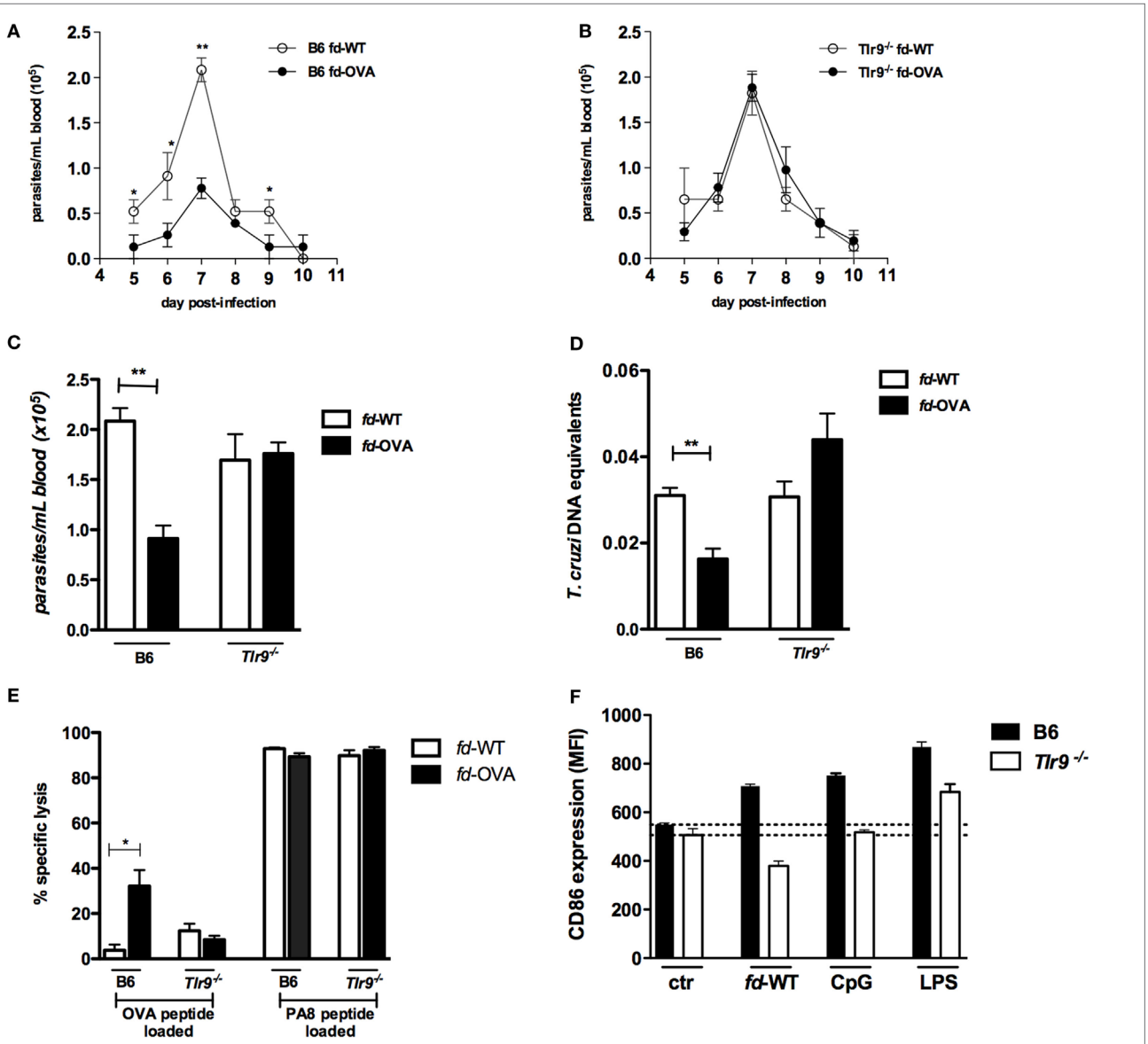

FIGURE 1 | Immunization with *fd*-OVA phages protects against infection with *T. cruzi* Y-OVA strain in TLR9-dependent manner. Male mice were immunized (as in Figure S1 in Supplementary Material) and infected on day 0 with 2×10^6 culture trypomastigotes of Y-OVA strain. Parasitemia curves of **(A)** B6 mice and **(B)** *Tlr9*$^{-/-}$ immunized with *fd*-OVA (black circles), or *fd*-WT phages as control (empty circles). Mean parasitemia values at day 7 pi. **(C)**. Parasite load in the myocardium at day 20 pi **(D)** and Ag-specific cytotoxicity *in vivo* against target cells loaded with OVA or ASP-2-derived (PA8) peptides, B6 and *Tlr9*$^{-/-}$ mice were immunized with *fd*-OVA (black bars) or *fd*-WT (white bars) (representative contour plots are shown in Figure S1B in Supplementary Material) **(E)**. CD86 expression (MFI) on bone marrow-derived dendritic cell (BMDC) after 20 h treatment *in vitro* with *fd*-WT (250 μg/ml), CpG (1.0 μg/ml), LPS (100 ng/ml) or left untreated (ctr). B6 (black bars) and *Tlr9*$^{-/-}$ (white bars) BMDCs **(F)**. Data represent mean values of individually analyzed mice ($n = 5$ per group) from control or immunized mice **(A–E)** or triplicate cultures **(F)**. Error bars = SEM, $^*p \leq 0.05$; $^{**}p \leq 0.01$ (two-tailed Student's *t*-test).

for 20 h in the presence of *fd*-WT particles or of the TLR9 and TLR4 ligands CpG and LPS, respectively. As shown in **Figure 1F**, *fd* phages were able to induce the upregulation of the CD86 costimulatory molecule in WT, but not in *Tlr9*$^{-/-}$ BMDCs. Note that, here, a 2.5 higher dose of *fd* phage particles was employed compared to the dose used in a previous study (1). Together, these data demonstrated that *fd*-OVA phages devoid of sc-αDEC also depend on the TLR9 pathway in order to induce the maturation of DCs and, consequently, a specific cytotoxic T cell (CTL) response, as previously shown for *fd*-OVA-αDEC virions (4).

Vaccination With r*fd* Phages Displaying the *T. cruzi* Immunodominant PA8 Peptide Protect B6 Mice Against Infection

The *T. cruzi* ASP-2-derived PA8 peptide was described as an immunodominant peptide, which induces high levels of CD8$^+$

CTLs (11). We then constructed r*fd* phages expressing the PA8 peptide and use it to vaccinate B6 mice, following the same previous protocol (Figure S1 in Supplementary Material). As shown in **Figure 2A**, the parasitemia was significantly diminished in mice previously vaccinated with *fd*-PA8, but not with *fd*-WT phages, when compared to parasitemia in non-vaccinated mice (PBS group). The *in vivo* cytotoxicity assay revealed an increased lysis rate against target cells loaded with the PA8 peptide in *fd*-PA8-vaccinated mice at day 13 pi, while no differences in the level of cytotoxicity against control (TSKB20) immunodominant peptide-loaded target cells was found between the groups (**Figure 2B**; Figure S2 in Supplementary Material). In accordance with the lower parasite load, the spleen of *fd*-PA8-vaccinated mice display lower numbers of $CD8^+$ T cells, as well as of total splenocytes, although the percentage of total $CD8^+$ T cells is equal in all the infected groups (**Figures 2C–E**). We also analyzed the expansion of PA8-specific $CD8^+$ T cells by staining with K^b-PA8 pentamers and found that their frequency was increased by *fd*-PA8 vaccination (**Figures 3A,E**; contour plots shown in Figure S3 in Supplementary Material). Then, we investigated the induction of CTL and cytokine-secreting $CD8^+$ T cells in the spleens of *fd*-PA8 or *fd*-WT vaccinated and infected mice, as well as in only infected (B6 infect) and non-infected (B6 ctr) mice. For this, the percentages (**Figures 3B–D**) and absolute numbers (**Figures 3F–H**) of $CD8^+$ T cells expressing IFN-γ, TNF, or GzB were assessed by *ex vivo* restimulation of splenocytes with the PA8 peptide, followed by intracellular staining. As shown in **Figures 3B,C**, vaccination with *fd*-PA8 increased the percentages of cytokine-secreting $CD8^+$ T cells, while the percentages of IFN-γ^+ $CD8^+$ and TNF^+ $CD8^+$ T cells is not different between *fd*-WT-treated and non-treated infected mice. On the other hand, no difference was found on the percentages of GzB^+ cells between the different groups of infected mice (**Figure 3D**). As the absolute number of $CD8^+$ T cells is lower in *fd*-PA8-vaccinated mice (**Figure 2E**), significant lower numbers of GzB^+ $CD8^+$ T cells are found in this group (**Figure 3H**).

Since it has been described that infected mice previously vaccinated with a recombinant adenovirus expressing ASP-2 display higher levels of polyfunctional $CD8^+$ T cells, compared to non-vaccinated infected mice (18), we also analyzed here the percentages and absolute numbers of $CD8^+$ T cells producing

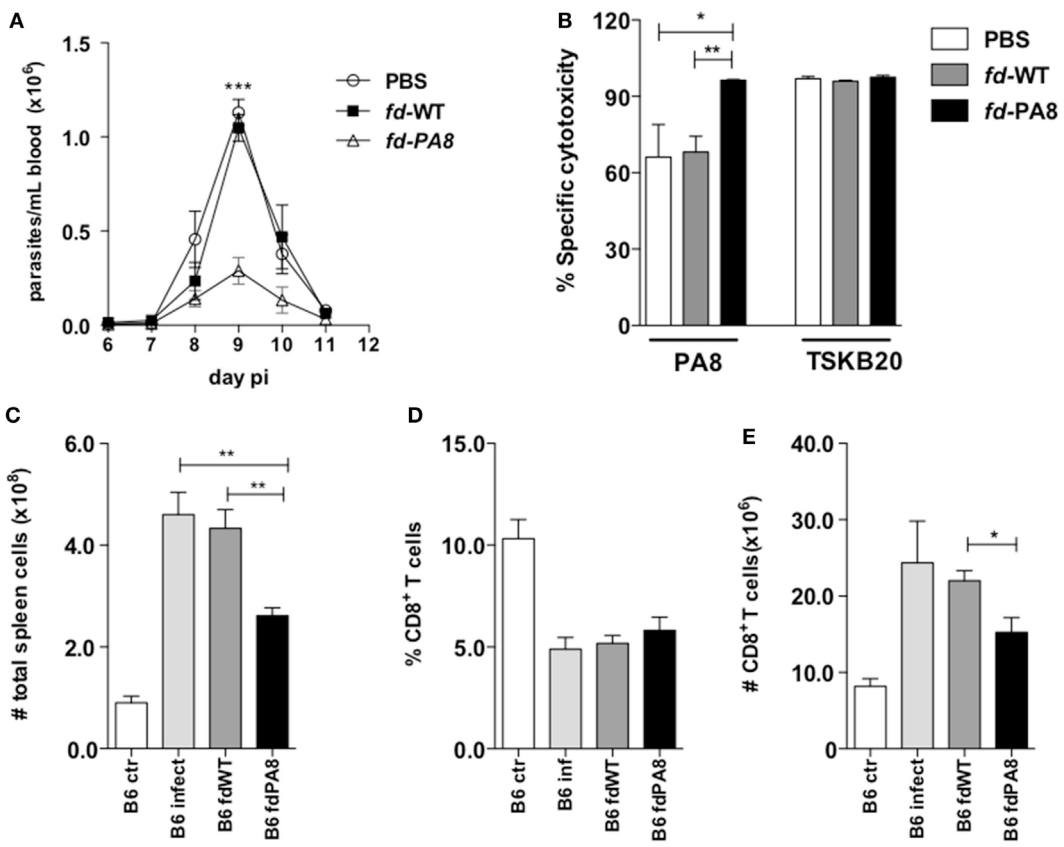

FIGURE 2 | Immunization with *fd*-PA8 phages protects B6 mice against infection with *T. cruzi* Y-strain and increments Ag-specific cytotoxicity. Male mice were immunized (as in Figure S1 in Supplementary Material) and infected on day 0 with 2×10^3 blood trypomastigotes of Y-strain. Parasitemia curves **(A)** and specific cytotoxicity against target cells loaded with PA8 or TSKB20 peptides (representative contour plots are shown in Figure S2 in Supplementary Material) **(B)**, of B6 mice immunized with *fd*-PA8 (triangles and black bars), or *fd*-WT phages (black squares and gray bars), or only infected [phosphate buffered saline (PBS)] (empty circles and white bars) as controls. Total spleen cell numbers **(C)**, $CD8^+$ T cell percentages **(D)**, and absolute $CD8^+$ T cell numbers **(E)**. Data represent mean values of individually analyzed mice ($n = 5$ per group) from control naïve (white bars), infected-only mice (light gray bars), *fd*-WT (dark gray bars) or *fd*-PA8 immunized mice (black bars). Error bars = SEM, $^*p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$ (two-tailed Student's *t*-test).

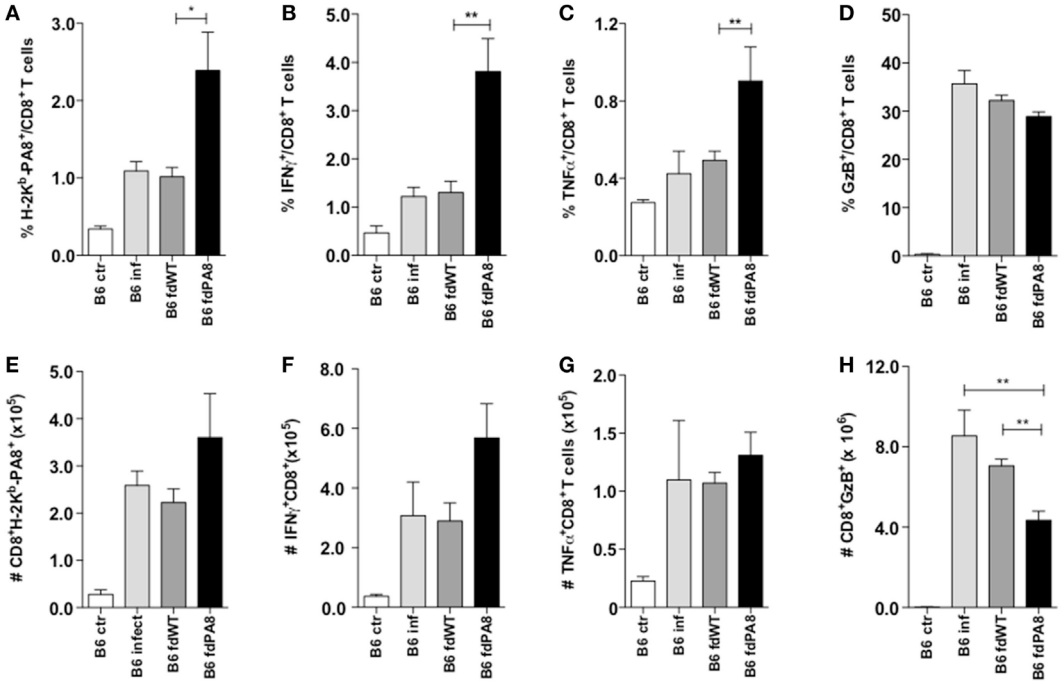

FIGURE 3 | Immunization with *fd*-PA8 phages increases the levels of Ag-specific and cytokine-secreting CD8$^+$ T cells in the spleen of B6 mice. Male mice were immunized (as in Figure S1 in Supplementary Material) and infected on day 0 with 2 × 10^3 blood trypomastigotes of the Y-strain. On Day 13 pi, splenocytes from control naïve (white bars), infected-only (light gray bars), *fd*-WT (dark gray bars), or *fd*-PA8 immunized mice (black bars) were stained following a 10-h *in vitro* incubation with PA8 peptide, as described in Section Materials and Methods. Mean percentages **(A–D)** and absolute numbers **(E–H)** of H-2K^b-PA8$^+$ **(A,E)**, IFN-γ$^+$ **(B,F)** TNF$^+$ **(C,G)**, and GzB$^+$ **(D,H)** CD8$^+$ T cells of individually analyzed mice (n = 5) are shown. Error bars = SEM, *$p \leq 0.05$; **$p \leq 0.01$ (two-tailed Student's t-test).

simultaneously TNF and IFN-γ, or TNF and GzB, or GzB and IFN-γ in the four experimental groups (naïve controls, infected-only, *fd*-WT + infection, and *fd*-PA8 + infection). As shown in **Figure 4**, all these subsets of double-positive CD8$^+$ T cells are increased in the spleens of infected mice previously immunized with *fd*-PA8 phages. Moreover, the frequency of triple positive (IFN-γ$^+$TNF$^+$GzB$^+$) CD8$^+$ T cells is also increased in the *fd*-PA8-vaccinated group, as shown in Figures S4A–C in Supplementary Material. Therefore, our results suggest that, contrary to what happens in non-vaccinated infected mice, vaccination with r*fd* phages causes the expansion of polyfunctional CD8$^+$ T cells, which correlates to protection against infection with *T. cruzi*.

Previous Immunization With *fd*-PA8 Induces Higher Levels of Peptide-Specific IgG Antibodies in Infected Mice

Since vaccination using *fd* phages as delivery system can also induce the production of Ag-specific antibodies (2, 19), we also quantified here the PA8-specific IgG in the sera of vaccinated and *T. cruzi*-challenged B6 mice. For this, sera of naïve controls, *fd*-WT + infection-, and *fd*-PA8 + infection-groups were collected at day 13 pi and first absorbed against immobilized *fd*-WT phages, in order to (at least partially) deplete anti-phage Igs. Then, each *fd*-absorbed serum was further tested in an ELISA assay against either immobilized *fd*-PA8 or *fd*-WT particles (**Figure 5A**). The difference in the obtained optical densities testing absorbed *fd*-PA8 sera against *fd*-PA8- and *fd*-WT-coated phages represents the titer of specific anti-PA8 IgG. We found that PA8-specific IgG Abs are also present in mice immunized with *fd*-WT and challenged with infection, although in these mice PA8-specific IgG Abs are at significantly lower levels to the ones found in mice vaccinated with *fd*-PA8 and infected, as quantified by ELISA assays (**Figures 5A,B**). In order to confirm that PA8 peptide-specific IgG antibodies induced by vaccination with *fd*-PA8 phages are capable of recognizing ASP-2 protein on the amastigote forms, sera from *fd*-PA8αDEC immunized-only (non-infected) mice were also employed in the immunofluorescence assays against the *in vitro* infected LLC-MK2 cell line (**Figure 5C**). These data indicate that the immune sera from vaccinated mice contain peptide-specific IgG Abs able to recognize the amastigote antigen. Since the ASP-2 molecule is a member of the *T. cruzi trans*-sialidase (Ts) superfamily, we hypothesized that other proteins of this family could also contain the same peptide sequence. We then searched the non-redundant NCBI Protein Reference Sequence Database using BLAST. The obtained result is displayed in Table S1 in Supplementary Material: the PA8 sequence (VNHRFTLV) was found at 100% coverage and 100% identity in six different putative Ts protein sequences (20). Search of the UniProtKnowledgebase using Peptide Search,[3] gave the same result (not shown). Hence, this epitope is also present in other

[3]http://www.uniprot.org/peptidesearch/ (Accessed: January 10, 2018).

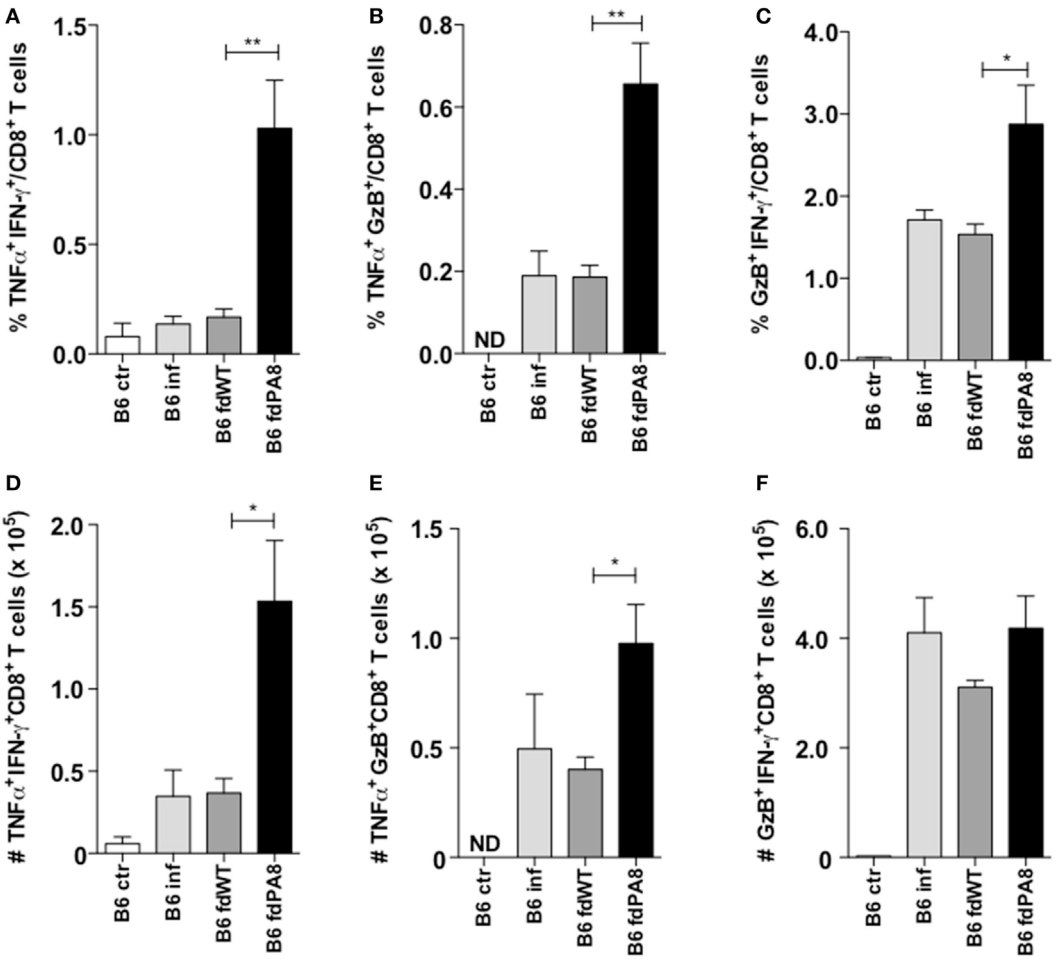

FIGURE 4 | Immunization with *fd*-PA8 phages increases the levels of polyfunctional CD8+ T cells in the spleen of B6 mice. Male mice were immunized (as in Figure S1 in Supplementary Material) and infected on day 0 with 2×10^3 blood trypomastigotes of the Y-strain. On Day 13 pi, splenocytes from control naïve (white bars), infected-only (light gray bars), *fd*-WT (dark gray bars), or *fd*-PA8 immunized mice (black bars) were stained following a 10-h *in vitro* incubation with PA8 peptide, as described in the Section Materials and Methods. Mean percentages **(A–C)** and absolute numbers **(D–F)** of TNF+IFN-γ+ **(A,D)**, TNF+GzB+ **(B,E)**, and IFN-γ+GzB+ **(C,F)** CD8+ T cells of individually analyzed mice ($n = 5$) are shown. Error bars = SEM, $^*p \leq 0.05$; $^{**}p \leq 0.01$ (two-tailed Student's *t*-test).

proteins potentially expressed at the surface of trypomastigotes (the parasite infecting forms) and, as such, might be recognized by neutralizing and/or opsonizing antibodies (21, 22).

Vaccination With r*fd* Phages Displaying the TSKB20 Peptide Reduces Mortality and Increases Specific Cytotoxicity Long Term After Infection

We next constructed r*fd* phages displaying the previously described K^b-restricted immunodominant TSKB20 peptide (ANYKFTLV) derived from the Ts of *T. cruzi* (12). In order to follow mortality, a higher inoculum of parasite (2×10^5 blood trypomastigotes) was employed in the experiment illustrated in **Figure 6**. Apart from that, mice were immunized twice with *fd*-TSKB20 following the same vaccination/infection scheme showed in Figure S1 in Supplementary Material. As shown in **Figure 6A**, vaccination with *fd*-TSKB20 significantly decreased parasitemia levels in B6 mice. Moreover, while only 10% of B6 mice immunized with *fd*-WT survived infection, around 50% of mice vaccinated with *fd*-TSKB20 were protected (**Figure 6B**). Then, we tested the *in vivo* cytotoxicity against TSKB20-loaded target cells both in B6 and *Tlr9*$^{-/-}$ vaccinated mice at an early time point (day 8 pi) after the challenging infection. As shown in **Figure 6C**, vaccination increased the specific cytotoxic response in WT, but not in *Tlr9*$^{-/-}$-vaccinated mice. It is known that the CTL response against certain immunodominant peptides last for hundred of days in B6 mice infected with the *T. cruzi* Y-strain (23). Accordingly to this, we could detect *in vivo* Ag-specific cytotoxicity against both PA8 and TSKB20 peptides at day 106 pi (**Figure 6D**). However, only the CTL response against TSKB20-pulsed target cells was significantly increased in mice vaccinated with *fd*-TSKB20 (**Figure 6D**). Finally, we have also investigated the early production of anti-TSKB20 IgG in vaccinated mice, at day 8 pi. As shown

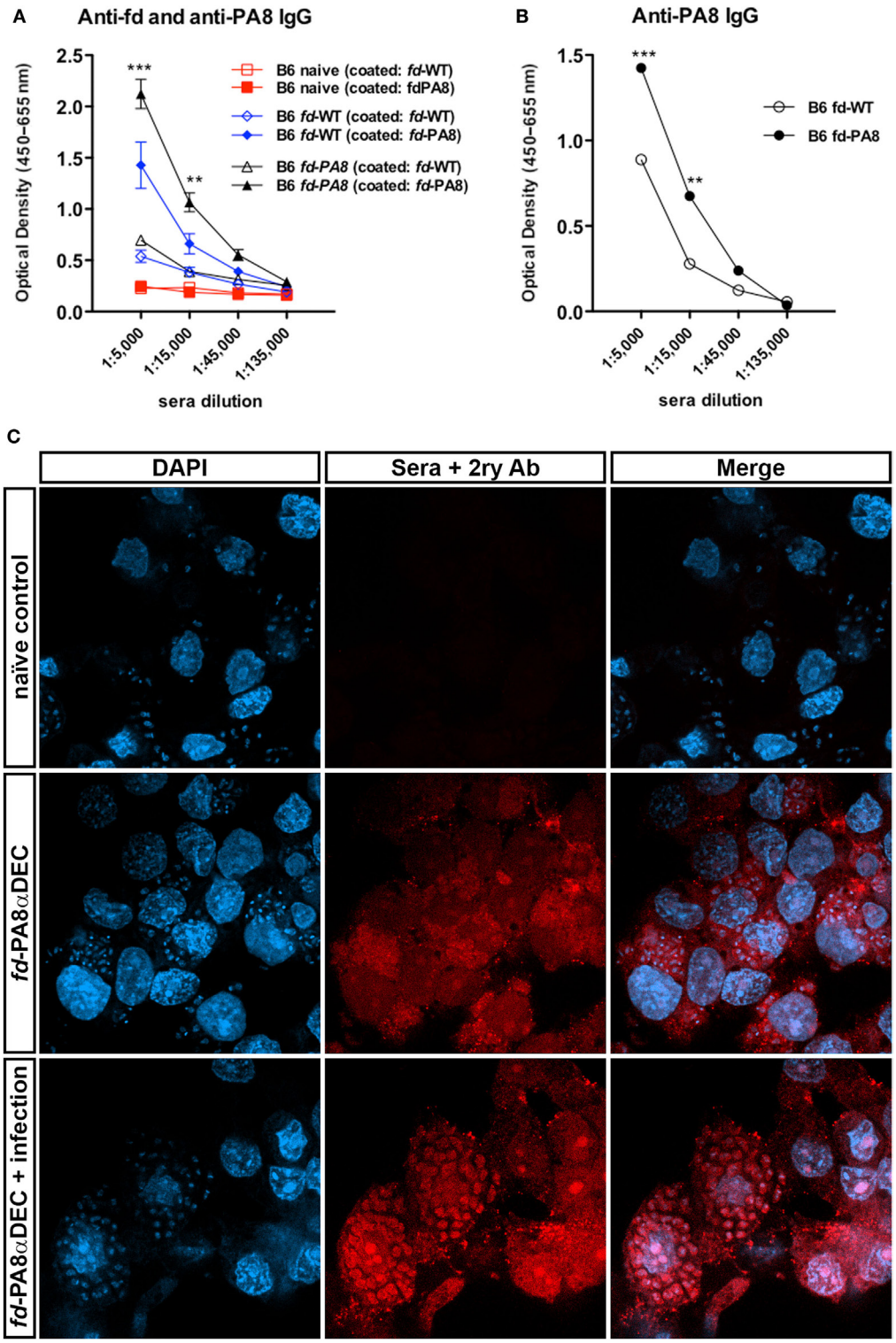

FIGURE 5 | Immunization with *fd*-PA8 phages increases the levels of PA8-specific IgG in the serum B6 mice**.** Male mice were immunized (as in Figure S1 in Supplementary Material) and infected on day 0 with 2×10^3 blood trypomastigotes of the Y-strain. On Day 13 pi, sera were collected from control naïve (red symbols and lines), *fd*-WT (blue symbols and lines), and *fd*-PA8-immunized mice (black symbols and lines), adsorbed against immobilized *fd*-WT phages and then tested on an ELISA assay against *fd*-WT and *fd*-PA8 phages, as described in the Section "Material and Methods." Mean values ($n = 3$), error bars = SEM **(A)**. OD curves obtained against *fd*-WT phage was subtracted from OD curves obtained against *fd*-PA8 phages; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$ (two-tailed Student's *t*-test) **(B)**. Confocal microscopy of LLC-MK2 cells infected with Y-strain at day 2 pi **(C)**. Cells were stained with serum from control naïve or *fd*-PA8αDEC-immunized-only or *fd*-PA8αDEC-immunized and infected mice, followed by secondary anti-mouse IgG Cy3-labeled Ab and DAPI, as detailed in the Section "Materials and Methods."

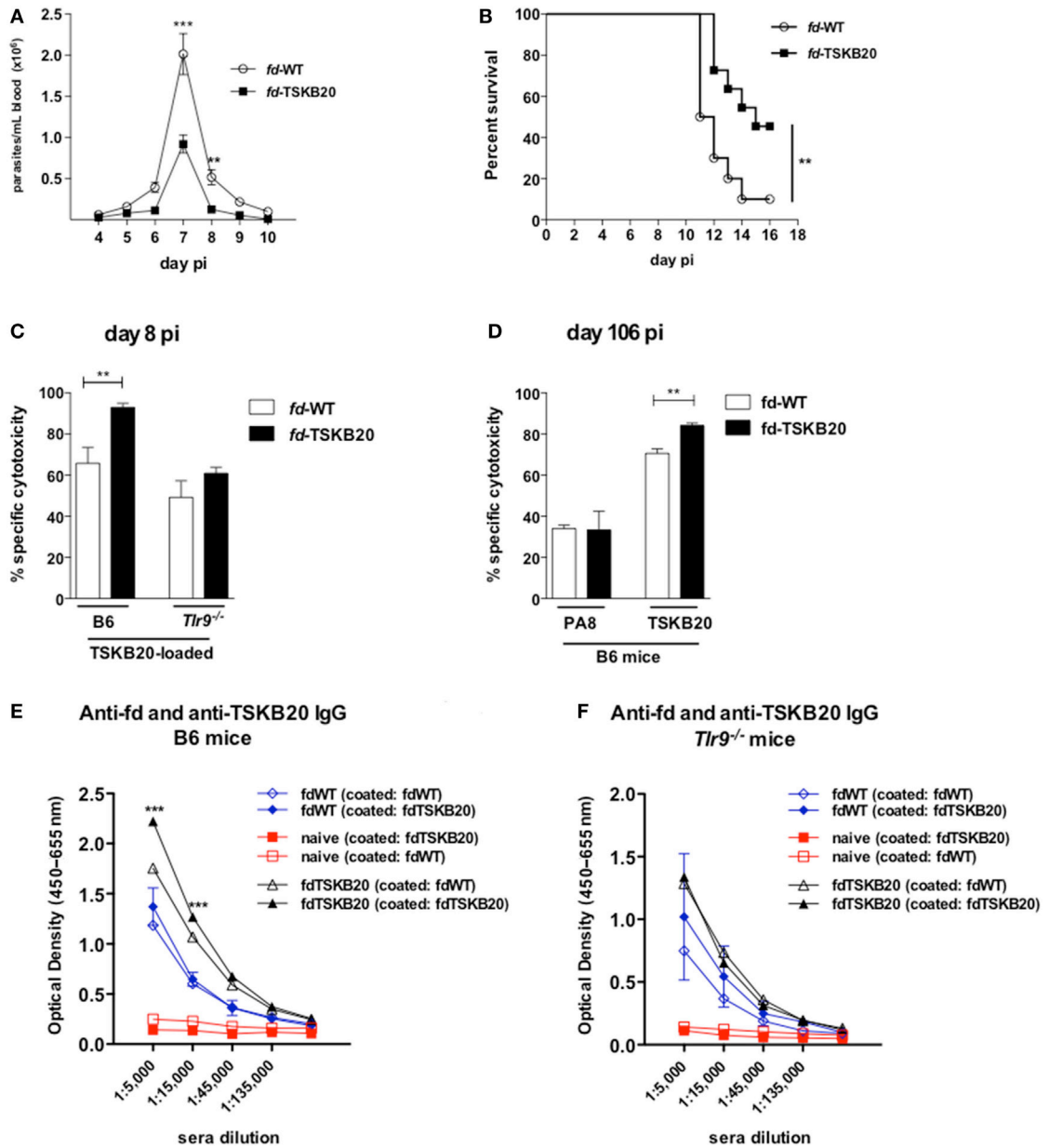

FIGURE 6 | Immunization with *fd*-TSKB20 phages protects against infection with *T. cruzi* Y-strain and increases IgG and Ag-specific cytotoxicity in TLR9-dependent manner. Male mice were immunized (as in Figure S1 in Supplementary Material) and infected on day 0 with 2×10^5 blood trypomastigotes of Y-strain. Parasitemia curves ($n = 6$ in each group) **(A)** and survival curves **(B)** of B6 mice immunized with *fd*-TSKB20 (black squares) or *fd*-WT phages (empty circles), as controls ($n = 10$ in each group) $^{**}p \leq 0.01$ (Gehan–Breslow–Wilcoxon Test). Specific cytotoxicity in B6 and *Tlr9*$^{-/-}$ mice at day 8 pi, against target cells loaded with TSKB20 peptide ($n = 4$) **(C)**. Specific cytotoxicity in B6 mice at day 106 pi against target cells loaded with PA8 or TSKB20 peptides. Bars represent mean values ($n = 3$) **(D)**. **(A,C,D)** Error bars = SEM; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$ (two-tailed Student's *t*-test). On Day 8 pi, sera were collected from control naïve (red symbols and lines), *fd*-WT (blue symbols and lines), and *fd*-TSKB20 immunized (black symbols and lines) B6 mice **(E)** or *Tlr9*$^{-/-}$ mice **(F)**, adsorbed against immobilized *fd*-WT phages and then tested on an ELISA assay against *fd*-WT and *fd*-PA8 phages, as described in the Section "Materials and Methods." Symbols represent mean values ($n = 3$), error bars = SEM; $^{***}p \leq 0.001$ (two-tailed Student's *t*-test).

in **Figures 6E,F**, TSKB20-specific IgG could be detected in the sera of vaccinated B6 but not *Tlr9*$^{-/-}$ mice. Therefore, together these results demonstrate that vaccination with *fd*-TSKB20, as well as with *fd*-PA8, confers protection against infection with the *T. cruzi*, as it diminishes parasitemia and increases survival, being able to induce a more robust long-lasting CTL response, as well as early IgG production, against specific parasite epitopes. Moreover, both effector anti-parasite mechanisms, CTL and IgG responses, induced by r*fd* phages, were shown to depend on the activation of the *Tlr9*-mediated innate pathway.

DISCUSSION

Filamentous *fd* phages are viruses which structure is formed by a cylindrical flexible protein scaffold, approximately 7-nm wide and 890-nm long, containing a single-strand DNA genome rich in CpG motifs (24). It has been previously shown that recombinant *fd* phages, expressing a CD8 epitope and directed to CD205$^+$ DCs (*fd*sc-αDEC), are potent inducers of Ag-specific CTL responses and are more effective than other immunization strategies for inhibiting the growth of the B16 tumor *in vivo* (1), reviewed in Ref. (24). In fact, DCs show an enhanced receptor-mediated binding and internalization of phage particles expressing the anti-DEC-205 scFv, when compared to *fd*-WT virions, using both *in vitro* and in *in vivo* assays (1). Moreover, it has been recently demonstrated that *fd*sc-αDEC virions are delivered to the late endosome/lysosome compartment and induce the activation of the TLR9 pathway in DCs, which, as a consequence, secrete different pro-inflammatory cytokines, including type I IFN, leading to DCs maturation (4). Although *fd* phages devoid of sc-αDEC are not as efficient as *fd*sc-αDEC in inducing the maturation of DCs, we have shown here, by employing phage doses that are 2- to 2.5-fold higher than the previously used (1, 4), that r*fd* phages devoid of sc-αDEC nevertheless induce the expression of the costimulatory molecule CD86 in WT BMDCs, but not in TLR9-deficient cells. Importantly, we have also shown here that vaccination with these r*fd* particles is capable to induce the protection of B6 mice against infection with the intracellular *T. cruzi* parasite, as it reduces parasitemia, parasite load in the myocardium and mortality in WT-infected mice. However, none of these infection parameters was reduced in vaccinated *Tlr9*$^{-/-}$ mice. This was shown using either *fd*-OVA followed by infection with the transfected Y-OVA strain of *T. cruzi*, as a proof of concept, or *fd* phages expressing the *T. cruzi trans*-sialidase-derived immunodominant epitope TSKB20 followed by infection with the *T. cruzi* Y-strain. It is not clear, at the moment, if TLR9-signaling is required only for the induction of type I IFN and costimulatory molecules in DCs or if it also increases the efficiency of the parasite-antigen cross-presentation. This is an interesting point and deserves further work in order to be elucidated.

Regarding the effector immune mechanisms induced by vaccination with r*fd* phages, which would be responsible for the protection against infection with *T. cruzi*, we have shown here that vaccination induces higher levels of Ag-specific and cytokine-secreting CD8$^+$ T cells, as well as higher levels of Ag-specific cytotoxicity *in vivo*. The CTL response, as well as the secretion of IFN-γ by CD8$^+$ T cells, has been known to play a fundamental role on the protection against infection with *T. cruzi* and with other intracellular pathogens; reviewed in Ref. (10). Of note, we have found that immunization with r*fd* phages increases the percentages and absolute numbers of polyfunctional CD8$^+$ T cells in the spleen of vaccinated mice. This phenomenon was previously observed in other models of experimental vaccination, such as against *T. cruzi,* employing adenovirus as a vaccine vector (18) and against malaria, using prime-boost immunization with modified vaccinia virus Ankara and adenoviral vectors (25). Moreover, polyfunctional human CD8$^+$ T cells are found at higher levels in HIV nonprogressors than in progressors and the presence of these multifunctional T cells negatively correlates with viral load in the latter group (26, 27). Nevertheless, until the present date, it has not been possible to clearly attribute a role to polyfunctional T cells as markers of protective immunity in other infections (28), and therefore, more studies are necessary to clarify this point. It is known that the parasite displays escape mechanisms that retard and somehow compromises the immune response toward it, which leads to the chronification of the infection. Thus, it is possible that vaccination is beneficial not only by inducing an immune response that precedes infection but also because it allows the establishment of a robust and qualitatively superior multifunctional CD8$^+$ T cell-mediated response, which correlates to protection in some infection models.

On the other hand, we have also found here that immunization with *fd*-PA8 and *fd*-TSKB20 phages induces increased levels of serum IgG Abs directed against these ASP-2- and *trans*-sialidase (Ts)-derived parasite epitopes. The capacity of r*fd* phages in inducing a humoral response to vaccine Ags has been previously described (2, 19). Of note, both the ASP-2 and Ts proteins belong to an extended multigene family, which members are expressed at the cell surface at different stages of the protozoan life cycle and against which Abs might cross-react. More than 1,400 copies of the Ts gene were found in the *T. cruzi* genome (of which almost the half are pseudogenes), encoding full length and partial non-enzymatically active Ts molecules, the exact function of which is still not clear (20). In the absence of active Ts enzymatic function, trypomastigotes invade host cells poorly and are highly sensitive to host complement-mediated lysis (29). Moreover, several studies have suggested an immunomodulatory role for proteins of the Ts family (30). Hence, it is possible that anti-PA8 IgG would also bind to other Ts protein family members expressed on the surface of the infective trypomastigote stage (as indicated by the search with PA8 peptide query against the NCBI database), and consequently, would lead to parasite opsonization and neutralization. In fact, IgG2a lytic and opsonizing Abs have been described as important effector mechanisms against infection with *T. cruzi* (21, 22). Most probably, both humoral and cell-mediated responses contribute to the effective protection induced by r*fd* vaccination. This issue is currently under investigation by our group.

Finally, it is interesting to note that the induction of not only the CD8$^+$ T cell-mediated cytotoxicity, but also the anti-TSKB20 IgG response induced by previous vaccination with *fd*-TSKB20 was defective in *Tlr9*$^{-/-}$ mice. This is probably the consequence of a poor innate activation of DCs in *Tlr9*$^{-/-}$ mice, leading to a defective activation of T-follicular helper cells, which in turn are required for helping the B cell response. It is tempting to speculate that *fd* phages might also directly interfere with the B-cell response through the B-cell intrinsic activation of the TLR9 pathway.

In summary, the results obtained in the present work further extend previous studies, demonstrating that r*fd* phages can be used as potent and innovative delivery systems, conferring both humoral and cellular-mediated protection against an important human parasite infection, through a TLR9-dependent mechanism.

ETHICS STATEMENT

All animal experiments were conducted in accordance with guidelines of the Animal Care and Use Committee of the Federal University of Rio de Janeiro (Comitê de Ética do Centro de Ciências da Saúde CEUA-CCS/UFRJ). Procedures and animal protocols were approved by CEUA-CCS/UFRJ license no.: IMPPG022.

AUTHOR CONTRIBUTIONS

JG-N, AN, PB, and MB conceived and designed the experiments. JG-N, RS, FC, C-HB, TA, AD, PA, GM, and MS performed the experiments. JG-N, FC, MS, AN, and MB analyzed the data. AO, CM, HG, MS, AN, PB, and MB contributed parasites/reagents/mice/materials/analysis tools. MB wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01173/full#supplementary-material.

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Vectorized Delivery of Alpha-GalactosylCeramide and Tumor Antigen on Filamentous Bacteriophage fd Induces Protective Immunity by Enhancing Tumor-Specific T Cell Response

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We have exploited the properties of filamentous bacteriophage *fd* to deliver immunologically active lipids together with antigenic peptides. Filamentous bacteriophages resemble for size, capability to be permeable to blood vessels, and high density antigen expression, a nature-made nanoparticle. In addition, their major coat protein pVIII, which is arranged to form a tubular shield surrounding the phage genome, has a high content of hydrophobic residues promoting lipid association. We conjugated bacteriophages to alpha-GalactosylCeramide (α-GalCer), a lipid antigen-stimulating invariant natural killer T (iNKT) cells and capable of inducing their anti-tumoral activities. We found that bacteriophage fd/α-GalCer conjugates could repeatedly stimulate iNKT cells *in vitro* and *in vivo*, without inducing iNKT anergy. Moreover, co-delivery of α-GalCer and a MHC class I restricted tumor-associated antigenic determinant to antigen-presenting cells *via* bacteriophages strongly boosted adaptive $CD8^+$ T cell response and efficiently delayed tumor progression. Co-delivery of a tumor antigen and iNKT-stimulatory lipid on the surface of filamentous bacteriophages is a novel approach to potentiate adaptive anti-cancer immune responses, overcoming the current limitations in the use of free α-GalCer and may represent an attractive alternative to existing delivery methods, opening the path to a potential translational usage of this safe, inexpensive, and versatile tool.

Keywords: vectorized alpha-GalactosylCeramide, filamentous bacteriophage, invariant Natural Killer T cells, anti-tumor immunity, $CD8^+$ T Cells

INTRODUCTION

Invariant natural killer T (iNKT) cells represent a unique subpopulation of T lymphocytes with both innate-like and adaptive functions mainly found in spleen, liver, and bone marrow. iNKT cells express NK lineage receptors and a semi-invariant T cell receptor (TCR) composed of Vα14–Jα18 chain in mice and Vα24–Jα18 chain in humans, paired with β chains encoded by a limited number of Vβ genes (1).

Invariant natural killer T cells recognizes (glyco)lipid antigens (Ag) presented by the CD1d molecule expressed by several types of antigen-presenting cells (APCs), including dendritic cells (DCs) (2).

The potential anti-tumoral function of iNKT cells was first discovered with the identification of alpha-GalactosylCeramide (α-GalCer), a synthetic derivative of agelasphin, a glycolipid originally isolated from the marine sponge *Agelas mauritianus*, as a strong stimulatory ligand during screening of anti-tumor compounds from natural sources (3–5).

In response to α-GalCer, iNKT cells rapidly secrete large quantities of cytokines, including IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IL-17, IL-21, IL-22, TGF-β, and TNF-α, that in turn activate a variety of other cell types, including NK cells, DCs, B, and T cells (6–9). Through this activation cascade, α-GalCer showed to exert potent anti-tumor and adjuvant activities *in vivo* in mouse models (5, 10, 11), rendering it a powerful candidate for adjuvant therapy in cancer.

Based on these pioneering reports, attempts have been made to exploit the anti-tumor property of α-GalCer. Although the drug was well tolerated, no or moderate clinical responses were observed among the patients repeatedly injected intravenously with α-GalCer (12). Studies performed in a mouse model demonstrated that α-GalCer induced a long-term anergy of iNKT cells, thus preventing proliferation and cytokine release upon a recall stimulation (13).

Even though the mechanism underlying iNKT cell anergy mediated by α-GalCer is still unknown, it is common knowledge that co-stimulatory signals and cytokines provided by antigen-presenting DCs are considered crucial for avoiding anergy (14).

Indeed, mouse studies demonstrated that the injection of α-GalCer-pulsed DCs induced a sustained cytokines production when compared with administration of free α-GalCer, suggesting that the type of α-GalCer administration is critical for iNKT cell stimulation (15).

A way to optimize iNKT cell responses may lie in actively directing α-GalCer to the appropriate APC using suitable delivery systems (16).

In previous studies, we found that the filamentous bacteriophage is an efficient antigen-delivery system because it is internalized by DCs and activates innate and adaptive immune responses in the absence of classical adjuvants (17–19).

The filamentous bacteriophage can be considered as a nature-made nanocarrier according to its nano-dimensions (5 nm in diameter and 1,000 nm in length), its capability to cross blood vessels and for the capacity of expressing very large amounts of recombinant protein antigen. Its major coat protein pVIII is present in 2,700 copies on the phage coat and is arranged to form a tubular shield surrounding the phage genome. The pVIII protein is composed of three specific domains: a hydrophobic core, an acidic N-terminal domain, and a basic C-terminal domain (20). Due to the high content of hydrophobic residues, the pVIII shows strong binding of lipids, a feature that we exploited by promoting association of the bacteriophage with α-GalCer. Here, we show that α-GalCer conjugated with bacteriophages could repeatedly stimulate iNKT cells *in vitro* and *in vivo*, without inducing iNKT cell anergy. Moreover, therapeutic vaccination with phages co-displaying α-GalCer and a tumor antigen delayed tumor progression in mice, showing improved adjuvanticity of the phage particles and leading to enhanced vaccine efficacy. These results suggest a novel approach to potentiate the efficacy of the bacteriophage delivery system as anti-tumor vaccine.

MATERIALS AND METHODS

Cells and Reagents

CD1d-restricted Vα14i NKT hybridoma FF13 (21) cells were a kind gift of Dr. De Libero (Department of Biomedicine, University of Basel and University Hospital Basel, Basel, Switzerland) and were cultured in RPMI 1640 (Lonza) medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum (FCS) (all from GIBCO, Milan, Italy).

OTI hybridoma cell line, recognizing the $OVA_{257–264}$ SIINFEKL determinant, was produced by infection of 54ζ17 hybridoma T cells (22) with a retrovirus encoding the OTI TCR V alpha 2 and V beta 5 chains. Recombinant retroviral particles were produced in HEK293T cells (ATCC CRL 1573) by transfection with pMXOTI, pEcotropic, and pVSVg plasmids. The virus-containing supernatant was collected 48 h after transfection. 5×10^5 54ζ17 cells were infected with 10 ml of viral supernatant and TCR expression was verified after 5 days by APC-anti-CD3ε (145-2C11, Biolegend) staining and FACS analysis. Positive cells were then sorted using a FACS ARIA (Becton Dickinson, Fullerton, CA, USA) and amplified in DMEM supplemented with 10% FCS, 25 U/ml penicillin G, 25 µg/ml streptomycin, and 0.05 µM β-mercaptoethanol.

B16-OVA melanoma cells (H2Kb), stably expressing chicken ovalbumin, were a kind gift of Dr. Dellabona (San Raffaele Scientific Institute, Milan, Italy). Cells were cultured in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 10% FCS, and 100 µg/ml Hygromicin (Sigma-Aldrich).

The synthetic peptide $OVA_{257–264}$ (SIINFEKL) was purchased from Primm (Naples, Italy).

Synthetic α-GalCer (KRN7000) (*2S,3S,4R*)-1-*O*-(α-D-galactosyl)-*N*-hexacosanoyl-2-amino-1,3,4-octadecanetriol (BML-SL232-1000) was purchased from Vinci Biochem.

Internal standard (IS) for mass spectrometric analysis D-Galactosyl-β-1,1'-*N*-Palmitoyl-D-erythro sphingosine was purchased from Avanti Polar Lipids.

MeOH and water for liquid chromatography–mass spectrometric analysis were purchased from Merck and were LC–MS grade.

Mice

Six- to eight-week-old female C57BL/6 were purchased from Charles River (Lecco, Italy) and housed in IGB "A. Buzzati-Traverso" Animal House Facility under standard pathogen-free conditions abiding institutional guidelines.

Purification of Bacteriophages Particles and Conjugation to α-GalCer

Wild type (fdWT) and hybrid fdOVA (expressing the recombinant $OVA_{257–264}$-pVIII proteins) filamentous bacteriophages were purified from the supernatant of transformed *Escherichia coli* TG1recO cells. Bacteria were grown in TY2X medium for 16 h and the bacteriophage virions were harvested from *E. coli* supernatant, Poly(ethylene glycol) 6000 (Sigma-Aldrich) precipitated, purified by ultracentrifugation (24,500 *g*) on cesium chloride (Sigma-Aldrich) gradient, and dialyzed against phosphate buffered saline (PBS) 1×. Elimination of lipopolysaccharides (LPS) from phage particles was performed using Triton X-114 (Sigma-Aldrich). Briefly, Triton X-114 was mixed to the phage preparations to a final concentration of 1% by vigorous vortexing. The mixture was incubated at 4°C for 5 min, then incubated for 5 min at 50°C, and centrifuged (20,000 *g*, 10 min) at 25°C. The upper aqueous phase containing the virions was carefully removed and subjected to Triton X-114 phase separation for more cycles. The resulting aqueous phase containing virions was subjected to cesium chloride gradient centrifugation, dialyzed against PBS 1×, and assayed for LPS contamination using the Limulus Amebocyte Lysate assay (Limulus Amebocyte Lysate QCL-1000 chromogenic modification, Lonza), according to the manufacturer's instructions.

The expression of the recombinant $OVA_{257–264}$-pVIII proteins was induced adding 0.1 mM isopropyl-beta-D-thiogalactopyranoside (Sigma-Aldrich) to the bacteria growing in TY2X medium.

The number of copies of pVIII displaying the $OVA_{257–264}$ peptide was estimated by N-terminal sequence analysis of the purified virions and resulted in 15–20% for each phage preparation.

Bacteriophages in PBS 1× pH 8 and KRN7000 in dimethyl sulfoxide (Sigma-Aldrich) were combined at a 10:1 ratio (μg phages: μg α-GalCer) and stirred at 4°C overnight. Virions were subjected to cesium chloride gradient ultracentrifugation (24,500 *g*), dialyzed against PBS 1× and the concentration of bacteriophages was determined using spectrophotometer. The presence of α-GalCer in the phage preparations was determined by the *in vitro* biological assay and its conjugated amount determined by mass spectrometric analysis, as described below.

Release of α-GalCer From the Conjugated Bacteriophage by Solvent Extraction

A small aliquot (50 μl) of a PBS solution containing the bacteriophage conjugated to α-GalCer at a concentration of 1.5 mg/ml was diluted 1:10 to a final volume of 500 μl with ultrapure water; 200 ng of IS in 20 μl of methanol (MeOH) were added and the suspension was extracted with 2 ml of MeOH/$CHCl_3$ (1:1) by sonication. After centrifugation, the organic phase was removed and the aqueous layer was re-extracted with MeOH/$CHCl_3$. The combined organic phases were dried under nitrogen, reconstituted in 1 ml of MeOH, and subjected to LC–MS analysis.

Quantitative LC–MS/MS Analysis of α-GalCer

A quantitative method was developed on a UPLC system (Acquity, Waters) coupled to a triple quadrupole mass spectrometer (API 3200, SCIEX). The chromatographic analysis was performed on an Acquity BEH Phenyl column (Waters, 100 × 2.1 mm, 1.7 μm), eluted with a short gradient program from 95:5 MeOH/H_2O to 100% MeOH in 1 min followed by an isocratic elution at 100% MeOH for 4 min. Flow rate was set at 0.4 ml/min and column temperature at 40°C. α-GalCer eluted at a Rt of 1.59 min, IS at 1.1 min.

A calibration curve was prepared by using five calibration points of α-GalCer standard (STD) (62.5, 125, 250, 500, and 1,000 ng/ml) spiked with 200 ng/ml IS and plotted as area ratio of STD/IS response vs concentration. Two MRM transitions were monitored for both STD and IS for quantitative purposes and to confirm analytical identification. The most intense transitions for each compound (i.e., m/z 856.7 > 178.9 for STD and m/z 698.5 > 89.2 for IS) were used as analytical responses.

In Vitro α-GalCer Presentation on CD1d and Stimulation of iNKT and OTI Hybridoma Cells

Mouse bone marrow-derived dendritic cells (BMDCs) were generated from C57BL/6 mice according to Ref. (23). At 7 day of culture, BMDCs were incubated in RPMI medium supplemented with 10% FCS, 5 μM 2-ME, 1 mM glutamine, and 1 mM sodium pyruvate for 2 h with different concentrations of free α-GalCer, fdWT bacteriophages, or fd/α-GalCer bacteriophages. The experiment OTI hybridoma cell experiment was performed by incubating BMDCs with different concentration of fdOVA, fdOVA/α-GalCer, or $OVA_{257–264}$ synthetic peptide. After the incubation, cells were washed and stained with PE-conjugated anti mouse α-GalCer:CD1d complex (L363, Biolegend) or co-cultured (50,000/well) with the mouse Vα14 iNKT hybridoma FF13 or OTI hybridoma (50,000/well) for 40 h.

PE-conjugated anti-mouse α-GalCer:CD1d complex (L363, Biolegend) antibody was used to stain DCs and fluorescence of stained cells was analyzed by FACSCanto II flow-cytometer and DIVA (Data-Interpolating Variational Analysis) software (Becton Dickinson). The IL-2 released into cell co-culture supernatants was measured by ELISA. Supernatants (0.1 ml/well) were assayed in duplicate using mouse IL-2 ELISA MAX™ Standard (Biolegend), according to the manufacturer's instructions.

Measurement of *In Vivo* and *In Vitro* Recall Response to α-GalCer

Mice were injected intravenously (i.v.) or intratumorally (i.t.) with 100 μl of PBS containing 5 μg α-GalCer, 50 μg of fd/α-GalCer bacteriophage conjugate, or with vehicle alone. Where indicated, 200 or 130 ng of α-GalCer was also used. After 24 h mice were sacrificed, spleens were harvested, and single-cell suspensions were prepared. Spleen cell suspensions were plated in U-bottomed 96-well plate at 2×10^5 cells per well in RPMI medium supplemented with 10% FCS, 5 μM 2-ME, 1 mM glutamine, and 1 mM sodium pyruvate in the presence of indicated doses of free α-GalCer or with medium alone. For proliferation assays, 1 μCi of [3H] thymidine (PerkinElmer Life Sciences) was added to the wells after 60 h of culture, and cells were cultured for an additional 12 h. Cells were then harvested using a semi-automatic cell harvester

FilterMate (PerkinElmer, CA, USA) and uptake of radioactivity was measured using the Top Count NTX microplate scintillation counter (PerkinElmer). Cell proliferation was expressed as a cpm fold increase vs unstimulated cells. IL-2 secretion was evaluated by ELISA using Mouse IL-2 ELISA MAX™ Standard, collecting cell culture supernatants (0.1 ml/well) after 60 h of culture.

Analysis of iNKT and NK *In Vivo* Activation

Mice ($n = 3$/group) were injected i.v. with 100 µl of PBS containing 5 µg free or 50 µg of *fd* and fd/α-GalCer. Mice were sacrificed 3 h post-treatment. Splenic iNKT cells were analyzed for intracellular IFN-γ secretion by culturing 7.5×10^6 spleen cells overnight in the presence of 10 µg/ml of the secretion inhibitor Brefeldin-A (BFA, Sigma-Aldrich). Cells were then harvested and IFN-γ production was evaluated by intracellular staining on gated $CD3^+NK1.1^+$ cells or $CD3^-NK1.1^+$ cells using APC-conjugated anti mouse CD3 monoclonal antibody (mAb) (17A2, Biolegend), PE-conjugated anti-mouse NK1.1 mAb (PK136, Biolegend), FITC-conjugated anti-mouse IFN-γ mAb (XMG1.2, Biolegend), and Leucoperm fixation and permeabilization kit (AbD Serotec, Oxford, UK). The IFN-γ release induced by 30 ng/ml of Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) plus 1 µg/ml of ionomycin (Sigma-Aldrich) was used as a positive control. Cells were analyzed using FACSCanto II flow-cytometer and DIVA software.

In Vivo $OVA_{257–264}$ T Cell Response Evaluation

Group of mice ($n = 4$) were primed (day 0) by subcutaneous injection with 100 µl of PBS containing fdOVA (SIINFEKL peptide) bacteriophages or fdOVA/α-GalCer bacteriophages (all delivering 1.5 µg of $OVA_{257–264}$ peptide) and boosted (day 14) with the same amount of fdOVA bacteriophages delivering or not the α-GalCer. As control, mice were inoculated twice with vehicle alone (PBS).

At day 21, splenocytes were isolated and the frequency of $OVA_{257–264}$ specific $CD8^+$ T cells was assessed using FITC or APC conjugated anti-mouse CD8a mAb (53–6.7, Biolegend) and PE-H2Kb SIINFEKL MHC dextramers (Immudex) staining. Stained cells were analyzed by flow cytometry using a FACSCantoII. Results are expressed as the percentage of $CD8^+$ gated cells that are positive for the MHC I/peptide dextramers.

IFN-γ-producing effector cells were evaluated by culturing 7.5×10^6 spleen cells with $OVA_{257–264}$ SIINFEKL synthetic peptide (10 µg/ml) for 5 h in the presence of BFA (Sigma-Aldrich). Cells were then harvested and IFN-γ production was evaluated by intracellular staining on gated $CD8^+$ cells using FITC or APC conjugated anti-mouse CD8a mAb, PE conjugated anti-mouse IFN-γ mAb (XMG1.2 Biolegend), and Leucoperm fixation and permeabilization kit. The IFN-γ release induced by 30 ng/ml of PMA plus 1 µg/ml of ionomycin was used as a positive control. Data were acquired on FACSCanto II flow-cytometer and DIVA software.

Therapeutic Vaccination Against B16 Tumor Cells

Naïve C57BL/6 mice were engrafted with 2×10^5 B16 melanoma cells subcutaneously in the left flank. When tumors were palpable, mice were vaccinated with 100 µl volumes containing PBS, 2.5 µg α-GalCer, 50 µg of fdWT bacteriophages, or 50 µg of fd/α-GalCer. In the experiment with B16-OVA tumor cell line, mice were injected intratumorally twice, on day 0 and day 5, with 100 µl volumes containing PBS, 2.5 µg α-GalCer, 50 µg of fdOVA bacteriophages, 50 µg of fd/α-GalCer, or 50 µg of fdOVA/α-GalCer. Tumor growth was assessed three times weekly using caliper and recorded as tumor volume (mm^3) according to the formula $(d^2 \times D)/2$, where d and D are the shortest and the longest diameters. Mice were culled once tumor size met or exceeded 1,500 mm^3, in accordance with established guidelines. Survival was recorded as the percentage of surviving animals.

In another set of experiments, mice were sacrificed on day 12 and tumors and spleens were collected and homogenized. Tumors and spleen cells were filtered through a 70-µm cell strainer nylon mesh, and erythrocytes were lysed. Cells were then washed once with medium, resuspended in PBS + 5% FCS to a concentration of 1.5×10^6 cells/ml and labeled with APC conjugated anti-mouse CD8a mAb and PE-H2Kb SIINFEKL MHC dextramers for flow cytometric analysis. 7-aminoactinomycin D (7-AAD) (BD) was used for live–dead cell discrimination.

Statistical Analysis

Comparative analyses were performed using the analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test or Bonferroni *post hoc* comparisons. Analyses of survival were performed using the log-rank (Mantel–Cox) test. All the analyses were performed with the GraphPad Prism 5 program (GraphPad Software, CA, USA). In all cases, differences were considered statistically significant when $p < 0.05$.

RESULTS

α-GalCer Conjugation to *fd* Filamentous Bacteriophage

In order to exploit the ability of the major coat protein pVIII of the filamentous bacteriophage *fd* to bind hydrophobic lipids, α-GalCer was conjugated to LPS-purified bacteriophage particles (**Figure 1A**) in a 10:1 ratio (1.5 mg of bacteriophages: 150 µg α-GalCer), followed by ultracentrifugation on cesium chloride gradient to remove unbound lipids.

Alpha-GalactosylCeramide rapidly distributed on the pVIII protein thanks to hydrophobic interactions between the hydrophobic domains of the protein and the acyl chains of the lipid. The amount of α-GalCer conjugated to the bacteriophage vector was determined by quantitative mass analysis by a UPLC-MS/MS method. To this aim, the lipid was released from the conjugated vector by solvent extraction and the free glycosphingolipid was measured by multiple reaction monitoring (MRM) analysis. β-galactosylpalmitoylsphingosine was added as IS before extraction. The organic extract obtained as reported in the Section "Materials and Methods," contained a measured absolute amount of α-GalCer of 0.196 µg/ml, indicating that 2.6% of the added galactosphingolipid was effectively loaded onto the phage particles (**Figure 1B**).

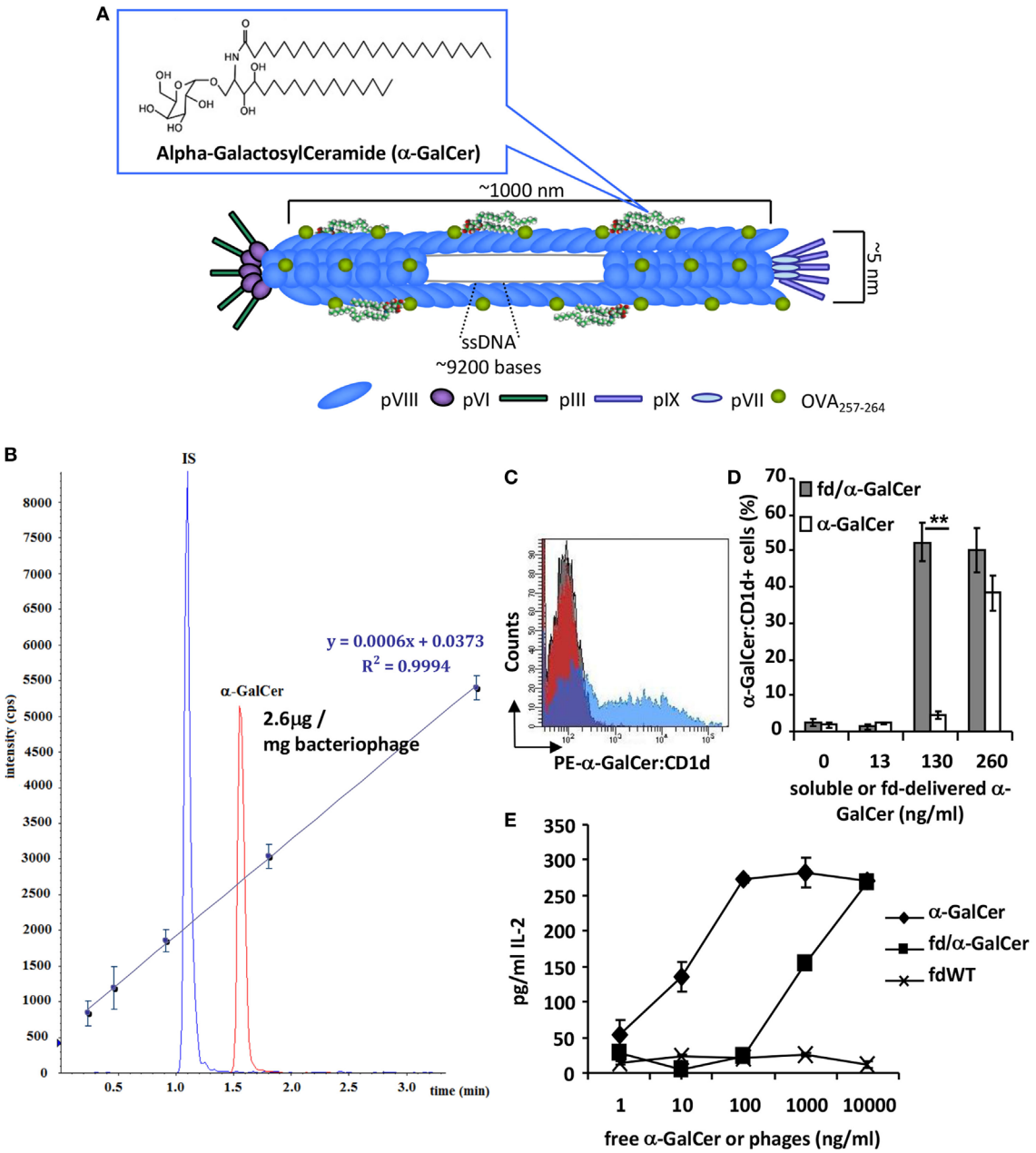

FIGURE 1 | fd/alpha-GalactosylCeramide (α-GalCer) conjugate characterization. **(A)** Schematic image representing filamentous bacteriophage engineered for the expression of antigenic peptide and α-GalCer on the coat surface. **(B)** UPLC-multiple reaction monitoring profile of the organic extract of a solution containing 1.5 mg/ml of the bacteriophage conjugated with α-GalCer; IS, internal standard. The calibration curve obtained from five calibration points of α-GalCer STD (62.5, 125, 250, 500, and 1,000 ng/ml) spiked with fixed amount of IS (400 ng/ml) is reported along with equation parameters. **(C,D)** Dendritic cells (DCs) were cultured with free α-GalCer or fd/α-GalCer at different doses. After incubation, cells were stained with anti-α-GalCer:CD1d antibody and analyzed by flow cytometry. **(C)** Representative histogram overlay of DCs incubated with free α-GalCer or fd/α-GalCer at a dose of 130 ng/ml α-GalCer or fdWT. **(D)** Percentage of anti-α-GalCer:CD1d positive cells. Mean ± SD of two different experiments is reported. $^{**}p < 0.01$ by unpaired two-tailed Student's *t*-test. **(E)** The chart represents the IL-2 release of mouse Vα14 invariant natural killer T (iNKT) hybridoma cell line FF13 to α-GalCer delivered by phage particles. LPS-free filamentous bacteriophages were conjugated to α-GalCer, and presented by mouse DCs to stimulate iNKT hybridoma cells. Free form of α-GalCer was used as positive control. Supernatants were diluted 1:10 and assayed in duplicate. Mean ± SD is reported, one representative experiment of four is shown.

fd/α-GalCer Ability to Activate iNKT Cells

We assessed the ability of α-GalCer loaded on phage particles to be presented by BMDCs and to activate iNKT cells *in vitro*. BMDCs were incubated with different doses of free α-GalCer or bacteriophage particles delivering α-GalCer. Cells were stained with a mAb recognizing the α-GalCer:CD1d complex and analyzed by flow

cytometry. We found that fd/α-GalCer is able to be internalized by DCs and to efficiently induce α-GalCer presentation on CD1d molecule. In addition, at the dose of 130 ng only the α-GalCer carried by fd was displayed on CD1d molecule (**Figures 1C,D**).

Moreover, to investigate the efficiency of the antigen presentation of α-GalCer delivered by phage particles on CD1d, we co-cultured fd/α-GalCer pulsed BMDCs with the mouse Vα14 iNKT hybridoma cell line FF13. Bacteriophage-vectorized α-GalCer was presented by BMDCs, triggering activation of iNKT hybridoma, as assessed by IL-2 release (**Figure 1E**). According to mass analysis data, it is noteworthy that the highest used dose of bacteriophage (10,000 ng/ml) contains about 26 ng/ml of α-GalCer. The IL-2 production by hybridoma cells was due to the α-GalCer delivered on phage particles as demonstrated by the lack of capacity to induce IL-2 production from BMDCs pulsed with bacteriophage particles not-conjugated to the glycolipid (fdWT).

We also tested the *in vivo* ability of bacteriophage/α-GalCer conjugates to activate a response, by injecting mice intravenously with 50 μg of fd/α-GalCer or 5 μg of free α-GalCer. We found that 3 h after the injection of fd/α-GalCer particles, $CD3^+NK1.1^+$ cells were activated, as measured by *ex vivo* analysis of IFN-γ production (**Figure 2A**). It is noteworthy that similar response was induced by the injection of the free α-GalCer and *fd*-conjugated α-GalCer even though the amount of lipid administered with the phage particles was less with respect to the free α-GalCer used. In fact, mass analysis demonstrated that approximately 130 ng of α-GalCer resulted bound to 50 μg of bacteriophages. Moreover, only the injection of vectorized glycosphingolipid was able to significantly increase the number of $CD3^+NK1.1^+$ in the spleen, compared to free α-GalCer injection (**Figure 2B**).

Importantly, notwithstanding the lower amount of lipid administered with bacteriophage particles, we found that fd/α-GalCer induced a higher percentage of NK cells able to produce IFN-γ compared to cells isolated from mice injected with free α-GalCer (**Figure 2C**).

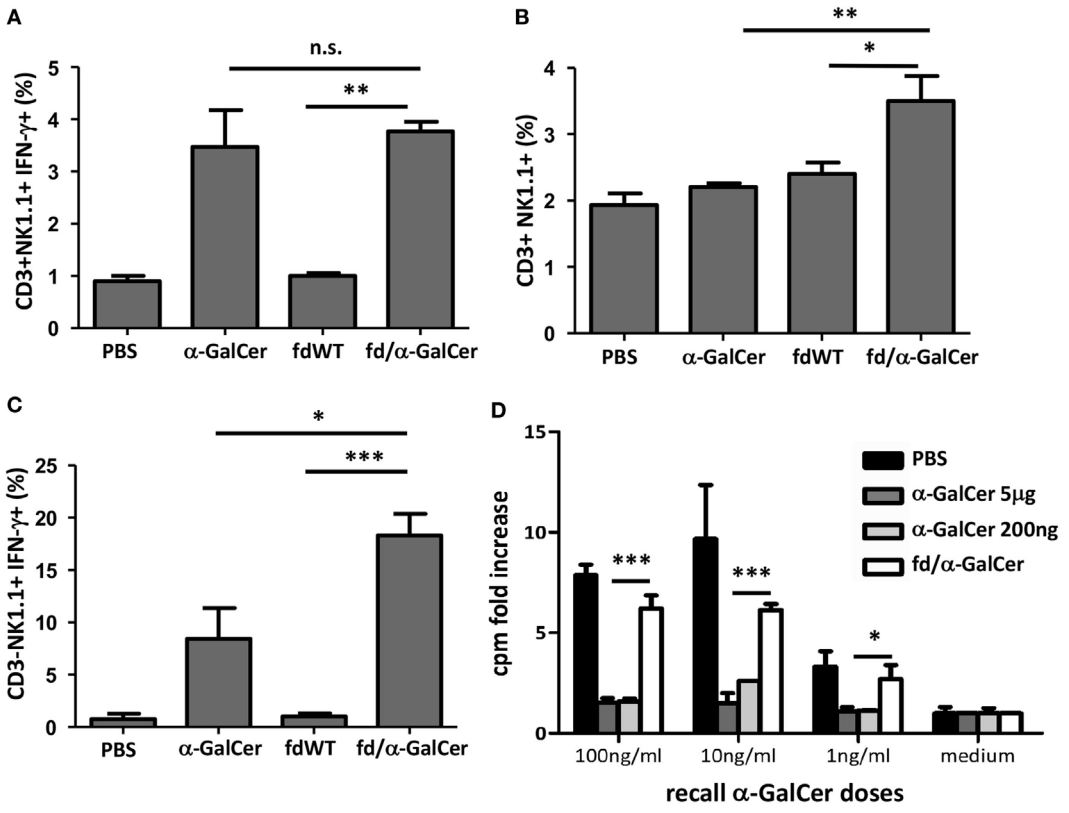

FIGURE 2 | *In vitro* recall response of mice to intravenous alpha-GalactosylCeramide (α-GalCer) administration. Mice (n = 3/group) were injected intravenously with 50 μg of *fd* bacteriophages or 5 μg of free α-GalCer/mouse or vehicle (phosphate buffered saline, PBS). After 3 h, mice were sacrificed, spleen cells were collected and stained with the reported antibodies. **(A)** Percentage of INF-γ-producing $CD3^+NK1.1^+$ cells; average + SEM is reported. $p < 0.001$ by one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test. **(B)** Percentage of $CD3^+NK1.1^+$ cells 3 h after intravenous injection of above-mentioned reagents. Average + SEM is reported. $p < 0.01$ by one-way ANOVA followed by a Dunnett's multiple comparison test. **(C)** Percentage of INF-γ-producing $CD3^-NK1.1^+$. Average + SEM is reported. $p < 0.001$ by one-way ANOVA followed by a Dunnett's multiple comparison test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Abbreviation: ns, not significant. **(D)** *In vitro* recall response of mice to α-GalCer immunization. Mice (n = 3/group) were intravenously injected with 5 μg or 200 ng of free α-GalCer, 50 μg of fd/α-GalCer or vehicle alone (phosphate buffered saline, PBS). The day after, splenocytes were cultured with graded doses of α-GalCer or medium. After 3 days, proliferation was assessed by [3H] thymidine incorporation. Average + SEM is reported. $p < 0.001$ by two-way ANOVA and the Bonferroni multiple comparison test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Recall Response to Intravenous α-GalCer Administration

It is known that free α-GalCer administered intravenously to mice, causes TCR down regulation on iNKT, and splenocytes from α-GalCer-injected mice loose their capacity to proliferate and produce cytokines upon *in vitro* re-stimulation with α-GalCer (13). In agreement with these reports we found that spleen iNKT cells isolated from mice injected with 5 μg of free α-GalCer were unable to proliferate when re-stimulated *in vitro* with the same lipid (**Figure 2D**). Since mass experiments showed that the amounts of α-GalCer bound to the phage particles is low, we also used a low dose of free α-GalCer in these experiments and similar unresponsiveness was observed (**Figure 2D**). In contrast, we observed that splenocytes from mice injected with vectorized α-GalCer, by conjugation to bacteriophages, were still responsive to α-GalCer re-stimulation *in vitro* in a dose-dependent manner (**Figure 2D**, empty bars).

Antigen-Specific Immune Response Is Increased by the Adjuvant Effect of α-GalCer/Bacteriophage Conjugate

We then investigated the adjuvant effect of α-GalCer delivered by bacteriophages on the immunogenicity of a displayed antigenic determinant. For this purpose, α-GalCer was conjugated on the surface of *fd* particles displaying the ovalbumin (OVA) determinant SIINFEKL ($OVA_{257–264}$).

In **Figures 3A,B** we show that OVA antigen and α-GalCer co-delivered by phage particles are presented by DCs. BMDCs were

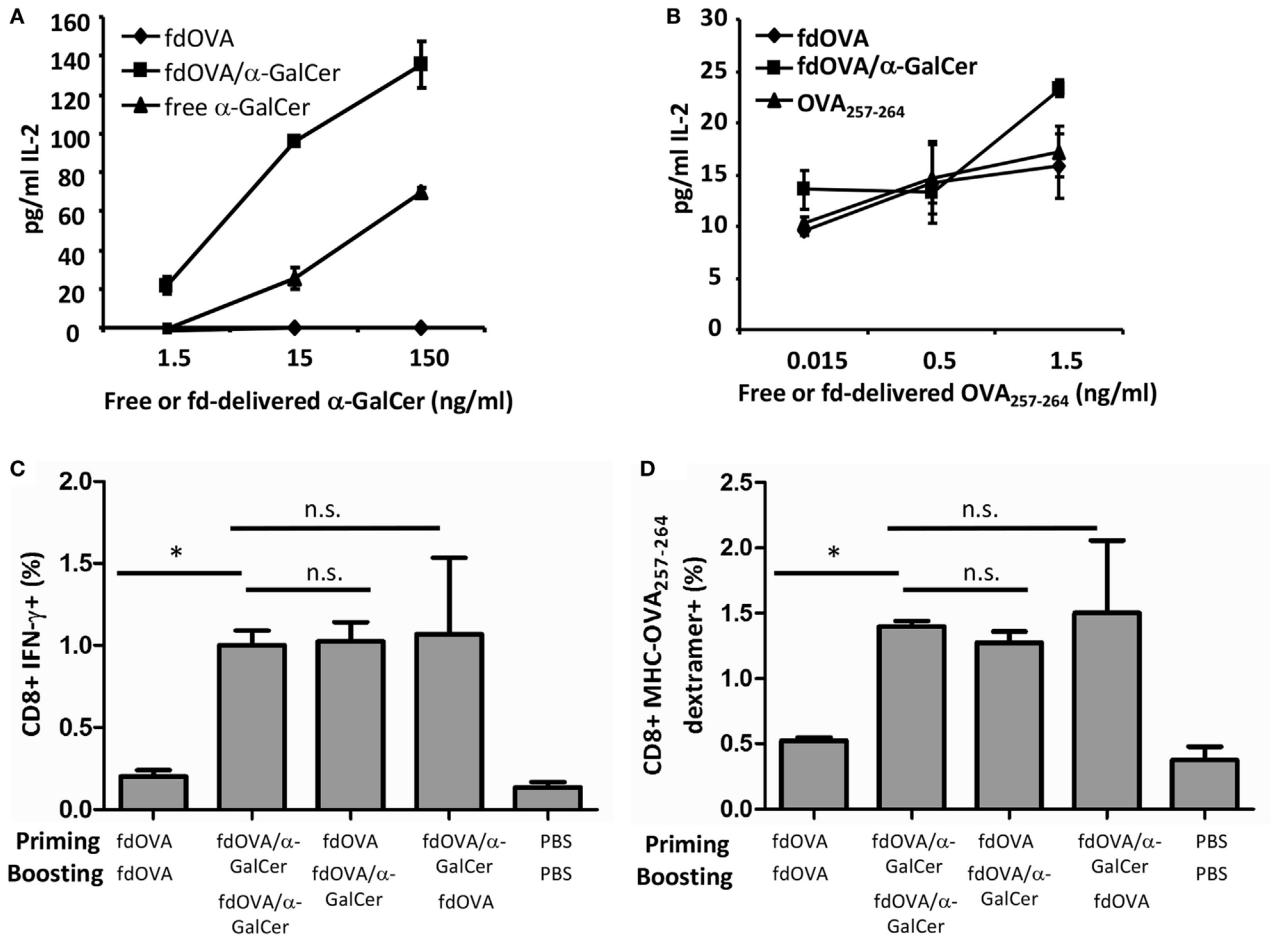

FIGURE 3 | Adjuvant effect of vectorized delivery of alpha-GalactosylCeramide (α-GalCer) on bacteriophages in antigen-specific adaptive immune response. IL-2 release of mouse Vα14 invariant natural killer T hybridoma cell line FF13 **(A)** or OTI hybridoma cell line **(B)** in response to α-GalCer or OVA SIINFEKL peptide delivered by phage particles. LPS-free fdOVA filamentous bacteriophages were conjugated to α-GalCer, and fdOVA or fdOVA/α-GalCer were presented by mouse bone marrow-derived dendritic cells to stimulate FF13 or OTI hybridoma cells. Soluble form of α-GalCer was used as positive control in **(A)**. Synthetic $OVA_{257–264}$ peptide was used as positive control in **(B)**. Supernatants were diluted 1:10 **(A)** or left undiluted **(B)** and assayed in duplicate. Mean ± SD is reported, one representative experiment of two is shown. **(C,D)** Group of mice (n = 4/group) were primed (day 0) and boosted (day 14) with fdOVA (SIINFEKL peptide) bacteriophages delivering or not the α-GalCer as indicated on the x-axis. As control, mice were inoculated twice with vehicle alone (phosphate buffered saline, PBS). At day 21, splenocytes were isolated and percentage of $OVA_{257–264}$-specific $CD8^+$ T cells producing IFN-γ **(C)** and percentage of H2Kb-SIINFEKL dextramer positive $CD8^+$ T cells **(D)** were evaluated. Average + SEM is reported. Difference were statistical significant by one-way analysis of variance followed by a Dunnett's multiple comparison test. *$p < 0.05$. Abbreviation: ns, not significant.

incubated with fdOVA/α-GalCer conjugate, or with fdOVA alone. As controls, DCs were incubated with free α-GalCer or synthetic $OVA_{257–264}$ peptide in the same amount as estimated on phage surface (see methods). BMDCs pulsed with fdOVA/α-GalCer are able to stimulate IL-2 production by either OTI hybridoma cells or iNKT hybridoma cells (**Figures 3A,B**). Of relevance, iNKT hybridoma cells produced higher amount of IL-2 when stimulated by DCs pulsed with fdOVAα–GalCer with respect to free α-GalCer.

C57BL/6 mice were immunized with bacteriophages expressing $OVA_{257–264}$ peptide (fdOVA) or with fdOVA delivering α-GalCer (fdOVA/α-GalCer) and after 14 days, mice were boosted with the same bacteriophage particles, delivering or not α-GalCer. As control, mice were inoculated twice with only vehicle (PBS). On day 21, mice were culled and spleen cells were isolated and assayed for $OVA_{257–264}$-specific cell response.

We found that immunization with fdOVA/α-GalCer particles developed an $OVA_{257–264}$-specific IFN-γ-secreting T cell response. As illustrated in **Figure 3**, mice that received two fdOVA/α-GalCer administrations, showed increased OVA-specific $CD8^+$ T cells producing IFN-γ compared to mice inoculated twice with fdOVA alone (**Figures 3C,D**).

Interestingly, the group of mice injected first with fdOVA and then with fdOVA/α-GalCer, showed similar results to two fdOVA/aGalCer injections, indicating that an adjuvant effect of α-GalCer administration *via* bacteriophage particles on the adaptive antigen-specific immune response can also be observed after a single administration of *fd* vectorized α-GalCer. As control, we treated a group of mice first with fdOVA/α-GalCer and then with fdOVA, obtaining comparable results to fdOVA/α-GalCer-treated group, suggesting that the fdOVA/α-GalCer administration either in priming or boosting is able to induce a higher $CD8^+$ response.

Therapeutic Vaccination With Bacteriophages in B16 Tumor-Bearing Mice

We also tested the ability of bacteriophage particles coated with α-GalCer to mediate protection in a therapeutic anti-tumor vaccination setting. For this purpose, C57BL/6 mice were injected subcutaneously with B16 melanoma cells (day −10), and when tumors were palpable (day 0), mice were treated intratumorally with free α-GalCer, *fd* particles (fdWT), or fd/α-GalCer bacteriophages (**Figure 4A**). Although intratumoral route of administration is not applicable to all the type of cancer, it has been demonstrated that intratumoral administration of drugs offers several advantages over traditional routes of immunization, as reduced systemic toxicity due to lower diffusion in the body and high local concentration, which permits use of smaller amount of drugs (24).

We found that the intratumoral injection of α-GalCer vectorized on phage particles (fd/α-GalCer) was able to increase the number of iNKT cells, as demonstrated by FACS analysis on spleen cells 24 h later, in comparison with the number of iNKT cells observed in the spleens isolated from mice treated with free α-GalCer (**Figure 4B**). We also found that spleen cells derived from mice primed *in vivo* with fd/α-GalCer still retained the capability to proliferate and to produce cytokines like IL-2 when re-stimulated *in vitro* with increasing doses of free α-GalCer (**Figures 4C,D**). This response was not observed with cells isolated from mice injected *in vivo* with free α-GalCer, and also re-stimulated *in vitro* with free α-GalCer. Moreover, we used for i.t. injection a dose of free α-GalCer (130 ng), normalized according to the amount retained in the phage particles. Similar unresponsiveness to *in vitro* restimulation was observed (**Figure 4D**).

An important anti-tumor effect was also clearly assessed. fd/α-GalCer treatment resulted in a significant delay in the tumor growth, while administration of free α-GalCer or *fd* wild-type bacteriophages were less efficient in delaying tumor growth (**Figures 5A,B**). No significant differences were noted between groups of mice treated with fdWT or free α-GalCer. This protective effect was confirmed by the longer survival of mice receiving fd/α-GalCer (**Figure 5C**).

Finally, to investigate the capacity of fd/α-GalCer to induce a tumor-specific adaptive response in a vaccination model, mice were injected with hybrid bacteriophages co-expressing the $OVA_{257–264}$ SIINFEKL peptide in the presence or absence of conjugated α-GalCer. C57BL/6 mice were engrafted (on day −10), with B16 melanoma cells engineered for the expression of ovalbumin protein. After tumor engraftment, mice received intratumorally fdOVA, fdOVA/α-GalCer, or fd/α-GalCer on day 0. The injection was repeated on day 5 and tumor growth was assessed over time (**Figure 6A**).

Priming and boosting mice with fdOVA/α-GalCer efficiently protected mice, as demonstrated by measuring tumor growth (**Figures 6B,C**). We observed a delay in tumor growth in mice immunized with fdOVA/α-GalCer phage particles, in comparison with mice treated with fdOVA or free α-GalCer.

Mice engrafted with B16-OVA cells after 2 injections with fdOVA/α-GalCer showed 80% survival rate at the end of the experiment, in comparison with 40% survival of mice treated twice with fd/α-GalCer in the absence of the specific $OVA_{257–264}$ peptide (**Figure 6D**). In this latter group of mice, a variable response was observed, suggesting the presence of responder and not responder mice (Figure S1 in Supplementary Material). No increased survival was observed in mouse groups treated with two injections of free α-GalCer or fdOVA particles not delivering the galactosylceramide.

The analysis of tumor-infiltrating cells at day 12 from intratumor vaccination showed the presence of an increased percentage and absolute number of $CD8^+$ T cells within the tumor bed in mice injected with fdOVA/α-GalCer compared to free α-GalCer and fdOVA-injected groups (**Figures 6E,G**), while the percentage of $OVA_{257–264}$ specific T cells, as stained by SIINFEKL-MHC dextramer, was the same in fdOVA or fdOVA/α-GalCer-treated cells, both in tumors and in spleens (**Figure 6F**; Figure S2 in Supplementary Material). Importantly, the absolute number of tumor infiltrating $OVA_{257–264}$-specific T cells was higher in mice treated with fdOVA/α-GalCer with respect to mice which received fdOVA (**Figure 6H**). These data indicate that fdOVA/α-GalCer bacteriophage particles are able to induce a general expansion of tumor-infiltrating T cells, probably due to the activity of α-GalCer, and then, an expansion of $OVA_{257–264}$-specific

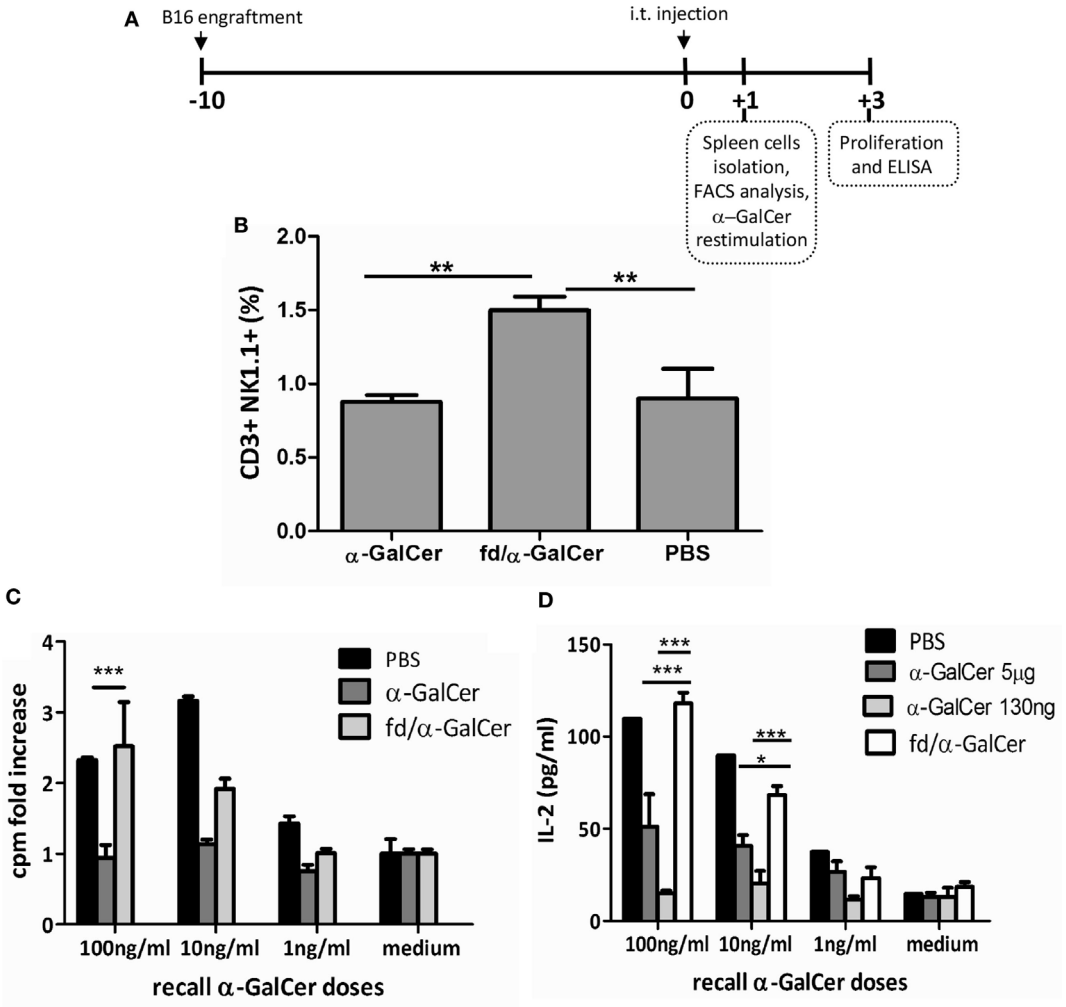

FIGURE 4 | Invariant natural killer T response in mice injected intratumorally with fd-vectorized alpha-GalactosylCeramide (α-GalCer). Mice were inoculated with B16 melanoma cells and when tumor was palpable, mice were intratumorally injected with free α-GalCer, α-GalCer delivered by phage particles or vehicle alone, and sacrificed after 24 h. **(A)** Schematic representation of the experiment schedule. **(B)** $CD3^+NK1.1^+$ cells were evaluated in freshly isolated spleen of injected mice by FACS analysis and staining with anti-NK1.1 and anti-CD3 antibodies. Percentage of $NK1.1^+CD3^+$ positive cells ± SD is reported. $p < 0.01$ by one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test. $^{**}p < 0.01$. **(C,D)** Splenocytes were cultured with graded doses of α-GalCer. After 3 days, proliferation was assessed by [3H] thymidine incorporation **(C)**, and culture supernatants were evaluated for IL-2 release by ELISA **(D)**. Average + SEM is reported. $p < 0.001$ by two-way ANOVA and the Bonferroni multiple comparison test. $^*p < 0.05$, $^{***}p < 0.001$.

cells, which are boosted by the phage particles co-displaying the $OVA_{257–264}$ antigenic peptide.

DISCUSSION

Filamentous bacteriophage is a well characterized, powerful antigen delivery system that has been demonstrated to evoke long and sustained adaptive immune responses toward the antigens displayed on its surface (17–19). In addition, bacteriophage is able to activate also innate immune responses mainly *via* toll-like receptor pathways (23, 25).

To further improve the immune responses elicited by bacteriophage particles delivering antigenic peptides, in this work we exploited the capacity of the partially hydrophobic pVIII structural protein expressed on the phage surface to interact and bind the immunostimulating glycolipid α-GalCer. We found that the activation of iNKT *via* fd/α-GalCer, also rapidly induced transactivation of NK cells, which is considered to enhance the anti-tumor effect of α-GalCer treatment (26).

Furthermore, iNKT cells activated *in vivo* by fd/α-GalCer remained responsive to repeated stimulations, in contrast to long-term anergy observed upon free α-GalCer injection (13). The reason of iNKT unresponsiveness remains unclear, although it is known that injection of free α-GalCer rapidly induces the overexpression of inhibitory co-stimulatory programmed death (PD)-1 receptor (27). Nevertheless, blockage of PD-1/PD-L1 interaction does not restore full iNKT functions, indicating the presence of additional inhibitory mechanisms.

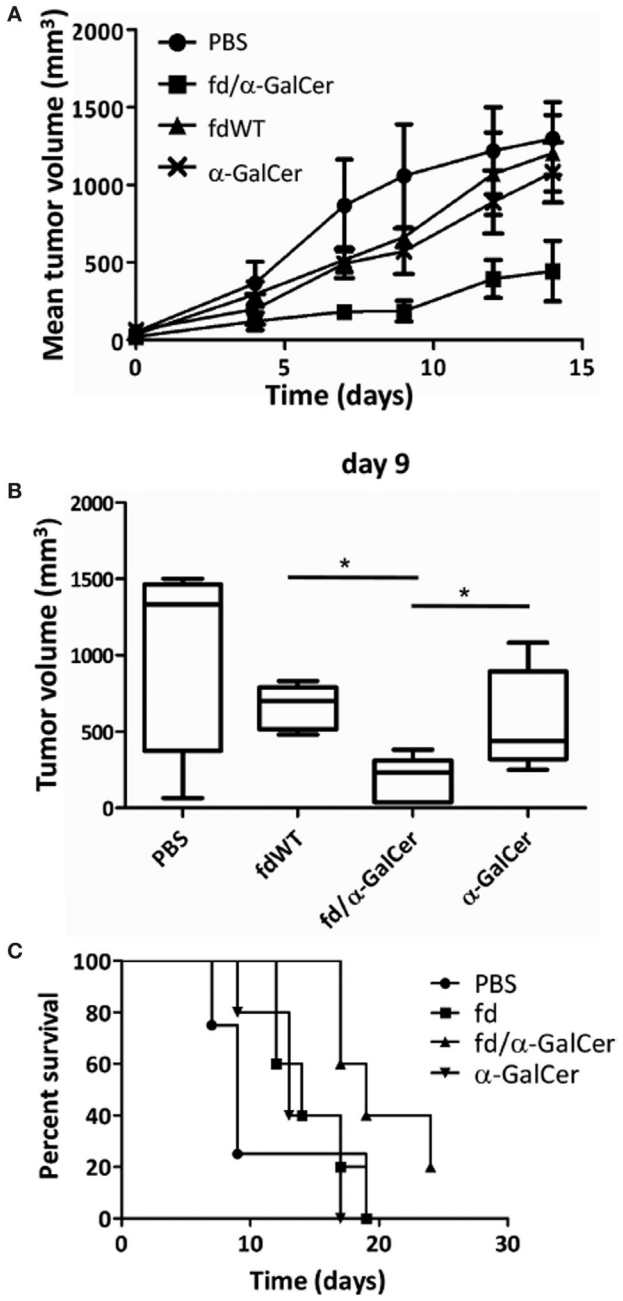

FIGURE 5 | Therapeutic vaccination with filamentous bacteriophages fd/alpha-GalactosylCeramide (α-GalCer) is protective against a subcutaneous tumor challenge. C57BL/6 mice (n = 5/group) were engrafted with B16 melanoma cell line and when tumor was palpable, mice were injected two times subcutaneously into the tumor with phosphate buffered saline, free α-GalCer, fdWT, or fd/α-GalCer. **(A)** The chart shows the mean tumor size reached in each group. **(B)** Tumor volumes recorded at 9th day after vaccination are reported as box plot, showing the group median, quartiles, and extreme values. Median values are represented by the horizontal line. *$p < 0.05$ by one-way ANOVA followed by a Dunnett's multiple comparison test. **(C)** Kaplan–Meier survival curves of B16-engrafted mice during the experiment. Differences among survival curves of α-GalCer-treated and fd/α-GalCer-treated mice are statistically different ($p < 0.01$) by log-rank (Mantel–Cox) test.

CD1d is expressed by many types of APCs which might all stimulate iNKT cells in principle. However, the APCs expressing appropriate levels of costimulatory molecules are those more efficient in inducing full activation of iNKT cells (13, 15, 28, 29). Studies involving conditional depletion of CD1d molecule on selected types of APC have demonstrated that different APCs have different capability to prime iNKT cells to glycolipid-specific responses.

In particular, when α-GalCer was presented by B cells a state of unresponsiveness was observed in iNKT cells (13, 30). In contrast, DCs resulted to be very active in stimulating iNKT cells with α-GalCer (31), and anergy could be further avoided by injection of α-GalCer-pulsed autologous DCs (15, 32). However, the dose of the injected lipid may be relevant in the induction of iNKT unresponsiveness by administration of α-GalCer-pulsed DCs (33). In addition, *ex vivo* manipulation of autologous human DCs appears to be a very expensive and time-consuming procedure.

For this reason, vectorizing α-GalCer on the surface of nanoparticles might be an interesting strategy to efficiently deliver the galactosylceramide to DCs in order to decrease the possibility of iNKT anergy. Indeed, nanoparticles are preferentially uptaken by DCs, and α-GalCer-coupled nanoparticles have been described to activate DCs without inducing anergy (34). The filamentous bacteriophage is composed by repeated and ordered subunits and for its particulate nature can be assimilated to a nature-made nanoparticle, and its use prevent unresponsiveness of iNKT.

Importantly, the simultaneous delivery of glycolipid and a MHC class-I-presented peptide to the same APC enhanced CTL cross priming as compared to separate administration of α-GalCer and protein antigen (35). We confirmed in our model that the co-delivery of tumor peptide and α-GalCer by phage particles increased the induction of antigen-specific $CD8^+$ T cells.

The exact mechanism how α-GalCer facilitates priming and expansion of OVA-specific $CD8^+$ T cells remain to be investigated. We envisage two, non-alternative mechanisms. The first is due to the recruitment of iNKT cells next to DCs that internalized fdOVA/α-GalCer. Recruited iNKT cells may immediately induce maturation of DCs and also the optimization of their Ag-presentation and priming capacities of $CD8^+$ cells. These effects might be facilitated also by the large numbers of iNKT cells and their immediate response (within a few minutes) to α-GalCer stimulation. The activation of iNKT cells might explain the reports in which it was found superior to those elicited by TLR agonists in inducing specific anti-tumor $CD8^+$ T cell responses (36).

The second mechanism may be ascribed to the fact that bacteriophage, after internalization localizes into the endocytic cellular compartments (23), where loading of glycolipids on CD1d molecule also occurs.

Overall, our findings suggest that filamentous bacteriophage is a powerful tool to deliver immunogenic lipid molecules together with antigenic peptides.

Growing interest is focused on the role of lipids as key stimulatory molecules of the immune response. Either naturally derived non-self lipids, microbial lipids (i.e., peptidoglycan, lipoteichoic acid, and lipopolysaccharides) or self lipids (i.e., phospholipids, sphingomyelin, and oxidized lipoproteins) are known to trigger the

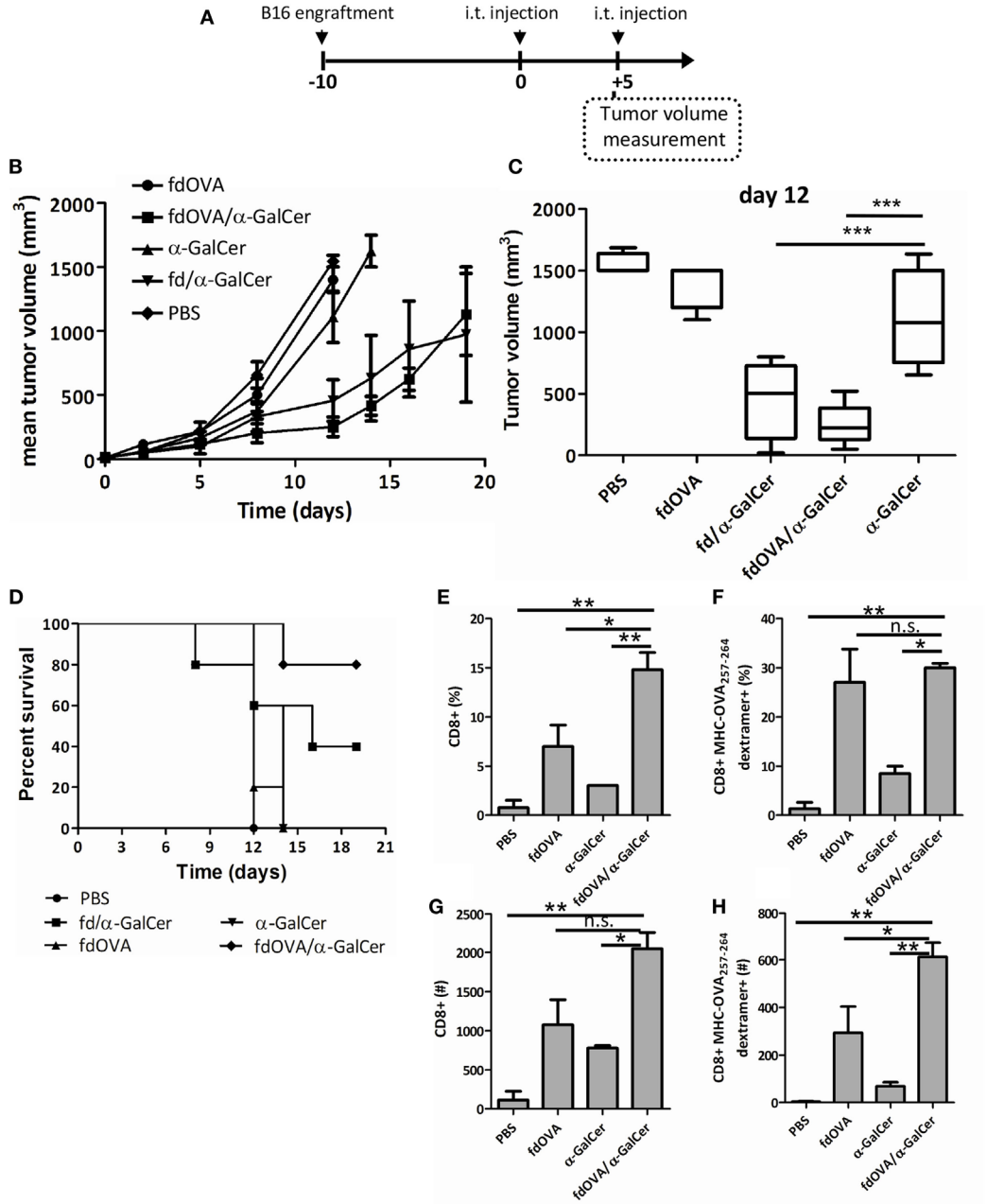

FIGURE 6 | Filamentous bacteriophage *fd* co-delivering tumor antigen and alpha-GalactosylCeramide (α-GalCer) induces a stronger anti-tumor response. Mice (n = 5/group) were challenged with B16-OVA expressing melanoma cells. When tumor was palpable (day 0) mice were immunized intratumorally with fd/α-GalCer, fdOVA/α-GalCer, fdOVA, free α-GalCer, or with phosphate buffered saline. The injection was repeated at day 5, and tumor volume was monitored with a caliper over time. **(A)** Schematic representation of the experiment. **(B)** Tumor growth recorded during experiment. The chart shows the mean tumor size reached in each group. Line for each group ends once more than half of the group is culled. **(C)** Box plot of tumor volumes recorded at day 12 post vaccination. Median values are represented by the horizontal line. $p < 0.001$ by one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test. ***$p < 0.001$ **(D)** Kaplan–Meier survival curves of B16-engrafted mice during the experiment. $p < 0.05$ between fdOVA/α-GalCer and free α-GalCer-treated mice group by log-rank (Mantel–Cox) test. **(E-H)** Groups of mice treated as above were sacrificed at day 12 post vaccination and tumor infiltrating lymphocytes were evaluated. Mean percentages **(E,F)** and absolute numbers **(G,H)** of $CD8^+$ T cells gated on 7-AAD negative cells **(E,G)** and of H2Kb-SIINFEKL dextramer positive gated on $CD8^+$ T cells **(F,H)** are reported. $p < 0.01$ by one-way ANOVA followed by a Dunnett's multiple comparison test. *$p < 0.05$, **$p < 0.01$. Abbreviation: ns, not significant.

immune system (37–41), and co-administration of stimulatory lipid molecules and antigens of interest in vaccination seems a promising manner to formulate novel vaccines including anti-tumor vaccines.

The use of filamentous bacteriophages complexed with lipid molecules might represent an efficient, fast, and cheap way to generate new vaccines capable of efficient priming and expansion

of T cells and also devoid of chemical adjuvants. Our approach can be further improved by developing phage-based antigen delivery systems loaded with α-GalCer and simultaneously expressing antigens target of the vaccine and molecules delivering the phage to specific cell subsets. Bacteriophages are generally considered safe (42, 43) and cheap to prepare and purify (44, 45), thereby they might represent new tools for the next generation of vaccines.

ETHICS STATEMENT

This study was carried out in accordance with European Union Laws and guidelines (European Directive 2010/63/EU). The study was approved by our institutional review board and the animal procedures (i.e., immunization, sacrifice) were performed according to rules approved by the ethics committee (permission no. 79/2014-PR).

AUTHOR CONTRIBUTIONS

Conceptualization: RS and PB. Methodology: RS, AC, and PB. Investigation: RS, LA, PB, DC, LG, and AC. Writing: RS and PB. Review and editing: LA and AC. Funding acquisition: PB. Supervision: PB. All authors read and approved the final version 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.2018.01496/full#supplementary-material.

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Conflict of Interest Statement: RS and PB are inventors of the patent no. PCT/IB2018/050525 "Phage conjugates and uses thereof."

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Comparison of Protein and Peptide Targeting for the Development of a CD169-Based Vaccination Strategy Against Melanoma

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CD169$^+$ macrophages are part of the innate immune system and capture pathogens that enter secondary lymphoid organs such as the spleen and the lymph nodes. Their strategic location in the marginal zone of the spleen and the subcapsular sinus in the lymph node enables them to capture antigens from the blood and the lymph respectively. Interestingly, these specific CD169$^+$ macrophages do not destroy the antigens they obtain, but instead, transfer it to B cells and dendritic cells (DCs) which facilitates the induction of strong adaptive immune responses. This latter characteristic of the CD169$^+$ macrophages can be exploited by specifically targeting tumor antigens to CD169$^+$ macrophages for the induction of specific T cell immunity. In the current study we target protein and peptide antigen as antibody-antigen conjugates to CD169$^+$ macrophages. We monitored the primary, memory, and recall T cell responses and evaluated the anti-tumor immune responses after immunization. In conclusion, both protein and peptide targeting to CD169 resulted in strong primary, memory, and recall T cell responses and protective immunity against melanoma, which indicates that both forms of antigen can be further explored as anti-cancer vaccination strategy.

Keywords: CD169, Siglec-1, sialoadhesin, tumor immunology, macrophage, T cell, antigen, cross-presentation

INTRODUCTION

Invasion of pathogens into the circulatory system can result in rapid development of disease. To prevent disease dissemination, the spleen, and lymph nodes sequester microbial, viral, and other nanoparticles from the blood or lymph fluid. In the marginal zone of the spleen and the subcapsular sinus of the lymph nodes, these entry sites are lined with phagocytic cells to trap invading pathogens (1, 2). One of the subsets of macrophages present at these locations are characterized by the high expression of CD169, also known as sialoadhesin or sialic acid-binding immunoglobulin-like lectin-1 (Siglec-1). CD169$^+$ macrophages produce multiple cytokines and stimulate a variety of innate lymphocytes and together with these cells form a first line of defense after infection (3–7).

Next to their functions in innate immunity, CD169$^+$ macrophages play a central role in the induction of both humoral and cellular adaptive immunity. Antigens captured by CD169$^+$ macrophages are directly presented in intact form on their surface to follicular B cells and thereby

stimulate germinal center B cell responses (8–13). We and others have observed antigen transfer from CD169$^+$ macrophages to Batf3-dependent classical DCs (cDC1s) which in turn stimulate subsequent CD8$^+$ T cell activation (14, 15). This antigen transfer between macrophages and DCs is facilitated by the CD169 molecule which functions as an adhesion molecule for sialylated molecules on DCs and binds strongly to cDC1s, (16). In addition, CD169$^+$ macrophages appear to be able to directly stimulate CD8$^+$ T cells after viral infection (15). As a result, antigen targeting to or infection of CD169$^+$ macrophages stimulates strong CD8$^+$ T cell responses that can lead to anti-tumor immunity (14).

Although the application of checkpoint inhibitors has dramatically improved the clinical outcome of several cancer types, most specifically melanoma (17, 18), still a significant portion of patients does not benefit from this form of immunotherapy. Since the response to checkpoint inhibitors is related to the presence of an anti-tumor T cell response, the induction of tumor-specific T cells by a vaccination approach could potentially increase the number of patients that will respond to the checkpoint inhibitor immunotherapy (18, 19). However, the optimal vaccination approach to induce CD8$^+$ T cells is not yet established.

Because DCs are crucial for the activation of T cells, several previous experimental approaches focused on the *in vitro/ex vivo* generation of DCs that loaded with tumor antigens were to be utilized as a cellular vaccine. However, these cellular vaccines are very laborious and have not shown very strong clinical responses so far. *In vivo* targeting approaches are being developed in which antigens are directed to DCs through antibodies that bind to surface receptors specifically expressed on DCs. Several mouse studies have demonstrated the applicability of this approach for a number of surface receptors on DCs, most notably DEC205 and Clec9A/DNGR-1 (20–23), but (pre)clinical studies in humans are still necessary to determine which markers on (which) human DCs are most optimal for the activation of T cells. In our previous studies, we have shown that antigen targeting to CD169$^+$ macrophages result in Ag presentation by DCs and the activation of strong CD8$^+$ T cell responses in mice. In humans, CD169$^+$ macrophages are also found in lymphoid organs and the numbers in tumor draining lymph nodes are positively related to longer survival in cancer patients. (24–28). Therefore, antigen targeting to CD169$^+$ macrophages may form an attractive strategy to activate anti-tumor T cell responses in humans.

While a number of *in vivo* targeting studies used whole protein conjugated to antibodies, other studies utilized peptides containing only a CD8$^+$ T cell epitope (21, 22, 29). Whole protein contains multiple epitopes to simultaneously induce CD4$^+$ T cells, CD8$^+$ T cell and B cell responses, while a peptide may only include single epitopes to induce CD8$^+$ T cells and/or CD4$^+$ T cells. Since helper CD4$^+$ T and B cells enhance CD8$^+$ T cell memory responses (30, 31), peptide targeting may lead to less than optimal long-term CD8$^+$ T cells responses. However, next to these immunological differences, more practical considerations should also be taken into account. Some melanoma proteins are difficult to produce while a peptide has the advantage that it can easily be synthesized and will allow quicker implementation for future clinical applications. This especially may be advantageous when neoantigens will be used for vaccination. Because of these considerations, it should be determined if a peptide is sufficient to evoke a protective long-term anti-tumor immune response. We therefore compared whether CD169-targeting of whole protein compared to single peptide differed in the induction of specific T cell responses and subsequent tumor eradication. Our experiments show that peptide targeting is as efficient as protein targeting and could be implemented in a vaccination strategy for melanoma.

MATERIALS AND METHODS

Mice

C57Bl/6 mice were bred at the animal facility of the VU University Medical Center (Amsterdam, The Netherlands). Females between the age of 8–12 weeks were used for the experiments unless indicated otherwise. All mice were kept under specific pathogen-free conditions and used in accordance with local animal experimentation guidelines. This study was carried out in accordance with the recommendations of and approved by the "dierexperimentencommissie" or the "centrale commissie dierproeven." Batf3 knockout mice were ordered form Jackson and bred in our facility.

OVA And SIINFEKL Conjugates

Ab-OVA conjugates were produced with SMCC-SATA mediated crosslinking as described previously (13, 14). In short, purified antibodies [αCD169 (MOMA-1), αDEC205 (NLDC-145), and a rat IgG2a isotype control (R7D4)] were functionalized with 5 equivalents of SMCC and endotoxin free OVA (Seikagaku) with 3 equivalents of SATA (N-succinimidyl S-acetylthioacetate, Thermo Fischer Scientific Breda) in phosphate buffer pH 8.5. Antibodies were desalted over PD-10 columns (GE Life Sciences Eindhoven) against phosphate buffer pH 7.2, and concentrated with centricon 30 (Merck Millipore Amsterdam) down to 300 μL. OVA-SATA was deprotected with 100 mM hydroxylamine hydrochloride (Thermo Fischer Scientific Breda) and desalted over PD-10 columns against phosphate buffer pH 7.2. After concentration of OVA-SATA with centricon 30 down to 200 μL, 6 equivalents OVA was added to antibodies while stirring. The antibody-OVA conjugates are incubated at room temperature for 1 h prior purification over sephadex 75 10/30 column.

Conjugation of SIINFEKL-eahx-lysine(biotin) peptide to antibodies was realized via a sulfhydryl based coupling. Briefly, antibodies were functionalized with 8 equivalents of SMCC [succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate, Thermo Fischer Scientific Breda] in phosphate buffer pH 8.5. After desalting over PD-10 columns (GE Life Sciences Eindhoven) against phosphate buffer pH 7.2 activated antibodies were concentrated with centricon 30 (Merck Millipore Amsterdam) down to 500 μL. 12 Equivalents of peptides in 50 μL DMSO was added to the antibodies and after 1 h incubation at room temperature conjugates were purified over sephadex 75 10/30 column (GE Life Sciences Eindhoven) according to manufacturer's HPLC settings.

Immunization

Mice were immunized i.v. with 1 μg Ab:Ag conjugates in the presence of 25 μg purified αCD40 Ab (1C10) and 25 μg Poly(I:C). On the indicated days after immunization spleens and/or blood were taken for processing. Boosts consisted of 1 μg free SIINFEKL peptide in the presence of adjuvants i.v. 28 days after primary immunization. 7 days after the boost spleens were taken for processing.

Flow Cytometric Analysis

Single cell suspensions were stained in 0.5% BSA in PBS for surface markers after blocking Fc receptors with clone 2.4G2. For intracellular staining 0.5% saponine buffer was used. For macrophages and DC staining spleens were digested with 2WU/ml Liberase TL (Roche Diagnostics) in PBS in the presence of 4mg/ml Lidocaine hydrochloride monohydrate (Sigma) and 50 μg/ml DNAse (Roche Diagnostics) at 37°C. Samples were measured on the Cyan (Backman Coulter) or the Fortessa (BD) and analyzed using FlowJo software (Tree Star).

Intracellular Cytokine Production

Splenocytes were incubated for 5 h with $OVA_{257-264}$ in the presence of GolgiPlug (BD) for CD8 T cells and with $OVA_{262-276}$ overnight with last 5 h in the presence of GolgiPlug (BD). Cells were fixed in 2% PFA and stained in saponine buffer for IL-2 and IFNγ.

Antibodies for Flow Cytometry And Immunofluorescence

Antibodies specific for: CD-8a-488 (clone 53-6.7 Biolegend), IL-2-488 (clone JES6-5H4 eBioscience), CD169-488 (clones SER4 and MOMA-1 in house), CD11a-FITC (clone M17/4 eBioscience), CD11c (clone N418 eBioscience), CD44-FITC (clone HI44a ImmunoTools), CD11c-PE (clone N418 eBioscience), CD38-PE (clone 90 eBioscience), CD4-PE (clone GK1.5 eBioscience), CD8a-PE (clone 53-6.7 eBioscience), GL7-biotin (eBioscience), B220-ef450 (clone 6B2 eBioscience), KLRG1-ef450 (clone 2F1 eBioscience), CD127/IL7Rα-APC (clone A7R34 Biolegend), CD8a-APC (clone 53-6.7 eBioscience), IFNγ-APC (clone xM61.2 eBioscience), CD62L-PECy7 (clone MEL-14 Biolegend), CD8a-PECy7 (clone 53-6.7 eBioscience), CD4-PERCPCy5.5 (clone RM4-5 eBioscience), CD8a-PERCPCy5.5 (clone 53-6.7 Biolegend). OVA-488 (Invitrogen). H-2Kb-SIINFEKL Tetramers (LUMC, Leiden). LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit (Invitrogen) was used according to manufacturers' protocol.

Tumor Experiments

200,000 B16OVA cells were injected s.c. in 100 μl PBS, 3, or 7 days later mice were treated i.v. with 1 μg Ab:Ag conjugates in the presence of 25 μg purified αCD40 Ab (1C10) and 25 μg Poly(I:C). Tumor outgrowth was monitored by measuring tumor size (length, height, width) 3 times per week using a caliper. Volume of the tumor was calculated using the formula for volume of an ellipsoid ($4/3 * \pi * (\frac{1}{2}l)(\frac{1}{2}h)(\frac{1}{2}w)$). Humane endpoints were chosen based on tumor size (max. 1,000 mm^3) or general appearance of mice. Tumor cell injection, i.v. vaccination, and tumor measurements were performed blinded, mice were appointed randomly to groups or were distributed according to an equal distribution of different tumor sizes among groups before treatment.

RESULTS

Efficient Peptide Ag Targeting to $CD169^+$ Macrophages *in vivo*

In previous studies we have shown that targeting of ovalbumin (OVA) to CD169 on macrophages can result in T cell responses and that these T cell responses can reduce tumor outgrowth (14). Here, we compare the conjugation of the immunodominant $CD8^+$ T cell epitope of OVA (SIINFEKL) to the whole protein, OVA, to verify whether a single peptide is sufficient to induce long-term $CD8^+$ T cell responses and to inhibit tumor outgrowth. OVA and SIINFEKL were chemically coupled to specific Abs for targeting to $CD169^+$ macrophages, to $DEC205^+$ DCs as a positive control or to isotype control Abs (14, 29). The functionality and specificity of the CD169- and DEC205-specific Abs after conjugation was confirmed by fluorescence microscopy or by flow cytometry (**Figures S1A–C**). For the induction of immune responses *in vivo,* mice were immunized with 1 μg of the different Ab:Ag conjugates in the presence of 25 μg anti-CD40 Ab and 25 μg Poly(I:C). We observed a strong induction of Ag specific $CD8^+$ T cells after both peptide and protein targeting to $CD169^+$ macrophages and DEC205 (**Figures 1A,B**, gating strategy **Figure S2A**). The isotype control antibody induced low levels of OVA-specific $CD8^+$ T cells as determined by intracellular IFNγ production or tetramer staining when compared to non-immunized naive mice (**Figure S1D**). The induction of specific $CD8^+$ T cells was dose dependent (**Figure S1E**). As shown recently for OVA targeting to CD169 (16), also the $CD8^+$ T cell responses induced by peptide targeting to CD169 relied on Batf3-dependent cross-presenting dendritic cells (DCs; **Figure 1C**). As expected only protein targeting induced OVA-specific $CD4^+$ T cell and B cell responses (**Figures 1D,E**). The T cells activated after targeting either protein or peptide exhibited equal affinity for the SIINFEKL epitope as analyzed using an *in vitro* titration of the peptide (**Figure 1F**, **Figure S2B**). These experiments indicate that both peptide and protein targeting to $CD169^+$ macrophages activate strong, cDC1-dependent, $CD8^+$ T cell responses, while only the protein targeting induced OVA-specific helper $CD4^+$ T cell and B cell responses.

Targeting of OVA Protein and Peptide to CD169 Results in Efficient Memory T Cell Induction

$CD4^+$ T cell responses have been recognized as essential for the maintenance and recall capacity of memory $CD8^+$ T cells (30, 32). To test if the absence of OVA-specific $CD4^+$ T cell help during the immunization influenced the memory pool of specific T cells, memory $CD8^+$ T cell responses were analyzed 28 days after immunization. At this time point the immune responses were still clearly measurable and we did

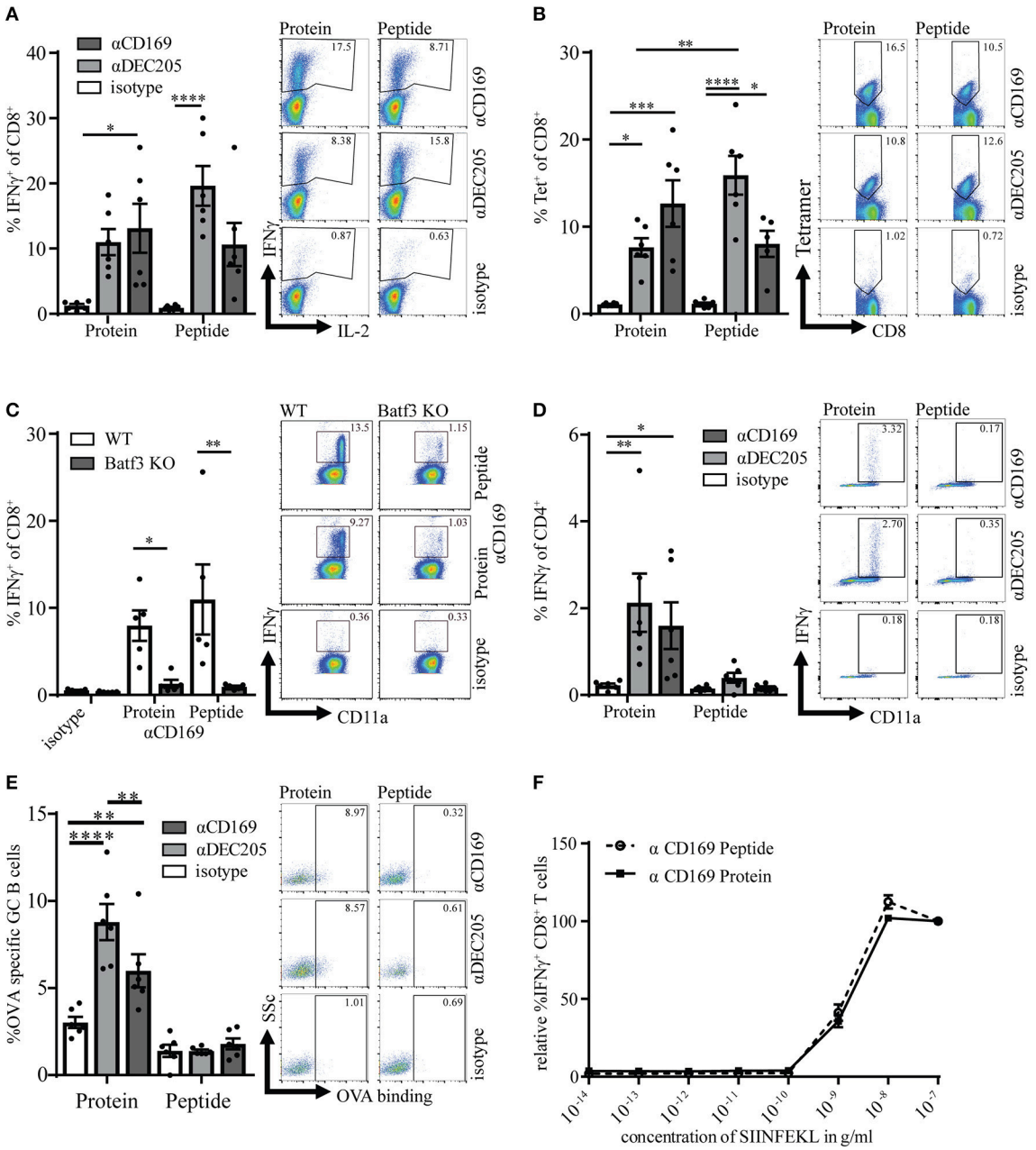

FIGURE 1 | Targeting of protein and peptide to CD169 results in strong primary T cell responses. (**A**) Percentage of IFNγ producing $CD8^+$ T cells after 5 h *in vitro* restimulation with SIINFEKL 7 days after immunization. (**B**) Percentage of $CD8^+$ T cells binding H-2Kb-SIINFEKL tetramers. (**C**) same as in A in WT and Batf3 KO mice (**D**) Percentage of IFNγ producing $CD4^+$ T cells after o.n. *in vitro* restimulation with I-Ab-restricted $OVA_{262-276}$. (**E**) Percentage of OVA-binding germinal center ($GL7^{hi}CD38^-$) B cells (**F**) Relative percentage of IFNγ producing $CD8^+$ T cells after 5 h *in vitro* restimulation with different concentrations of SIINFEKL. 100% is the IFNγ production after restimulation with the highest SIINFEKL concentration. (**A–F**) Splenocytes were taken 7 days after immunization with 1 μg Ab:Ag conjugates in the presence of 25 μg anti-CD40 Ab and 25 μg Poly(I:C). 1 representative experiment of 2–3 experiments is shown with 4–6 mice per group with one representative dotplot of each group. Statistical analysis one-way ANOVA with bonferroni's multiple comparison test $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

not observe any difference between targeting of protein or peptide (**Figures 2A,B**, **Figures S3A,B**). Furthermore, similar percentages of $CD8^+$ T cells could produce IL-2 on day 7 and day 28 (**Figures 2A** right panel, **C**) which is described to be indicative for memory T cells (33, 34). On day 7 most (∼90%) of the specific T cells that were induced with this vaccination strategy showed an effector phenotype ($CD44^+$ and $CD62L^-$), while a small percentage (∼10%) showed a central memory phenotype as shown by CD44 and CD62L coexpression (**Figure 2D**). Central memory phenotype CD8 T cells have been

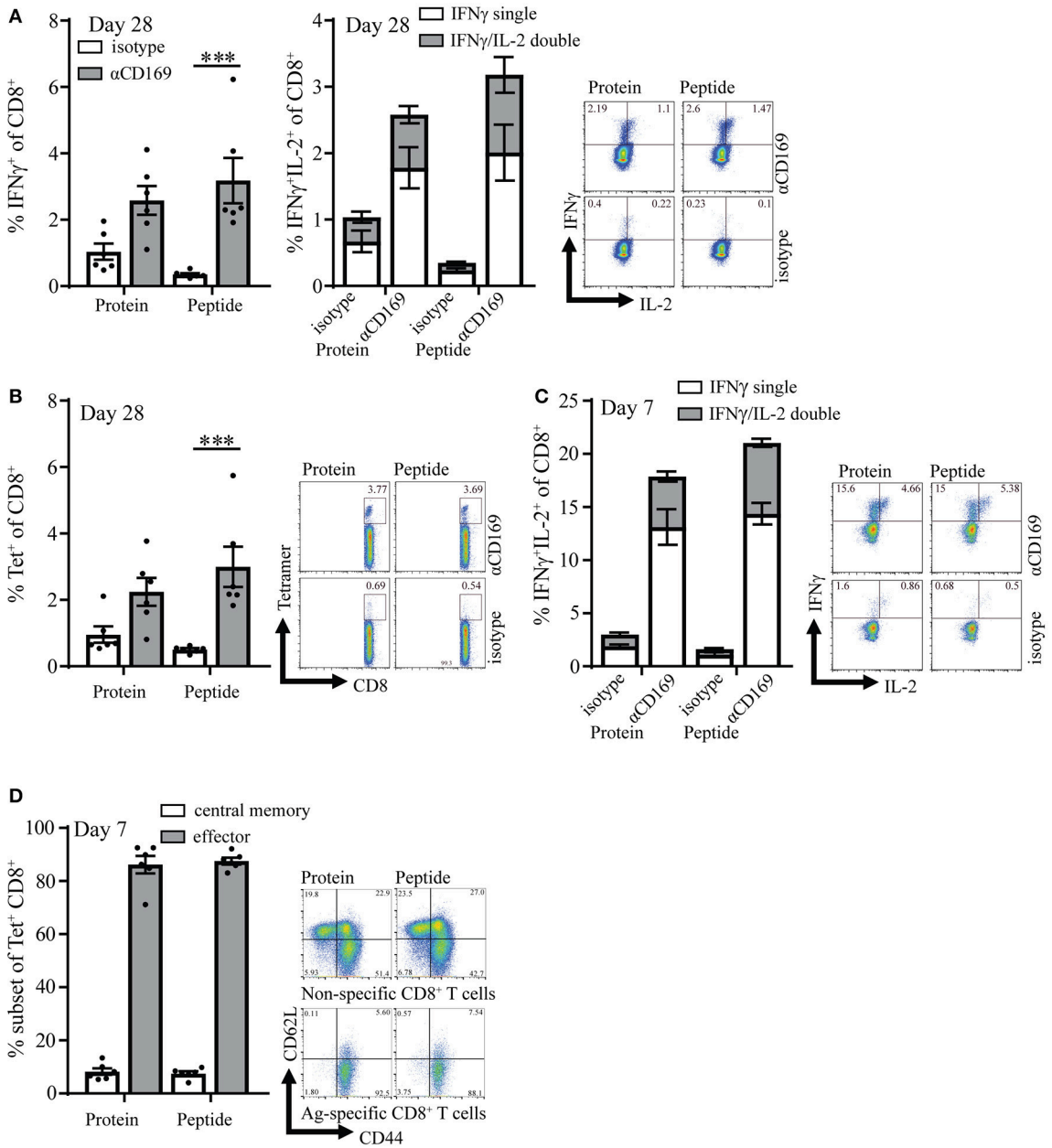

FIGURE 2 | Targeting of protein and peptide to CD169 results in long-lasting T cell responses. **(A)** Percentage of IFNγ producing $CD8^+$ T cells (left panel) and IFNγ single and IFNγ /IL-2 double producing $CD8^+$ T cells (right panel) after 5 h *in vitro* restimulation with SIINFEKL 28 days after immunization. **(B)** Percentage of $CD8^+$ T cells binding H-2Kb-SIINFEKL tetramers 28 days after immunization. **(C)** Percentage of IFNγ single and IFNγ /IL-2 double producing $CD8^+$ T cells after 5 h *in vitro* restimulation with SIINFEKL 7 days after immunization. **(D)** Percentage of central memory ($CD44^+CD62L^+$) and effector ($CD44^+CD62L^-$) antigen-specific $CD8^+$ T cells at day 7 after immunization and representative dotplots for Tet^+ and Tet^- $CD8^+$ T cells are depicted. **(A–D)** 1 representative experiment of 2 is shown with 6 mice per group with a representative dotplot for each group. Statistical analysis one-way ANOVA with bonferroni's multiple comparison test $^{***}p < 0.001$.

shown to have more proliferative capacity and better protective capacity than effector cells in infectious models (35). We did not observe differences in the generation of central memory $CD8^+$ T cells when peptide and protein targeting was compared (**Figure 2D**).

Four weeks after the primary response the mice were boosted with SIINFEKL peptide in the presence of adjuvant to determine the capacity to raise a secondary response. Primary immunization with peptide as well as protein led to memory T cell responses that generated strong recall responses as shown by intracellular IFNγ production and SIINFEKL-tetramer binding to $CD8^+$ T cells (**Figures 3A,B**). The percentage of IFNγ/IL-2 double producing $CD8^+$ T cells was similar for protein and peptide targeting and there was no difference

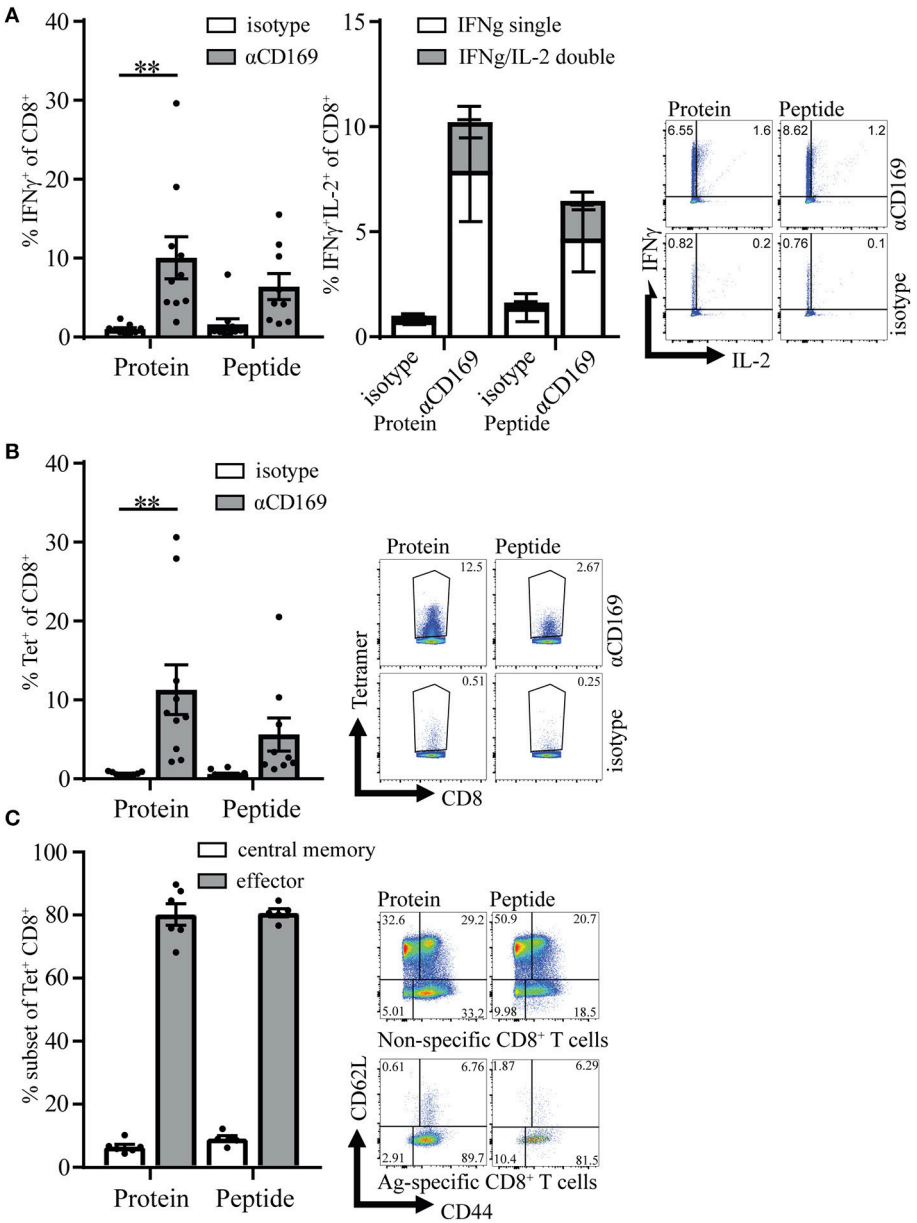

FIGURE 3 | Memory CD8$^+$ T cell responses after targeting antigen to CD169. Mice were initially immunized with indicated Ab:Ag conjugates and the immune response was boosted 28 days later with 1 μg free SIINFEKL peptide in the presence of adjuvants, 7 days after boost splenocytes were used for analysis. **(A)** Percentage of IFNγ producing CD8$^+$ T cells (left panel) and of IFNγ single and IFNγ/IL-2 double producing CD8$^+$ T cells (right panel) after 5 h *in vitro* restimulation with SIINFEKL peptide. **(B)** Percentage of CD8$^+$ T cells binding H-2Kb-SIINFEKL tetramers 28 days after immunization. **(C)** Percentage of central memory (CD44$^+$CD62L$^+$) and effector (CD44$^+$CD62L$^-$) of tetramer$^+$ CD8$^+$ T cells and representative dotplots for Tet$^+$ and Tet$^-$ CD8$^+$ T cells are depicted. **(A–C)** Combined results of 2 experiments is shown with 4–6 mice per group with a representative dotplot for each group. Statistical analysis one-way ANOVA with bonferroni's multiple comparison test $^{**}p < 0.01$.

in percentages of Ag specific T cells central memory and effector phenotype (**Figures 3A** right panel, **C**). These results indicate that peptide as well as protein targeting to CD169$^+$ macrophages stimulate strong CD8$^+$ T cell responses with equal potential for proliferation upon secondary encounter with antigen.

Targeting of Protein and Peptide to CD169 Results in Efficient Anti-tumor T Cell Responses

To test if the CD8$^+$ T cells induced by this vaccination strategy are able to kill tumor cells *in vivo*, we used a therapeutic vaccination setting. Mice were injected s.c. with melanoma

B16OVA tumor cells and 3 days later the mice were immunized with OVA protein or SIINFEKL conjugated to isotype control Ab or antiCD169 Ab. Immunization with protein or peptide targeting to $CD169^+$ macrophages was equally able to suppress outgrowth (**Figures 4A,B**). Since mice were sacrificed when the tumor size reached 1,000 mm^3, prolonged survival was observed in those groups that received a vaccination (**Figure 4C**). The number of mice that had an established tumor (size > 2 mm^3) during the course of the experiment was highest in non-treated and isotype-treated groups, while the number of mice that did not develop any tumor during the course of the experiment was highest in the protein targeted group. Interestingly, tumors initially grew in all the CD169-targeted groups the first 10 days (**Figure 4D**), but their growth was inhibited at day 10 which coincided with the peak in the $CD8^+$ T cell response, as measured by the SIINFEKL-specific $CD8^+$ T cells in the blood (**Figure 4E**). This was especially clear in the peptide targeted group (**Figure 4D**). To test if the vaccination strategy targeting protein and peptide to CD169 could result in T cell responses strong enough to suppress established tumors, mice with a visible tumor (average tumor size of 30 mm^3) were treated 7 days after tumor inoculation. The progression of established tumors into a fast-growing tumor was suppressed after targeting protein or peptide to CD169 (**Figure 4F**, **Figures S4A–C**). Together these data show that the induction of SIINFEKL-specific $CD8^+$ T cell responses by either protein or peptide vaccination results in efficient control of tumor outgrowth.

DISCUSSION

Vaccination approaches to induce anti-tumor $CD8^+$ T cell responses should fulfill a number of requirements. First of all, sufficient numbers of effector $CD8^+$ T cells should be activated to eradicate the existing primary tumor and/or metastases. Adoptive T cell transfer studies in both patients and mouse models have indicated that the number of transferred T cells is correlated with tumor regression (36, 37). Similarly, the number of T cells induced by vaccination approaches is predictive for their capacity to induce regression of existing tumors (38). Secondly, the differentiation stage of the activated $CD8^+$ T cells is important. Central memory or memory stem cell T cells are better in eliminating tumors than terminal differentiated effector cells that are obtained by multiple rounds of restimulation (36, 39). Thirdly, vaccination approaches should induce T cells with the capacity to efficiently home to tumors, such as resident memory T cells (40, 41) and should not induce T cells that home back to the vaccination site (42, 43). Finally, vaccination should result in long-lived $CD8^+$ T cell memory that will continuously eliminate outgrowth of tumor cells. Long term $CD8^+$ T cell memory is critically dependent on $CD4^+$ T cell help. Activation of $CD8^+$ T cells in the absence of CD4 T cells can result in effector cells when sufficient inflammation is present, but these cells have defects in restimulation, do not generate secondary responses, and are called "helpless" T cells (44–46). Vaccination approaches using only MHC class I restricted tumor epitopes may have the risk of not generating long-term $CD8^+$ T cell memory.

In our studies, we have evaluated the targeting of OVA protein and peptide antigen to $CD169^+$ macrophages to induce anti-tumor $CD8^+$ T cell responses. We observed very high frequencies of OVA-specific $CD8^+$ T cells (more than 10% of total $CD8^+$ T cells) after just one intravenous vaccination and targeting to $CD169^+$ macrophages was as good as targeting to $DEC205^+$ dendritic cells. This excellent induction of $CD8^+$ T cell responses, could potentially be due to the fact that $CD169^+$ very efficiently filter the blood and bind targeting antibodies. In addition, one of the unique characteristics of $CD169^+$ macrophages is the capacity to preserve intact antigen on their surface for days, which enables presentation and transfer to B cells and DCs (11, 13, 14, 16). Together these characteristics may enhance the amount of antigen presented during a longer period of time than that obtained during direct targeting to DCs. The activation of high numbers of $CD8^+$ T cells in just one immunization is also beneficial for their differentiation status as multiple rounds of Ag encounter lead to terminally differentiated cells with decreased potential for tumor regression (36).

Although $CD4^+$ T cells specific for OVA were not generated in the peptide vaccination and are considered essential for long-term $CD8^+$ T cell memory, we did not observe differences in the percentage of IL-2 producing or central memory phenotype $CD8^+$ T cells after priming and restimulation. Also similar memory $CD8^+$ T cell responses were generated when peptide and protein targeting were compared. Apparently our vaccination did not lead to a defect in memory T cell generation when peptides were used for targeting which could potentially be explained by two factors. First of all, we utilized a very strong adjuvant that mimics CD4 T cell help (anti-CD40 plus poly I:C) which makes additional CD4 T cell help dispensable (47–49). Indeed $CD8^+$ T cell responses are efficiently elicited in MHC class II-deficient mice with our vaccination approach, demonstrating the dispensable role for $CD4^+$ T cells with this adjuvant (data not shown). Several clinical trials in which agonistic anti-CD40 Ab is tested for its effect in solid tumors such as melanoma, non-small cell lung cancer and pancreatic duct adenocarcinoma sometimes in combination with checkpoint inhibitors have been started. The combination of agonistic anti-CD40 Ab with a vaccination strategy has not yet been explored in clinical trials and may be considered for neoantigen peptide vaccination strategies.

Secondly, we used rat antibodies to target our antigens to the $CD169^+$ macrophages. These rat antibodies are likely to be immunogenic in mice and could contain helper epitopes for $CD4^+$ T cells as has been described for anti-Clec9A/DNGR-1 antibodies (50). Cloning of the CD169-specific antibodies and the generation of recombinant mouse antibodies would be necessary to exclude this additional immunogenicity. However, although potential rat IgG2a-specific $CD4^+$ T cells could be induced in the vaccination procedure, these helper $CD4^+$ T cells were not involved in the OVA-specific anti-tumor response.

Both peptide and protein targeting to CD169 stimulated potent anti-tumor $CD8^+$ T cell responses that were as efficient to prevent outgrowth of B16-OVA melanoma cells, indicating that the generated OVA specific $CD8^+$ T cell homed to the tumor. Interestingly, Batf3-dependent cDC1 have been shown to

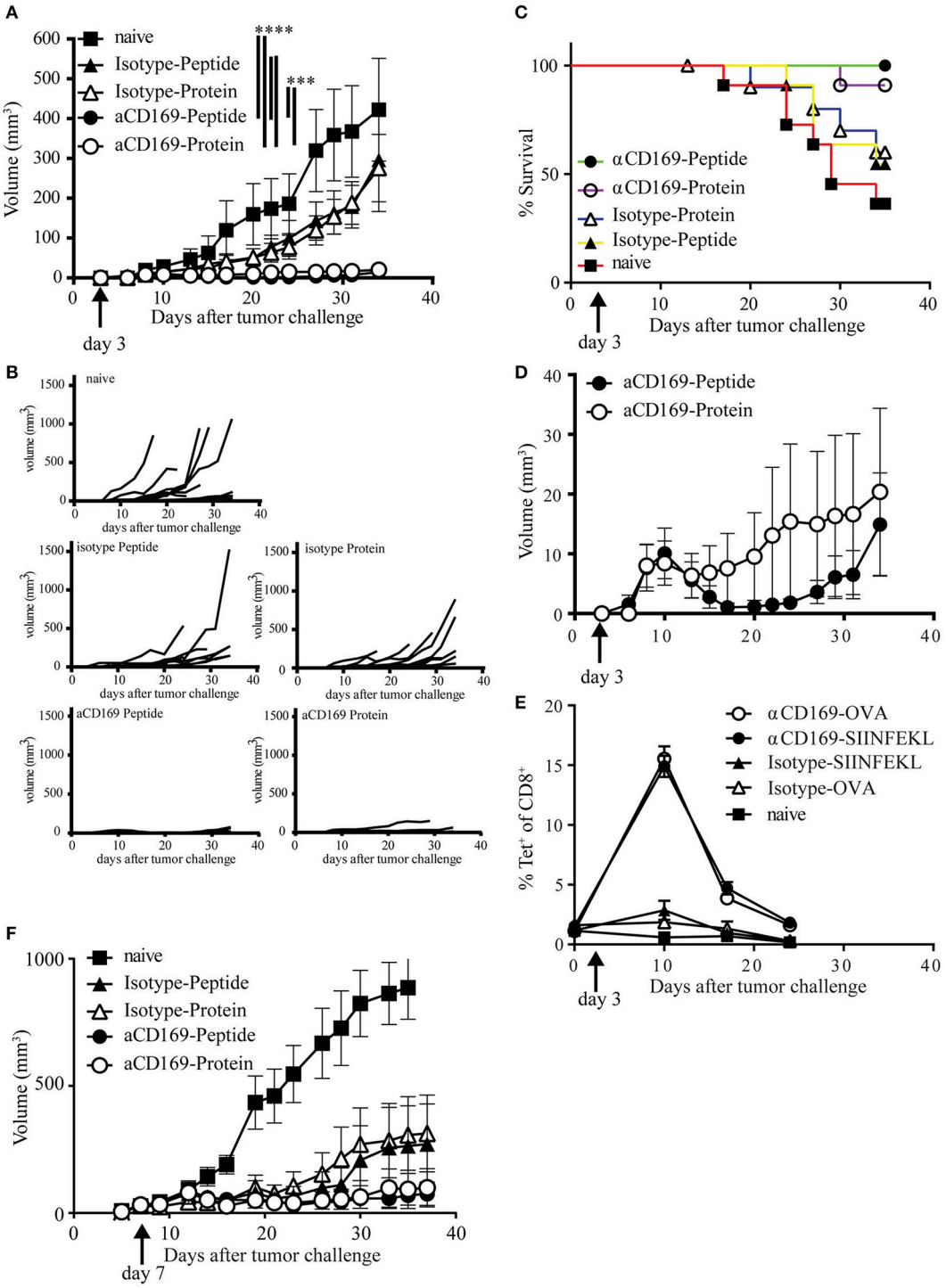

FIGURE 4 | Targeting of protein and peptide to CD169 results in the induction of tumor reactive T cell responses. **(A–E)** Mice were inoculated with 200,000 B16OVA tumor cells at day 0, on day 3 mice were immunized with indicated Ab:Ag conjugates in the presence of anti-CD40 Ab and Poly(I:C). Tumor size was monitored three times a week and mice were sacrificed based on physical appearance or tumor size. **(A)** Tumor size on different days after tumor inoculation, mean ± SEM is shown. Two-way ANOVA with Bonferroni correction comparing all groups on the last day of the experiment, only significant differences are depicted. **(B)** Tumor size per group of each mouse after tumor inoculation **(C)** Percentage of surviving mice on indicated days after tumor inoculation. **(D)** Tumor volume as in **(A)**, but only showing the groups treated with the anti-CD169:Ag conjugates on a fitting scale. **(E)** Percentage of $CD8^+$ T cells binding H-2Kb-SIINFEKL tetramers at indicated time points during the tumor experiment. **(A–E)** One experiment with 11 mice per group is shown. **(F)** Same as in **(A)**, with treatment on day 7 after tumor cell inoculation, when all mice had a visible tumor. Data of one experiment with 11–12 mice per group is shown. Statistical analysis one-way ANOVA with bonferroni's multiple comparison test $^{***}p < 0.001$, $^{****}p < 0.0001$.

promote the generation of tissue resident memory T cells (40, 51). Batf3-dependent cDC1 also are known to facilitate $CD4^+$ T cell help and efficient long-term memory $CD8^+$ T cell generation (52, 53). Apparently, antigen presentation by cDC1 is crucial for optimal memory $CD8^+$ T cell responses with the capacity to home into tissues. We previously showed that targeting protein antigens to $CD169^+$ macrophages transferred antigen to cDC1 and cross-primed $CD8^+$ T cells (14, 16). Here, we show that also peptide targeted to $CD169^+$ macrophages required Batf3-dependent cDC1s to stimulate $CD8^+$ T cell responses, which may explain the observed similar capacity of peptide and protein antigens to stimulate effector, memory, recall $CD8^+$ T cell responses and the capacity to prevent tumor outgrowth.

A recent study has demonstrated that mouse tumors contain increased numbers of $CD169^+$ antigen presenting cells with characteristics of both DCs and macrophages that are able to home to lymph nodes and to cross-present tumor cell-derived Ag (54). Since conventional DC numbers may be limiting in tumors, Ag targeting to $CD169^+$ cells in tumor-bearing individuals may potentially have more impact than Ag targeting to conventional DCs. Although the structure of the human spleen is different from mouse spleen and lacks a marginal zone, CD169-expressing macrophages are found in perifollicular sheaths surrounding small capillaries, also located in close contact with B cells (28). These structures would also be optimally suited for capture of antigens from blood by macrophages and presentation to B cells and potentially also to DCs. Strategies that target to human CD169 molecules may therefore also be efficient in directing antigens to the right lymphoid structure for the activation of anti-tumor immune responses and should be further explored as a vaccination strategy in humans.

AUTHOR CONTRIBUTIONS

DvD, HV, and JdH experimental design. Experiments were conducted by DvD, HV, MR, EB, LH, KO, and JG. HK synthesized conjugates. Data analysis by DvD, HV, and MR. The manuscript was written by JdH and DvD and edited by all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.01997/full#supplementary-material

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REVIEW
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Dendritic Cells in the Cross Hair for the Generation of Tailored Vaccines

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Vaccines represent the discovery of utmost importance for global health, due to both prophylactic action to prevent infections and therapeutic intervention in neoplastic diseases. Despite this, current vaccination strategies need to be refined to successfully generate robust protective antigen-specific memory immune responses. To address this issue, one possibility is to exploit the high efficiency of dendritic cells (DCs) as antigen-presenting cells for T cell priming. DCs functional plasticity allows shaping the outcome of immune responses to achieve the required type of immunity. Therefore, the choice of adjuvants to guide and sustain DCs maturation, the design of multifaceted vehicles, and the choice of surface molecules to specifically target DCs represent the key issues currently explored in both preclinical and clinical settings. Here, we review advances in DCs-based vaccination approaches, which exploit direct *in vivo* DCs targeting and activation options. We also discuss the recent findings for efficient antitumor DCs-based vaccinations and combination strategies to reduce the immune tolerance promoted by the tumor microenvironment.

Keywords: dendritic cells, vaccination, pattern recognition receptors, antigen delivery, adjuvants

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INTRODUCTION

Vaccines represent one of the most effective Copernican revolutions for humankind and world health. This innovative discovery by Edward Jenner in the late years of the XVIII century allowed for control or complete eradication of infectious diseases as smallpox (1979) and rinderpest virus (2011) (1). This immunization strategy posed the bases for current remarkable therapeutic approaches against not only infections but also cancer. In evolutionary terms, pathogens have acquired the capability to circumvent the immune system with several evasion mechanisms, revised elsewhere (2), that prevent pathogen clearance and the establishment of immune memory. Vaccines represent the unique tool we have to impede pathogen spread; therefore, the urgent need for efficient vaccines is as relevant as before. *Mycobacterium tuberculosis*, which causes tuberculosis, is currently one of the most feared infectious agent due to its capability to evade the immune system, leading to death of more than one million of people per year. Unbelievably, the only licensed vaccine against *Mycobacterium tuberculosis* is bacillus Calmette-Guérin (BCG) conceived about 100 years ago. Nonetheless, BCG has displayed some degree of inefficacy in humans, thus raising the need for new tailored vaccination strategies that are currently under investigation (3). Moreover, every year, new cases of human immunodeficiency virus (HIV) infections lead to the necessity of a vaccine to control and prevent the spread of the virus. Up to now, vaccines against HIV have not passed phase II clinical trials due to poor protection conferred, requiring revision of delivered antigens (ags) and strategy to improve T cell response (4). Moreover, the recent outbreaks of Ebola virus and Zika virus infections clearly demonstrate that still nowadays

more than few infectious diseases need to be overwhelmed, as reported by the World Health Organization. On the other hand, vaccines represent also a therapeutic tool against cancer. One of the hallmarks of cancer is the capability of tumor cells to evade immune-mediated destruction (5) by promoting a tolerant milieu. Therefore, the immune system has to be pushed to respond specifically and robustly against tumors cells.

To address this purpose, it is becoming more and more evident that dendritic cells (DCs) stand out as a potent tool in our hands, being the mediators of cellular and humoral responses (6). DCs have been discovered in 1973 by R. Steinman and Z. Cohn that divided phagocytic cells (discovered by E. Metchnikoff in 1887) in macrophages and DCs on the basis of different effector functions: microbial scavenging activities for macrophages and antigen-presenting function for DCs (7, 8). Since then, DCs have emerged as the most potent antigen-presenting cells capable of shaping adaptive responses both during infections and cancer. Moreover, the broad spectrum of DCs activation makes them suitable for fine shifting of the type of response the context needs. Taking advantage of new adjuvants, innovative ags-delivery carriers and targeting strategies, it is now feasible to optimize the activation and ag presentation processes by the specific DCs subset that is the most effective in the initiation of the adaptive response needed in a given context. Here, we discuss the diverse phenotypical and functional properties of DCs subtypes that are exploited by recently developed vaccine approaches, dealing with advances in the use of ags, adjuvants, carriers and DCs-expressed molecules, object of targeting.

DCs IDENTITY: A MULTIFACETED FUNCTIONAL FAMILY

Dendritic cells are the primary professional antigen-presenting cells (APCs) that reside in both lymphoid and non-lymphoid organs (9–11). DCs encompass several heterogeneous subsets whose subdivision relies on ontogeny, expression of surface-receptors, and transcription factors (12–14). Much effort has been done in the identification and characterization of tissue-specific DC subsets to unravel the correlation between phenotype, localization, and functional properties, both in health and disease. Initially, DCs have been classified into conventional DCs (cDCs) and plasmacytoid DCs (pDCs). Briefly, cDCs prime naïve T cells and orchestrate ag-specific adaptive responses, while pDCs intervene during viral infections producing type I interferons (IFNs). Advanced approaches have extremely pushed our understanding of DC biology, resulting in a recent readapted taxonomy (12, 15, 16). Indeed, Villani and colleagues identify six subsets of DCs and monocytes in human (**Figure 1**): DC1 (CLEC9A$^+$CD141$^+$ DCs), DC2 and DC3 (CD1c$^+$ DCs), DC4 (FCGR3A/CD16$^+$ DCs), DC5 (AXL$^+$SIGLEC6$^+$ DCs) and DC6 (pDCs). DC1 represent the cross-presenting CD141$^+$/BDCA3$^+$ DCs while D2 and D3 correspond to cDCs displaying antigen uptake and processing capabilities. DC4 seem to be more prone to respond to viruses and are phenotypically close to monocytes. DC5 represent a newly defined subset that share features with both pDCs and cDCs, even though they appear to be functionally different from pDCs and more similar to cDCs. Indeed, DC5 localize in T cell zone of tonsils, probably promoting fast adaptive immunity. Due to this fine clustering, DC6 correspond to a more pure pDCs population (12). This precise classification opens the way for a more accurate view of DCs role in pathologies and provides cues for more specific targeting in immunotherapies. Indeed, it is reasonable to assume that this extreme phenotypical diversity correlates with different intrinsic functional properties of DCs, as emerged in Villani's work (12, 17, 18). In addition, environmental cues dictate DC activation and drive specific T cell responses (19, 20). Indeed, DCs display a plethora of pattern recognition receptors (PRR) that are specifically bound by microbe- or damage-associated molecular pattern (PAMP and DAMP, respectively) (21). Upon receptors engagement in peripheral tissues, the transduction signals lead to DC maturation with the upregulation of co-stimulatory molecules

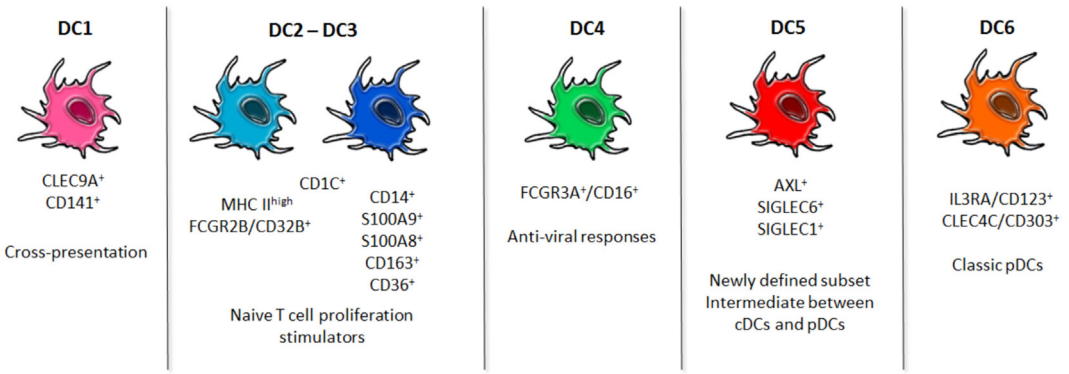

FIGURE 1 | Dendritic cells (DCs) readapted taxonomy. Newly identified populations of blood human DCs are shown. DC1 subset is clearly distinct by the expression of CLEC9A, and it is specialized in cross-presentation of ags. DC2 and DC3 constitute the conventional DCs pool, even though they appear to be phenotypically slightly different and, upon stimulation with TLR ligands, their diversity emerges. DC4 is a population characterized by an upregulated Type I Interferon pathway for antiviral responses. DC5 has emerged as a new population whose specific functions are still unexplored. DC6 corresponds to the classic plasmacytoid DCs. These advances in the fine characterization of DCs in humans may shed light on the best subset to be targeted to incentivize the desired immune response.

(referred to as "signal 2") and the pivotal chemokine receptor CCR7 that allows DCs migration through afferent lymphatic vessels to the draining lymph node (LN) (22–24). In parallel, DCs mediate ag proteolysis to present intracellular peptides on major histocompatibility complex (MHC) class I to $CD8^+$ T cells and exogenous peptides on MHC II to $CD4^+$ T cells (referred as "signal 1"). DCs can present exogenous ags on MHC class I through the so-called cross-presentation, allowing them to induce $CD8^+$ cytotoxic T lymphocytes (CTLs) against viruses and tumor cells. Indeed, once in the LN, mature DCs encounter cognate naïve T cells and initiate adaptive responses (25). In the absence of maturation, as in steady-state conditions, the ag presentation and consequent migration to LN promote peripheral tolerance *via* T cell anergy or regulatory T cell formation (26–28). Depending on the receptors engaged, DCs display different maturation states and produce different inflammatory mediators (often referred to as "signal 3") that impact on the following cellular and humoral responses. The three signals released by DCs drive T helper (Th) cell differentiation. Briefly, DCs educate $CD4^+$ T cells against intracellular bacteria by promoting their polarization into IFN-γ-producing Th type 1 (Th1) cells. Upon infection by multicellular parasites, DCs, with the help of basophils, polarize $CD4^+$ T cells into Th type 2 (Th2) cells that produce mainly IL-4. For specialized mucosal and skin immunity, DCs drive the activation of Th type 17 (Th17) (29). Thus, polarization of T cells is a crucial event that provides mechanisms specifically orchestrated to restore physiological homeostasis. DCs undergo apoptosis once they have fulfilled their functions. The rapid DC turnover after activation is necessary to avoid excessive T cell activation (30) and to maintain self-tolerance (31, 32). T lymphocyte activation culminates with the establishment of the immunological memory, providing the host with T cells more prone and efficient in responding to a reinfection by the same pathogen or upon tumor relapses (33). Besides, DCs are key players in humoral responses too. Indeed, they directly interact with B cells and indirectly support them by activating $CD4^+$ T cells, leading to humoral memory. All these notions strengthen the idea that DCs represent an optimal target for immunotherapies and vaccines, acting at the interface of innate and adaptive immunity.

ADJUVANTS SHAPING DC FUNCTIONS

To harness robust responses through DC-targeting vaccinations, DC maturation is essential. Adjuvants become compulsory complement of inactivated or subunit vaccines that may promote suboptimal responses. Furthermore, they improve DC migration, ag availability, and specific targeting. Although it seems clear that immunization could benefit from adjuvant uses, the solely adjuvant licensed in clinics, until recently, was alum (34). Despite alum has been used in vaccination practice since the beginning of the last century, the mechanism through which it activates innate immunity for the subsequent activation of adaptive immune responses remains elusive. The adjuvant properties of alum were initially attributed to the activation of NLRP3 inflammasome (35, 36); nevertheless, further studies have clearly shown the dispensability of NLRP3 and caspase-1 for the generation of responses in the presence of this adjuvant (37, 38). TLR signaling is also dispensable for alum adjuvanticity (39) as well as mast cells, eosinophils, or macrophages (40). Recently, it has been proposed that upon contact with alum, DCs produce IL-2 through the activation of src and Syk kinases, Ca^{2+} mobilization, and NFAT nuclear translocation. IL-2, in turn, is required for optimal T cell priming, activation, and antibody production (41). In addition to alum, other chemical adjuvants have been tested in preclinical models, showing a clear heterogeneity in the responses driven by different adjuvants, independently of the ag (42). This underlies the need of deepening our knowledge on these powerful tools to drive immune responses. Indeed, MF59, an oil-in-water emulsion adjuvant, that allows long-lasting ag retention in draining LN and enhanced ag uptake by LN-resident DCs, promotes robust humoral responses *via* follicular DC activation (43) and $CD4^+$ T cell immunity induction (44). Conversely, IC31 adjuvant, which consists of an antibacterial peptide and a synthetic oligodeoxynucleotide (ODN), elicits IFN-β release by human DCs *via* engagement of endosomal TLRs supporting immunity against intracellular pathogens and cancer (45).

In the last decades, attention has been focused on TLR ligands as adjuvants. Currently, several compounds are under investigation: Pam2CSK4, Pam3CSK4, or analogs as TLR2/6 or TLR2/1 ligands (46, 47), Poly(I:C) and similar compounds acting on TLR3 (48, 49), TLR4 agonists (50), Flagellin acting on TLR5 (51), Imiquimod and other TLR7 ligands (52, 53), TLR8 agonists (54), CpG ODN binding TLR9 (55, 56). Due to the possible reactogenicity that may be induced by administering TLR agonists, some compounds are chemically modified to reduce toxicity or are delivered specifically to the DC subsets of interest, avoiding TLR ligand dissemination. Monophosphoryl lipid A, a low-toxicity molecule derived from lipopolysaccharide (LPS), displays promising effects for vaccine design (57) even though it promotes terminal differentiation of $CD8^+$ cells, leading to reduced memory protection (58). Another LPS-analog is 7-acyl lipid A that has emerged as potent inducer of IFN-γ-mediated ag-specific responses when co-delivered with poorly immunogenic tumor ags (59).

To improve the effectiveness and strength of immunity, in addition to the efficiency of APC, activation and ag processing and presentation of other aspects should be taken into account. The importance of DC-derived IL-2 in the activation of adaptive responses has been shown not only in alum-driven immune responses and in mouse models of infections (60, 61) but also in tests of human T cell priming in the presence of activatory DCs. During the first few hours after interaction with T cells, activatory monocyte-derived DCs (MoDCs stimulated with the cytokine cocktail, TNF-α, IL-6, IL-1β, and PGE_2) produce IL-2 and CD25 (62). DC-derived IL-2 is, in turn, trans-presented to T cells at the immunological synapse *via* CD25. Since naïve T cells start to express CD25 only many hours after ag encounter, the DC-mediated presentation of the IL-2/CD25 complex is indispensable for an efficient T cell priming (62). It has been proposed that this is the reason why approved therapies based on the use of anti-CD25 antibodies to avoid the acute phases of autoimmune diseases, or acute rejection of kidney, heart, and

hand transplants, are so efficient in interfering with T cell priming or T cell reactivation (62). Since IL-2 is produced in NFAT-dependent manner to improve the adjuvanticity of PRR agonists for vaccination purposes, the capacity of selected PRR agonists to induce NFAT signaling pathway activation and IL-2 production should be considered. Many PRR ligands have been shown to activate the NFAT transcription factor family members in innate immune cells (63). The NFAT pathway is activated in neutrophils, macrophages, and DCs in response to curdlan (64, 65), it is also activated in DCs in response to LPS (30) downstream of CD14, it is activated in response to mannose-capped lipoarabinomannan (Man-LAM), a major lipoglican of *Mycobacterium tuberculosis* (66), and downstream of TLR9 in response to β-glucan bearing fungi (67). The production of IL-2 by innate immune cells during inflammatory responses is relevant not only for an efficient T cell priming but also for the skewing of T cell activation toward type I responses. In mice, DC-derived IL-2 is one of the cytokines required to elicit IFN-γ production from NK cells both in LPS-mediated inflammatory conditions and during fungal infections (68–70). IFN-γ potently activates macrophages and favors Th1 commitment of $CD4^+$ T cells. Therefore, early IFN-γ release by NK cells is not only crucial for controlling a variety of primary bacterial and fungal infections but also for the induction of type I immunity and memory, fundamental for the protection against bacterial, fungal, and viral infections and in antitumor immune therapies.

Another important reason for considering the capacity to activate the NFAT pathway in adjuvant selection tests is represented by the fact that NFATs regulate also the production of the prostanoid PGE_2 by activated DCs (71). PGE_2 promotes activated DC migration (72) and sustains vasodilation and local edema formation during the inflammatory process. This is particularly relevant for vaccination purposes since the increase of the interstitial pressure generated by the edema forces the fluids into the afferent lymphatics and favors a first wave of antigen arrival to the draining LN (71). Intriguingly, LN drainage of proteins or antigens occurs very rapidly after subcutaneous, intradermal, and intramuscular immunization (73–75), thus permitting an extremely fast uptake by phagocytes strategically localized in close proximity to the subcapsular sinus or lymphatic sinus of draining LN (76–79).

Antigen-presenting cells in LN then maintain the homeostasis of LN themselves and activate adaptive immune responses. In the last decades, the long-held paradigm of migratory DCs, resident in peripheral tissues as the skin, as unique APCs involved in T cell immunity has dramatically changed. Indeed, $CD169^+$ subcapsular sinus macrophages, medullary macrophages, and LN-resident DCs are LN sentinels that avoid excessive pathogen dissemination (80, 81) and mediators of immune responses (76).

Concerning migratory DCs and considering the skin, which represents the site of utmost importance for vaccination strategies due to the ease accessibility and the extremely high presence of DCs, skin-resident DCs have been subdivided into epidermal-resident Langerhans cells (LCs), which are $Langerin^+$ and two diverse subsets of dermal (d)DCs: $Langerin^+$ $CD103^+$ and $Langerin^-$ $CD103^-$ (14, 82). Upon infection, dDCs migrate to the LN within 10–24 h while LCs within 48–72 h, supporting long-lasting ag-presentation. Several works reveal the intrinsic differences between the two subsets in inducing Th or CTL responses, due to the particular cross-presenting capabilities of $CD103^+$ dDCs, for instance (19, 20, 83). Once in the LN, whose strategical architecture enhances the probability of encounter between migratory DCs and cognate naïve T cell, adaptive immunity is initiated. Of note, LN-resident DCs are sufficient to promote early adaptive responses independently of migratory DCs when pathogens or antigens directly access the lymphatic conduits (76, 84, 85). In antiviral responses, $CD8\alpha^+$ LN-resident DCs play a crucial role, thanks to their intrinsic capability of cross-presentation to $CD8^+$ CTL (86, 87) that may be supported by pDCs (88). In Herpes Simplex Virus (HSV) skin infection, $CD8\alpha^+$ LN-resident DCs uptake cargo-antigens, ferried by skin-resident migratory DCs in order to elicit CTL (89). Indeed, LCs and dDCs synergize with $CD8\alpha^+$ LN-resident DCs, which stand out as the most potent CTL inducers, preferentially sustaining $CD4^+$ Th responses both in influenza (90) and HSV cutaneous infections (91). In addition to $CD8\alpha^+$ LN-resident DCs, $CD103^+$ dDCs display intrinsic capability of cross-presentation, as their human counterpart, $CLEC9A^+$ $CD141^+$ DCs (92–96). Besides, some authors demonstrated that blocking DC migration from the skin hinders $CD4^+$ T cell activation in response to subcutaneous bacterial (97) or soluble antigen challenge (98). Ablating $Langerin^+$ dDCs reduced T cell immunity strength, corroborating the notion that migratory DC complement LN-resident DC effects on adaptive responses (99–101). Nonetheless, the roles of LCs in activating T cells are still uncertain, probably due to the controversial functional properties of this innate subset (102–104). Despite this, the synergic effects of LN resident and migratory DCs seem to be undoubted (25, 105). Indeed, Allenspach and colleagues reported that ag presentation by LN-resident DCs few hours after the infection is required to entrap ag-specific T cells in the draining LN and to favor an optimal activation of T cells by migratory DCs that arrive at the LN many hours later (106).

It emerges, therefore, that another aspect to be considered for the identification of efficacious adjuvants concerns the type of DC subset to be targeted and the consequential effects that adjuvants imprint on that subset. Adjuvants play a pivotal role in determining tissue-resident DC mobility to draining LN and efficiency of T cell polarization. Indeed, dDCs acquire mobility after subcutaneous injection of Th1-specific adjuvants as CpG and LPS, but not with Th2-specific ones, as papain, or following contact sensitization with dibutyl phthalate and acetone. Moreover, dDCs are sufficient to promote Th1 and Th2 responses, while LCs are only supportive of Th1 (107). This evidence underscore that, in addition to the polarizing capabilities of adjuvants, also the targeted DC subset must be considered to elicit specific adaptive immunity. Indeed, Antonialli and colleagues reported differential immune responses when $CD8\alpha^+$ and $CD8\alpha^-$ DCs were targeted with the same ag and adjuvant, either CpG ODN or Flagellin (108).

In addition, to enhance the efficacy of vaccination, the coincident delivery of ag and PRR adjuvants to APCs plays a crucial role. Encouraging evidence highlights the importance of conjugation of ag with PRR adjuvants, since it improves ag uptake, humoral

and cellular responses when compared to vaccination with ag co-delivered with free TLR ligands (109). These findings strengthen the notion that adjuvants are formidable chiefs in shaping immune responses and must be selected for the outcomes they promote, in chemical association to the ag of interest.

NOVEL STRATEGIES OF VACCINATION: MULTITASKING CARRIERS

The traditional vaccination approaches consisted in the administration of live or attenuated micro-organisms. Up to now, several innovative strategies have emerged to address the need for efficient vaccines, especially against diseases that are critical to treat, as cancer and the infectious diseases already mentioned. The main purpose is to convey ag, adjuvant, and targeting-molecule in a unique compound to increase the efficacy of the ag-specific immune response. To address this issue, different approaches have been explored or are currently under investigation, as shown in **Figure 2**.

Recombinant antibody (rAb) represents a feasible option. This approach exploits the possibility to chemically fuse peptide ag, adjuvant, and targeting-molecule to Ab to tailor DCs targeting (110–112). In addition to rAb, single-chain fragments variable (scFv) revealed to be an appealing strategy due to their reduced size and enhanced infiltration into tissues, as in solid tumors (113).

Other approaches involve the use of nano-carries as vehicles. The most promising solution to target phagocytes is indeed the use of particulate materials (114, 115). Nanoparticles (NPs) are the best candidates as delivery system, since they can be manipulated to efficiently and predominantly target phagocytes. This is possible, thanks to the versatility of NPs due to: (i) the large amounts of existing different nanomaterials; (ii) the possibility to adjust their size, morphology, and deformability with great precision; (iii) the possibility to load virtually any different type of drug molecules (116).

Viral vectors-based vaccines or virus-like particles rely on the intrinsic capability of viruses to infect cells and exploit their protein-encoding machinery, allowing expression in the cytosol of the engineered plasmid-genes, as ag, costimulatory molecules, cytokines, and adjuvants, providing the bases for strong CTL induction (117). On the other hand, naked DNA can be directly injected or conjugated to nano-carriers to favor specific targeting. The easy designing of nano-carriers-based vaccines along with their multi-component loading feature improve targeting of specific subsets (118) and shape immune responses (119, 120), favoring their application in several fields. In a cancer setting, nano-carriers allow to avoid killing of healthy cells, by delivering tumor ags or DNA encoding these peptides to APCs, inducing specific antitumor responses. Indeed, NPs allow endocytosis and MHC presentation on both class I and II (121) eliciting broad adaptive immunity, even against cancer cells. Rosalia and colleagues designed a polymer-based biodegradable poly(lactic-co-glycolic acid) PLGA NPs loaded with ag, Pam3CSK4, and Poly(I:C) and coated with an agonistic αCD40-monoclonal Ab (NP-CD40). This multi-functional strategy resulted in efficient and selective delivery of NPs to DCs *in vivo* upon s.c. injection and induced priming of $CD8^+$ T cells against tumor associated ags, increasing tumor-bearing mice survival (109). PLGA NPs carrying the poorly immunogenic melanoma-derived antigen tyrosinase-related protein 2 along with 7-acyl lipid A, manage in breaking the immunotolerance acting against tumor-antigens. Indeed, administration of the abovementioned NPs resulted in antigen-specific $CD8^+$ CTL responses, characterized by IFN-γ production and increase of pro-inflammatory cytokines in the tumor microenvironment (TME) (59). Another nano-carrier-based approach relies on liposome, self-assembled vesicles composed by lipid bilayers with high functionalizing properties. Besides, Maji and colleagues reported that after uptake by DCs, cationic liposomes localize in endosomal compartments that allow ag presentation preferentially on MHC I but do not exclude MHC II ag

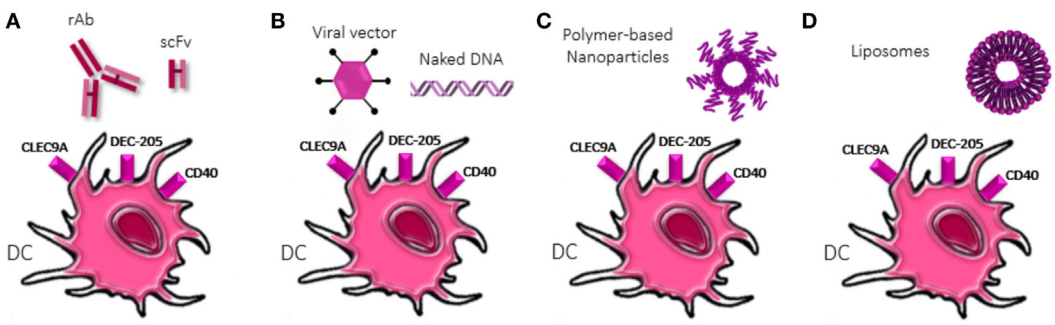

FIGURE 2 | Strategies of dendritic cells (DCs) targeting. Diverse approaches to deliver antigens to DCs are shown. **(A)** Recombinant antibody or single-chain variable fragment (scFv) specific for DC receptors are chemically conjugated with antigen and adjuvant molecules. scFv reduced dimension confers them higher tissue-penetrating properties. **(B)** Viral vector-based vaccines or naked DNA exploit the encoding machinery of DCs to translate antigens, adjuvants but also co-stimulatory molecules ("signal 2") and cytokines ("signal 3") increasing the activatory profile of DCs. Naked DNA could be delivered conjugated to nanoparticles (NPs) and liposomes. **(C)** Polymer-based NPs display physical and chemical properties that allow encapsulation or conjugation of antigens and adjuvants as well as ligands for specific DC receptors. Different polymer compositions provide diverse properties and dimension, allowing easy diffusion and/or retention in lymph node. **(D)** Liposomes allow both the encapsulation and intercalation in the phospholipid bilayer of antigens and adjuvants, depending on their chemical properties, as well as the functionalization of the surface with ligands of DC receptors.

presentation (122), suggesting a crucial role in antitumor or antiviral immunity supported by Th responses.

In addition to the use of NPs, targeting DC-specific receptors has become an attractive strategy for vaccine development due to the enforced efficiency of immune responses when compared to generic-delivering approaches. Here, we report the more characterized DCs receptors, currently under investigation in the scenario of tailored-vaccination, as shown in **Table 1**.

CLEC9A or DNGR1 is a C-type lectin receptor that mediates endocytosis, but not phagocytosis, with low pH endosomes promoting the drift toward cross-presentation. Importantly, CLEC9A binding of antigens induces antigen presentation on both MHC I (cross-presentation) and MHC II. It is highly and specifically expressed on $CD11c^+CD141^+XCR1^+$ cDCs and $CD14^+CD16^-$ monocytes in human and in murine pDCs and $XCR1^+$ $CD8a^+$ LN resident but not $CD103^+XCR1^+$ migrating DCs (123, 124). Indeed, $CD141^+XCR1^+$ DCs constitute the human counterpart of $CD8\alpha^+$ $XCR1^+$ murine DCs (125). They share XCR1, the receptor of XCL1. XCL1 is released by activated T cells and the axis XCR1–XCL1 is necessary for robust CTL responses (126). $CD141^+XCR1^+$ DCs are the main cross-presenting DCs in human, thus they appear promising for CTL-mediated responses, in tumors and viral infections (127). This specific subset is characterized by the expression of TLR3 that may be exploited to fully activate $CLEC9A^+XCR1^+$ DCs since antibody binding of CLEC9A leads to its rapid internalization but not TLR-pathway activation, preventing pro-inflammatory cytokine production and full maturation of DCs (127). Conversely, Caminschi and Li independently demonstrated the potentiality of targeting Clec9A that resulted in enhanced humoral immunity independently of TRIF-MyD88 or TLR4 pathway, even in the absence of adjuvants (128, 129). Targeting Clec9A induces enhanced $CD4^+$ T cell proliferation *in vivo*, which supports B cell immunity, when compared to the targeting of another endocytic receptor, discussed later, DEC-205, independently of the use of adjuvants as CpG (130). Some years later, different authors demonstrated that this strong humoral response is endorsed by the establishment of follicular T helper cells memory, even upon vaccination with glycoprotein D of HSV, both in mice and non-human primates (128, 131, 132). These promising results were confirmed also in a human *in vitro* setting, on $CD141^+$ DCs (133). Finally, the efficacy of targeting Clec9A has been evaluated in the delivery of poorly immunogenic virus-derived antigens. Park and colleagues managed in conferring specific humoral response, protective upon reinfection (134). Thus, exploiting the specific expression of this receptor on the most specialized DCs in cross-presentation in combination with TLR3 ligands, will enhance antiviral and anticancer responses (135), combined with robust humoral immunity.

DEC-205 or CD205 is a 205 kDa endocytic receptor that has a cysteine-rich domain, a fibronectin type II domain, and 10 C-type lectin-like domains, as well as an internalization sequence in its cytoplasmic tail (136). Thus, it mediates cross-presentation through clathrin- and dynamin-dependent receptor-mediated endocytosis. Indeed, it is expressed by the most professional cross-presenting DCs, the $CD8\alpha^+$ DCs subtype, while $CD8\alpha^-$ DCs display very low level of this receptor. In addition, DEC-205 is found on dermal/interstitial DCs and LCs (137), thus guaranteeing ag delivery to both skin-resident and LN-resident professional APCs. In humans, DEC-205 is shared among cDCs, monocytes, and B cells, while pDCs, granulocytes, NK cells, and T lymphocytes express low levels of this receptor (138). In addition, DEC-205 regulates molecule recycling through late endosomes, promoting also MHC II presentation to $CD4^+$ T cells in LCs (139). Steinman and Nussenzweig have addressed this molecule to improve vaccine efficacy since 2000 (140). By taking advantage of anti-DEC-205 rAb conjugated to OVA peptide, they demonstrated that s.c. injections of this compound lead to a strong IFN-γ and IL-2-mediated immunity only when DCs activation was supported by αCD40 mAb, otherwise, tolerance against the OVA peptide occurs (141). Indeed, diversely from PRR agonists, antibody crosslinking the DEC-205 does not induce DCs maturation (142). Furthermore, few years later, the combined strategy of anti-DEC-205 and αCD40 was reported to confer protection against melanoma and intranasal influenza infection (112).

TABLE 1 | Targeted receptors for tailored ags delivery.

	Receptor	Expression	Activity	Clinical trials
CLEC9A	C-type lectin receptor	Human: $CD11c^+$ $CD141^+$ $XCR1^+$ conventional DCs (cDCs) $CD14^+$ $CD16^-$ monocytes MOUSE: plasmacytoid DCs (pDCs) $XCR1^+$ $CD8\alpha^+$ lymph node-resident dendritic cells (DCs)	Major histocompatibility complex (MHC) class I MHC class II Ag presentation	–
DEC-205	Endocytic receptor	Human: cDCs, monocytes, B cells MOUSE: $CD8\alpha^+$ DCs Dermal/interstitial DCs Langerhans cells	MHC class I MHC class II Ag presentation	NCT03358719: recruiting NCT01834248: completed NCT02166905: recruiting NCT01522820: completed
CD40	Transmembrane glycoprotein Surface receptor	Human/mouse: cDCs and pDCs, monocytes, B cells, endothelial cells	DCs activation	NCT03329950: recruiting[a] NCT02706353: recruiting NCT03214250: recruiting NCT03389802: recruiting NCT03418480: recruiting NCT03123783: recruiting

[a]*There are currently more than 30 clinical trials involving the anti-CD40 antibody. Here, the more recent trials regarding DCs-based vaccination strategies are reported.*

In a viral setting, anti-DEC-205 rAb chemically coupled with HIV p24 gag protein tested *in vitro* on blood cells derived by 11 HIV-infected donors has revealed efficient expansion of IFN-γ-producing $CD8^+$ T lymphocytes (143) from all the different donors. This indicated that DCs and CD205 can lead to the generation of different peptides from a single protein. Moreover, vaccines based on the filamentous bacteriophage fd presenting an αDEC-205 scFv, efficiently induce DCs maturation *via* the activation of the TLR9-MyD88 pathway (144), without adjuvants and further elicit potent antitumor responses when compared to non-tailored ag delivery (145). Intriguingly, DEC-205, orphan of a specific ligand, has been proven to be necessary for CpG uptake and eventual DC activation (146).

CD40 is a molecule belonging to the TNF receptor family, expressed by several cell types and among these, DCs. It has emerged as a receptor for the human chaperone Heat shock protein (Hsp) 70 that mediates the internalization of peptides bound to Hsp70 itself (147). Moreover, upon activation, T cell transiently expresses CD40L allowing cross-linking of CD40 on DCs and completing their maturation. From these notions, CD40 appeared an interesting molecule to target for DC-based vaccination strategies. Indeed, by engineering antibody chemical structure, Schjetne and colleagues demonstrated the efficacy of CD40 engagement conferring protection against myeloma- and lymphoma-derived ags (148). Moreover, through the co-administration of two DNA-based vaccines encoding either CD40 and the foot-and-mouth disease-derived ags, the transient increase of endogenous αCD40 antibodies allows an efficacious DCs activation and an efficient development of ag-specific T cell immunity, if compared to the administration of DNA encoding ags alone (149). Further promising results have been obtained in a vaccine against cyclin-D1 that is overexpressed by mantle cell lymphoma (MCL). Thanks to algorithm analysis, Chen and colleagues identified three cyclin-D1-derived peptides that efficiently bind to MHC class I of DCs, potentially overexpressed in all MCL patients. By generating a rAb targeting CD40, they efficiently delivered these tumor associated ags to DCs and mounted IFN-γ-specific T cell responses in patients-derived peripheral blood mononuclear cells (150). Thus, CD40 represents a specific DC-targeting molecule that has been used in combination with other targeting approaches to support specific DCs activation, avoid tolerance, and induce robust T cell immunity (110, 141).

DCs AND CANCER

When evaluating vaccination strategies for cancer patients, it is compulsory to take into account one of the hallmarks of cancer: avoiding immune destruction by promoting tolerance and disarming the immune system (5). The orchestration of antitumor responses involves multiple protagonists and mediators, among these, cytotoxic T cells and NK cells, whose activation is supported by DCs (151). Furthermore, DCs-based vaccines has emerged as more efficient in promoting T cell immunity if compared to peptide-based vaccination approaches (152). Thus, much effort has been made to improve strategies of DCs-based vaccination in neoplastic diseases, to ameliorate the prognosis or eradicate both primary tumor and metastases. Up to now, two different approaches have been addressed: *ex vivo* generation of autologous pulsed DCs and direct *in vivo* targeting of DCs, as previously discussed. The former strategy provides a better control of the maturation and activation state of DCs and a specific load of the ag to the selected DCs subset. Despite this, intense work is needed to generate this vaccine, since it is personalized for each patient and only few subsets of DCs are feasibly generated *in vitro* or collected *ex vivo*, limiting the access of ags to other more functionally driven subsets. Diversely, the *in vivo* targeting methods allow the generation of large amount of vaccine in a one-step procedure, and the targeting of diverse DCs subsets in their natural environment.

Once the DCs-based vaccine is generated, the efficacy of antitumoral responses has to be evaluated. It is mainly related to (i) the capability to establish specific antitumor-associated ag (TAA) immunity and (ii) the overcome of the tolerogenic status promoted by the TME.

To select highly immunogenic ags, multiple solutions have been tested: whole tumor lysate or killed tumor cells, synthetic long peptides (SLPs), full length proteins, transfection or electroporation with DNA or mRNA coding for TAA, transduction with lentiviral vectors and neoantigens. The availability of an elevated number of antigens through the incubation of DCs with whole tumor lysates or autologous tumor cells allows the presentation of multiple epitopes, loaded on both MHC class I or II, which leads to Th and cytotoxic responses. Indeed, several clinical trials are currently evaluating the benefits obtained by using this approach (NCT01875653; NCT00045968; NCT02496520). SLPs are 28–35 aa long peptides cross-presented by DCs (153), currently under investigation in both preclinical and clinical setting. Compared to short synthetic peptides, the use of SLPs lacks the necessity to know the patients' HLA haplotype, thus permitting their full exploitation in a larger cohort of people. Moreover, SLPs administration to DCs leads to an enhanced $CD8^+$ T cells activation since, once engulfed, they rapidly escape from the endolysosome to follow the path of MHC class I presentation, fundamental in antitumor responses. Indeed, SLPs and DCs-based vaccines are showing promising results in terms of safety and immunogenicity, in both preclinical and clinical settings (154). They have gained attention in the context of human papilloma virus cervical (155), ovarian (156), and colorectal cancer (157, 158), displaying immunogenic capacities, in terms of antibody production and $CD4^+$ and $CD8^+$ T cell activation, when delivered with adjuvants, as poly ICLC, Montanide-ISA-51 (NCT02334735), and IFNα. When comparing SLPs and full length proteins, it has emerged that DCs process SLPs better that full length protein, due to the slower processing route the latter display (154). Concerning transfection or electroporation of DCs with mRNA or DNA encoding, not only for TAA but also for costimulatory molecules and cytokines, to enforce adaptive immunity, has proven to be efficacious in inducing antitumor $CD4^+$ and $CD8^+$ T cells expansion, mediated by DCs targeting (159). A similar approach regards *in vivo* lentiviral transduction of DCs, which displays versatility for gene delivery and efficient transduction for non-dividing cells, as DCs. Indeed, Bryson et al. conceived a multifunctional vaccine

composed by a modified lentivirus, whose glycoproteins can directly target DC-SIGN on DCs, loaded with breast cancer ags, alpha lactalbumin, and erb-b2 receptor tyrosine kinase 2. Single injections of the compound provided tumor self-ags-specific $CD8^+$ T cell immunity, reducing tumor growth (160). Despite the improvements derived by these advanced strategies, in the last years, neoantigens are becoming more and more appealing (161). During tumor progression, cancer cells give rise to neoantigens, novel ags different from the self-tumor ags, derived by the tumor-specific mutations. Therefore, prediction tools, RNA mutanome, and deep-sequencing have allowed the identification of specific non-self-ags that are fundamental in strong T cell immunity (162–164). Indeed, several clinical trials are currently investigating the potential of neoantigens (NCT0235956; NCT01970358; NCT02149225; NCT02348320; NCT02316457). As emerged, different strategies of ags selection have been explored and, even though one strategy may result in a more enforced antitumor immunity if compared to another, still the issue of the TME negative influence on the immune system has to be faced. Indeed, the TME actively suppresses the activation of the immune system. Tumor cells secrete immunosuppressive cytokines, as vascular endothelial growth factor (165, 166), macrophage colony-stimulating factor (167), transforming growth factor β (TGF-β) (168), and IL-10 (169, 170). Even though some of these cytokine display controversial roles, depending on the pathological context, they generally promote DCs tolerogenicity, by limiting their activation and increasing their expression of pro-tumor molecules, such as programmed cell death 1 (PD-1) and indoleamine 2,3-dioxygenase (IDO). Therefore, tolerogenic DCs lead to T cells anergy, Tregs expansion, and Th1 responses inhibition. Phenotypical characterization of immune cells isolated from breast cancer patients, highlighted the functional alteration in DCs, T, and NK cells in promoting antitumor responses (171). Furthermore, tumor cells retain DCs into the TME, preventing their migration to draining LNs and promoting metastatization (172). To address this issue, some *ex vivo* generated DCs-based vaccines are directly administered intranodally, as for the $CD1c^+$ DCs pulsed with HLA-A2.1-restricted tumor peptides administered to patients with stage IV melanoma (NCT01690377), which generated tumor-specific $CD8^+$ T cells responses and further improvement of survival (173). To reduce the tolerogenic influence of the TME on DCs, the positive role of GM-CSF in improving DCs survival and responsiveness is currently exploited in some clinical trials like a phase I/II trial with a DC/tumor cell fusion vaccine administered in association with GM-CSF to treat renal cancer (NCT00458536). Similarly, others are focusing their attention on FMS-like tyrosine kinase 3-ligand (FLT3L), another crucial DCs growth factor, in combination with other compounds (NCT01811992; NCT01976585; NCT02129075; NCT02839265). FLT3L has, indeed, been shown to increase the efficacy of proteins- and RNA-based vaccines, due to a maturation effect on DCs (174–176). Additional efforts made to counteract the tolerogenic influence of the TME include the use of PD-1 and IDO inhibitors. Co-administration of anti-PD-1 molecules increases the efficacy of DCs-based vaccines, in terms of enforced intratumoral $CD8^+$ T cell responses and trafficking of $CD8^+$ memory T cells, as observed in a preclinical model of glioblastoma (177). In parallel, several clinical trials are aiming at evaluating the efficacy of DCs-based vaccines combined with anti-PD-1 agents (NCT03014804; NCT03325101; NCT03035331). The other tolerogenic marker addressed in cancer immunotherapy and DCs-based vaccine is IDO. Indeed, silencing approaches to reduce the expression of IDO in DCs for vaccination in preclinical models, have resulted in decreased T cell apoptosis, reduced numbers of Tregs, decreased tumor size when compared to mice that had received ags-loaded DCs without IDO silencing (178). IDO inhibitors in DCs vaccination are currently being tested in phase II clinical trials (NCT01560923; NCT01042535).

All these approaches have explored different scenarios to evaluate the more efficient therapeutic combination that seems to move toward personalized vaccinations for cancer patients.

CONCLUDING REMARKS

In this review, we have underscored the crucial role of DCs in orchestrating immune responses and; therefore, the great interest in targeting these cells in novel vaccination strategies. We have reported examples of different approaches aimed at amplifying the efficiency of immunizations against cancer or infectious diseases. Indeed, the urgent need of vaccines is as relevant as before because of newly emerging diseases with ineffective current therapies. Deepen the mechanisms underlying these pathologies may provide cues on the more appropriate design of vaccines and by merging diverse tailoring strategies we could enforce the immune system. As a matter of fact, it is suggested to act on different fronts when designing new vaccines, since several factors must be considered: (i) targeting DC subsets specialized in initiating the desired cellular or humoral immunity/memory; (ii) adjuvants that strengthen and drive T and B cell responses; (iii) fine and optimized selection of the immunogenic ags to drive enforced responses; (iv) novel strategies to convey ags and adjuvants to DCs; (v) route of administration. Starting from these notions, in the last decades, enormous efforts have been made to tailor vaccination strategies. New technologies as well as recent advances have allowed extreme flexibility in designing vaccines and shaping the following outcomes. Nowadays, researchers do have smart tools to manipulate immune responses with prophylactic or therapeutic vaccinations. The abovementioned findings pave the way for possible therapeutic approaches, theoretically applicable to all pathological contexts. Despite this encouraging evidence, several limitations or issues still have to be overcome. Indeed, more than a few vaccines do not pass phases I of clinical trials either for toxicity issues and lack of immunogenicity in some individuals. What is missing? Part of the answer to this question could sit on human genetics and population variability. Syngeneic animal models are ideal settings in which the systems are pushed although they constitute a necessary and useful step preceding clinical trials.

Moreover, when translating vaccine testing from *in vivo* experiments on animals to *ex vivo* on human cells, often the opted choice are blood human cells, while in most of the cases vaccines will be administered in the skin, having a complete

different DCs-based milieu (15). Crucially, Idoyaga and colleagues dissected the interindividual variability in skin-resident DCs, stressing the need of shedding light on the effects that genetics and environment imprint on DCs. It is compulsory to decode the complex scenario of human diversity to provide personalized therapies with increased efficacy. In the Omics era, systems biology and computational modeling integrate huge data-sets to address the urgent need of information on the global behavior. Indeed, Genome-wide association studies have provided insights into human genetics variants associated to the immunogenicity of vaccines (179, 180). Therefore, integration of "wet" evidence and "dry" notions may fasten the designing process and provide both efficient vaccine strategies and their predictive efficacy.

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

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REVIEW
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Dendritic Cells and Programmed Death-1 Blockade: A Joint Venture to Combat Cancer

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Two decades of clinical cancer research with dendritic cell (DC)-based vaccination have proved that this type of personalized medicine is safe and has the capacity to improve survival, but monotherapy is unlikely to cure the cancer. Designed to empower the patient's antitumor immunity, huge research efforts are set to improve the efficacy of next-generation DC vaccines and to find synergistic combinations with existing cancer therapies. Immune checkpoint approaches, aiming to breach immune suppression and evasion to reinforce antitumor immunity, have been a revelation in the immunotherapy field. Early success of therapeutic antibodies blocking the programmed death-1 (PD-1) pathway has sparked the development of novel inhibitors and combination therapies. Hence, merging immunoregulatory tumor-specific DC strategies with PD-1-targeted approaches is a promising path to explore. In this review, we focus on the role of PD-1-signaling in DC-mediated antitumor immunity. In the quest of exploiting the full potential of DC therapy, different strategies to leverage DC immunopotency by impeding PD-1-mediated immune regulation are discussed, including the most advanced research on targeted therapeutic antibodies, lessons learned from chemotherapy-induced immune activation, and more recent developments with soluble molecules and gene-silencing techniques. An overview of DC/PD-1 immunotherapy combinations that are currently under preclinical and clinical investigation substantiates the clinical potential of such combination strategies.

Keywords: dendritic cell, programmed death-1, cancer immunotherapy, combination therapy, programmed death ligand 1/2

INTRODUCTION

Dendritic cells (DCs) are key antigen-presenting cells capable of presenting tumor antigens to T lymphocytes (1) and promoting innate immunity *via*, e.g., natural killer (NK) cells (2) and γδ T cells (3). To obtain and engineer DCs for therapeutic approaches, they can be generated *ex vivo* from multiple sources such as monocytes [monocyte-derived DCs (moDCs)] and $CD34^+$ hematopoietic progenitor cells, or they can be enriched from peripheral and cord blood (4–7). Exploiting their

antigen-specific and immunoregulatory qualities, DCs can be furnished with tumor antigens and other targeted molecules *via* different techniques (7–9). More than two decades after the first implementation of DCs as an immunotherapy to treat cancer (10), it can be ascertained that DC-based vaccination is safe, well tolerated, and capable of inducing antitumoral immune responses. Objective clinical responses, however, are amenable to substantial improvement (11). To date, scientists believe that the full potential of DC-based immunotherapy has not yet been reached (11–13). This is evidenced by the profound and multidimensional exploration of ways to invigorate the immunotherapeutic potential of DCs, both at the level of DC vaccine engineering and combining DC therapy with other synergistic antitumor (immuno)therapies (14–20). Core objectives of this common quest are to improve DC immunopotency to promote cytotoxic and long-lasting antitumor immunity and to overcome the tumor-mediated immunosuppressive environment (9, 20). In relation to this, interfering with immune checkpoint inhibitory pathways has been on the rise. Since its second-place ranking as a potential target for immunotherapy at the Immunotherapy Agent Workshop of the National Cancer Institute in 2007 research on the inhibitory checkpoint programmed death-1 (PD-1)/programmed death ligand (PD-L) pathway has boosted massively. Due to superior antitumor effects of anti-PD-1- and anti-PD-L1-blocking antibodies, these molecules even climbed to the first position as potential targets for immunotherapy at the 29th Annual meeting of the Society for Immunotherapy of Cancer in 2015 (21). Next to exploiting the systemic monoclonal antibody (mAB) strategy, other promising PD-1-/PD-L-targeted approaches are under development. As acknowledged for DC-based vaccination, combination strategies of PD-1-targeted inhibitors with other immune (checkpoint) modulators, cell vaccines, or standard-of-care therapies will likely hold the future (22). In this review, we discuss the role of the PD-1/PD-L pathway in DC-mediated antitumor immunity and the progress of emerging strategies combining DC-based therapy with PD-1/PD-L pathway interference.

PD-1/PD-L IN HEALTH AND DISEASE

The PD-1/PD-L axis is one of the most studied pathways to gain understanding of immunoregulatory signals delivered by immune checkpoint receptor/ligand interaction the past few years (23, 24). Originally discovered as a mechanism of the organism to protect itself against T cell reactions toward self-antigens, interaction of PD-1 with one of its ligands (PD-L1 or PD-L2) can induce peripheral tolerance by limiting T cell activity, contributing to protection against tissue damage in case of an inflammatory response (25), prevention of autoimmune diabetes (26), or promotion of the fetal–maternal tolerance (27). Infected and malignant cells that evade immune surveillance have been ascribed to employ the inhibitory PD-1/PD-L pathway (24). Indispensable in healthy immune responses (28, 29), overexpression or induction of PD-1 and its ligands PD-L1 and PD-L2 on both immune and target cells, has been associated with immune deficiency, such as exhausted T cells, dysfunctional NK cells, expanded functional regulatory T (Treg) cells, and immune evasion and suppression (30, 31). PD-L expression can also be indispensable for the establishment of T cell immunity in other settings (28, 29). This ambiguity could be explained by findings that PD-L2 also possesses a costimulatory role (32, 33), possibly through interaction with repulsive guidance molecule b (34). Arising from either intrinsic or adaptive immune resistance (35), PD-1 and PD-L1 surface expression or secretion in different malignancies has been mostly related to poor prognosis (36–42), although discordant data have been reported (43, 44), reflecting the need to improve our understanding of the host immune system and disease-specific microenvironment.

Inhibitory PD-1/PD-L signaling not only occurs between immune cells interacting with malignant cells, but is also effective between different immune cell types shaping the tumor immune environment. This provides a strong impetus to target this inhibitory axis to breach immune inhibition and promote durable immunity. In various solid and hematological tumors, blockade of the PD-1/PD-L1 pathway has proven to reverse this immune inhibition by restoring both antitumor function and number of tumor-infiltrating $CD8^+$ effector T cells, resulting in reduced tumor size and increased overall survival (45–49). While PD-1-/PD-L-targeted research predominantly focuses on effector T cells, interest in other cell types is growing. A study in metastatic melanoma patients showed that, in addition to $CD8^+$ T cells, tumor-infiltrating B cells and myeloid-derived suppressor cells (MDSCs) were increased by PD-1 therapy (50). With regard to innate immunity, it has been evidenced that also NK cells are negatively regulated by PD-1 signaling during chronic infections (*Mycobacterium tuberculosis* and HIV-1) (51, 52) and in cancer (multiple myeloma, glioblastoma multiforme, ovarian carcinoma, digestive cancers) (53–59), directly relating to NK cell cytotoxic and regulatory dysfunction, immune suppression, and poor prognosis. As for T cells, blockade of this inhibitory pathway by means of mABs could restore dampened NK cell functions, at the level of both interferon (IFN)-γ response (52) and cytotoxic capacity (57). In addition, antitumor immunity mediated by invariant NK T (iNKT) cells was also shown to be improved by blockade of the PD-1/PD-L pathway (60, 61). Expression of PD-1 is also demonstrated on γδ T cells (62) and resulted in γδ T cell exhaustion that could be overcome by administration of a blocking anti-PD-L1 antibody (63, 64). A subset of γδ T cells also expresses PD-L1 conferring them with tumor-promoting characteristics by inhibiting αβ T cells (65). Therefore, PD-L1-blocking antibodies could also restore antitumor immunity by inhibiting PD-1/PD-L1 interactions between γδ and αβ T cells. With regard to immunoregulatory cells, PD-1 is also highly expressed on Treg cells (66). As shown by Sauer et al. (67) and Francisco et al. (68), interaction between PD-1 and its ligands blocks the Akt/mTOR pathway leading to an increased FoxP3 expression, resulting in Treg cell differentiation and maintenance. Furthermore, blocking the PD-1 pathway combined with antitumor vaccination showed a significant decrease in the number of intratumoral Treg cells and reduced tumor growth (69). In addition to Treg cells, a role for the PD-1/PD-L pathway has been put forward for other regulatory cells including tumor-associated macrophages (TAMs), MDSCs, and mucosal-associated invariant T (MAIT) cells (61, 70–75). While research into the effect of PD-1/PD-L blockade in these cells is limited, preclinical anti-PD-1 therapy has been shown to

reduce the number of immune suppressive TAMs and MDSCs (73) and was able to increase the IFN-γ production by MAIT cells (71), indicating the valuable effect of PD-1/PD-L blockade on immune cells beyond the immune-activating $CD8^+$ CTLs.

THE ROLE OF PD-1/PD-L IN DC-MEDIATED ANTITUMOR IMMUNITY

As orchestrators of the immune system bridging innate and adaptive immunity, DCs are key players in directing antitumor immunity. Capable of expressing both the PD-1 receptor and its ligands, DCs can virtually interact with any PD-1 and PD-L-positive cell (**Figure 1**). In this context, the most acknowledged interaction is between DCs and T cells. PD-L surface expression on DCs [myeloid DC (mDC), plasmacytoid DC (pDC), and *in vitro* generated vaccine DC] is highest upon maturation with pro-inflammatory cytokines, Toll-like receptor (TLR) ligands, or (parts of) bacterial strains, often used to enhance the expression of costimulatory molecules on DCs (76–78). This PD-L surface expression has been demonstrated to suppress $CD4^+$ and $CD8^+$ T cell activity in various disease models, such as tuberculosis (79–81), HIV (82), and cancer (76, 83–88). Comparably, PD-1 expression on tumor-infiltrating mDCs has also been shown to suppress $CD8^+$ T cell activity and decrease T cell infiltration in mouse models for advanced ovarian cancer (89) and hepatocellular carcinoma (90). In addition to suppression of immune activation, DC PD-L expression was also shown to be involved in the promotion of $CD4^+CD25^+FoxP3^+$ Treg cell expansion and function (68). Tumor growth factor-beta in the tumor microenvironment promotes PD-L1 expression on DCs, further maintaining Treg cell populations (87, 91) and *de novo* generation of Treg cells (92) in favor of the immunosuppressive tumor microenvironment (84).

The role of PD-1/PD-L signaling in the crosstalk between DCs and NK cells remains largely unexplored. It has been shown that disruption of the PD-1/PD-L pathway is able to restore NK cell functions, mostly, but not exclusively in multiple myeloma (53, 55, 57, 93). Only few studies suggest a role of this pathway in DC-NK cell crosstalk and controversy remains. Ray et al. (57) demonstrated that NK cell function was abrogated by PD-L1 interactions on pDCs and PD-1 on NK cells and that NK cell functions could be restored by anti-PD-L1 treatment. On the other hand, in a preclinical mouse model, the expression of PD-L on NK cells was demonstrated to negatively regulate DC activity *via* interaction with PD-1 on DCs (94). To gain more conclusive insights in the contribution of PD-1/PD-L interactions in the crosstalk between DCs and NK cells, more research is warranted. Similar to DC-NK cell crosstalk, little is known about the role of PD-1 signaling in DC-γδ T cell crosstalk (3, 95) and how PD-1/PD-L blockade in combination with DC-based immunotherapies can further empower γδ T cells with antitumor capacities. Other innate immune cells that are able to crosstalk with DCs include iNKT cells, MAIT cells, and MDSCs (96–100). Blockade of PD-1/PD-L interactions between DCs and iNKT cells were shown to increase activation and release of T helper 1 cytokines by the latter resulting in the activation of NK cells and amplified antitumor responses (60, 101). Research on PD-1/PD-L interactions between DCs and MAIT cells or MDSCs is lacking.

Ligation of PD-1 to PD-L1/2 can also exert intrinsic effects on DCs by reverse signaling. Kuipers et al. (102) reported decreased expression of maturation markers in $PD\text{-}L^+$ DCs and increased interleukin (IL)-10 production upon treatment with soluble PD-1 (sPD-1), suggesting that through reciprocal signaling a suppressive DC phenotype is attained. In another study, upregulation of PD-1 on DCs was found to be a consequence of DC maturation, especially after TLR-mediated DC activation. Blocking PD-1 during DC maturation resulted in enhanced DC survival and increased immunostimulatory properties (103). In both studies, interference with the PD-1/PD-L pathway increased the immunostimulatory properties of the DCs toward T cell activation.

The interplay of PD-1 and PD-L in DC crosstalk with (virtually all) activating and regulatory adaptive and innate immune cells impacts the productivity of antitumor immunity (**Figure 1**). Other than monitoring PD-L expression on tumor cells, it has been suggested that monitoring PD-L expression on infiltrating myeloid cells is more predictive for response to blockade of PD-1 signaling (104). Building on the successes of DC-based therapy (11) and PD-1-blocking strategies (105), the exploration of its combinatorial therapeutic use is rationalized to empower the clinical response rates and efficacy of these targeted approaches (7, 16).

STRATEGIES TO LEVERAGE DC IMMUNOPOTENCY BY INTERCEDING PD-1/PD-L SIGNALING

It is generally agreed that the therapeutic potential of DC-based immunotherapy could be improved by tackling the immunosuppressive tumor microenvironment that contributes to ineffective or suboptimal responses (106, 107). Employing intrinsic and adaptive immune resistance mechanisms, PD-1 is a top-ranked checkpoint contributor to blunting immune responses. In a comprehensive review on the molecular and immunological hallmarks and prerequisites for next-generation DC vaccines, Garg et al. (20) discourses its combinatorial use with immune checkpoint inhibitors to enforce efficient antitumor activity. Based on the expression pattern of PD-1 and PD-L on immune cells and cellular contacts between DC and a myriad of immune effector and regulatory cells, blocking PD-1/PD-L interactions will likely impede tumor cell-mediated immune suppression, enhance T cell and NK cell activation and effector functions, and inhibit conversion or activation of Treg cells. However, these actions depend also on the way of implementation of PD-1/PD-L blockade with DC vaccination. Here, we elaborate on the currently applicable strategies (**Figure 2**) and clinical trials (**Tables 1** and **2**) that particularly interfere with the PD-1/PD-L pathway in the context of DC-based immunotherapies.

Systemic Receptor-Ligand Blockade

The use of mABs that block immune checkpoints, particularly cytotoxic T lymphocyte antigen-4 (CTLA-4), PD-1, and PD-L1, has made a profound impact in the field of cancer immunotherapy

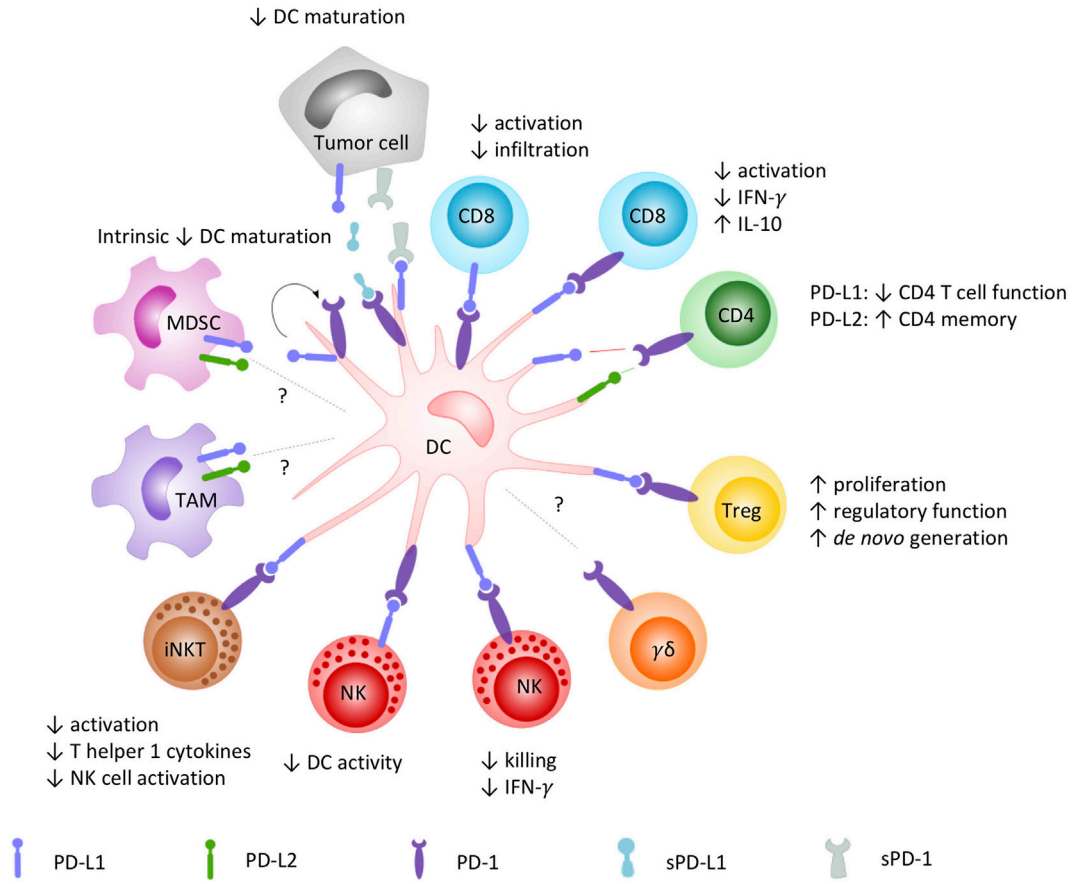

FIGURE 1 | How the PD-1/PD-L signaling axis plays a role in DC-mediated orchestration of innate and adaptive immunity. DCs are renowned for their pivotal role in regulating the immune response through interaction with a variety of immune cells. DC-moderated PD-1 signaling has been demonstrated to prototypically result in an inhibitory crosstalk with effector cells, evidenced by (1) reduced infiltration and activation capacities, decreased pro-inflammatory, and increased inhibitory cytokine release by $CD8^+$ and $CD4^+$ T cells; (2) impaired killing, regulatory and reciprocal DC activation properties of NK cells; and (3) impaired activation, Th1-cytokine secretion, and downstream NK cell activation by iNKT cells. On the opposite, a costimulatory role for particular interactions promoting $CD4^+$ T cell memory has been described as well. In crosstalk with Tregs, PD-1 engagement was shown to mediate their proliferation, regulatory function, and *de novo* generation, contributing to an immune suppressive environment. The role of PD-1-signaling in DC crosstalk with other emerging PD-1-sensitive effector (γδ T cells) and regulatory cells (MDSC, TAM) remains to be elucidated. Abbreviations: DC, dendritic cell; IFN-γ, interferon-γ; iNKT, invariant NK T cell; MDSC, myeloid-derived suppressor cell; NK, natural killer cell; PD-1, programmed death-1; PD-L1, programmed cell death ligand 1; PD-L2, programmed cell death ligand 2; sPD-1, soluble PD-1; sPD-L1, soluble PD-L1; TAM, tumor-associated macrophage; Treg, regulatory T cell.

(108). As of 2011, treatment of several malignancies with anti-CTLA-4- (ipilimumab), anti-PD-1- (nivolumab and pembrolizumab), and anti-PD-L1- (atezolizumab, durvalumab, and avelumab) blocking antibodies has been approved by the US FDA and EMA and a series of new inhibitors is being assessed in late stage clinical trials (105). With the experience that anti-CTLA-4 therapy comes with higher toxicity and lower response rates (16, 109, 110), the focus of research is propelling toward the PD-1/PD-L pathway as evidenced by the myriad of publications on fundamental, preclinical, and clinical PD-1/PD-L research and on its prognostic and predictive biomarker value. As an example, one of the latest developments is to extend the systemic antibody-blocking function with antibody-dependent cellular cytotoxicity (ADCC) properties. The majority of mABs bear a mutation in their Fc portion, making target cells insensitive to ADCC mediated through the FcγRIIIa on NK cells. Keeping the Fc part not mutated, avelumab resulted in ADCC-mediated clearance of $PD\text{-}L1^+$ tumor cells (111).

In combination with DC vaccination, systemic blockade with anti-PD-1 mABs (112, 113) or anti-PD-L mABs (114–116) resulted in increased activation of cytotoxic $CD8^+$ T cells and decreased Treg cell numbers (112) and showed better therapeutic efficacy compared with either monotherapy by preventing tumor growth and prolonging survival in tumor-bearing mice [glioblastoma (113), breast cancer (114), and melanoma (116)]. Recent studies evaluated the effect of different immune checkpoint inhibitors on human T cell responses after co-culture with allogeneic moDCs. In this setting, PD-1 and B and T lymphocyte attenuator (BTLA)-blocking antibodies could increase IFN-γ production and proliferation by T cells. Combined with anti-PD-1, other emerging immune checkpoint inhibitors such as anti-T cell immunoglobulin and mucin-domain containing-3 (TIM-3),

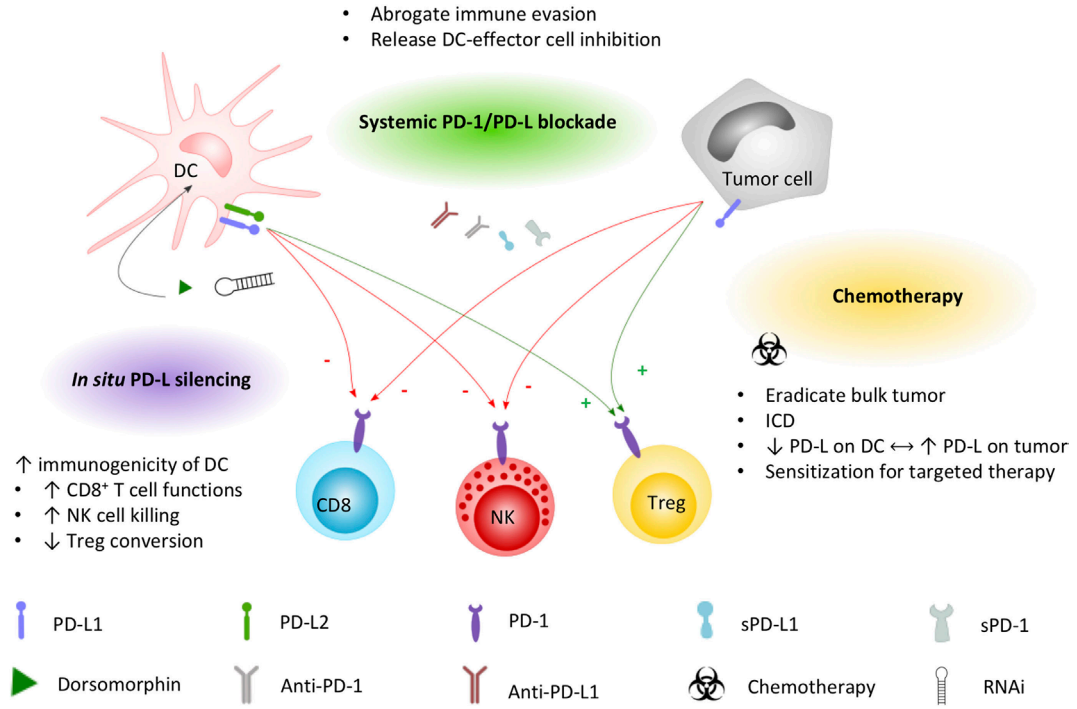

FIGURE 2 | Applied strategies to leverage DC immunopotency by interfering PD-1/PD-L signaling. DC and tumor cell PD-L1 and/or PD-L2 expression exerts direct inhibitory effects (–, red arrows) on CD8+ T cells and NK cells, while promoting (+, green arrows) regulatory T cell functions. Current strategies to increase the immunogenicity of DC vaccines by interfering the PD-1/PD-L signaling axis include combined systemic blockade by means of PD-L1-blocking moieties. Chemotherapy triggers different mechanisms that can promote DC vaccine efficacy, including the induction of immunogenic cell death favorable for DC activation. Exploiting the PD-1 pathway, platinum-based chemotherapeutics have been demonstrated to lower PD-L expression on DCs while increasing tumor cell PD-L expression, sensitizing the tumor for systemic blockade approaches. *In situ* engineering of DC vaccines by silencing PD-L with the small molecule dorsomorphin or RNAi constructs was demonstrated to successfully improve the immunopotency of DC vaccines. Abbreviations: DC, dendritic cell; ICD, immunogenic cell death; NK, natural killer cell; PD-1, programmed death-1; PD-L1, programmed cell death ligand 1; PD-L2, programmed cell death ligand 2; RNAi, RNA interference; sPD-1, soluble PD-1; sPD-L1, soluble PD-L1; Treg, regulatory T cell.

anti-lymphocyte activating gene-3 (LAG-3), anti-CTLA-4, and anti-BTLA were able to further increase the IFN-γ-producing and proliferative capacity of T cells, while ineffective on their own (117, 118). These findings further underscore the strength of the PD-1-/PD-L-signaling axis relative to other immune checkpoint pathways.

Over the past 8 years, a select number of phase I/II clinical trials combining DC vaccination with anti-PD-1 or anti-PD-L1 antibodies in a range of malignancies have been initiated and are currently all ongoing (**Table 1**). With the first clinical results expected in the near future, the challenges of conceptualization of such combination therapy are already subject of discussion (20). The growing portfolio of both next-generation DC vaccines and available PD-1 and PD-L targeting mABs makes the possible treatment regimens infinite. Moreover, knowledge is growing that tumors are differentially sensitive to either DC therapy or antibody-mediated checkpoint blockade, either intrinsically or dependent on the stage of the disease. While DC-mediated therapy is consistently proven safe (7), systemic mAB therapy has to deal with several immune-related adverse effects such as skin and mucosal irritation, diarrhea, hepatotoxicity, and endocrinopathy (110, 119). Today, we are learning how to recognize and manage immune-related adverse events and toxicities and gaining knowledge on which therapeutic combinations could be applied best at what time point (120, 121). As an alternative to human(ized) mABs, different blocking moieties with advanced target specificity and affinity and reduced toxicity profiles are under investigation, including chimeric fusion proteins (AMP-224, extracellular domain of PD-L2, and an Fc portion of IgG) and nanotechnologies [nanoparticles (122) and nanobodies ((123), Theravectys, Ablynx)]. Although research in this area is limited, these alternative blockers have interesting features because of their size, stability, and pharmacodynamical properties (124), which might pave the way for implementation in combination therapy with DCs.

Soluble PD-(L)1

Comparable to the systemic antibody approach is the use of sPD-1 receptor, which only contains the extracellular domain of the PD-1 molecule and can ligate to PD-Ls, making them inaccessible for interaction with PD-1 molecules on immune effector cells. Binding of sPD-1 to surface PD-L on DCs was demonstrated to enhance proliferation of lymphocytes *in vitro*. In addition, after administration of a vector encoding for sPD-1, tumor growth was inhibited or delayed in a murine model of hepatocarcinoma (125). Similar results were found by Song et al. (126) who additionally

TABLE 1 | Active clinical trials combining DC-based anticancer immunotherapy with PD-1/PD-L-targeted therapy (clinicaltrials.gov, January 14, 2018).

Intervention		Therapy schedule	Comparator(s)	Condition	Phase	*N*	Trial identifier	Status
PD-1-/PD-L-targeted therapy	**Type of DC vaccine**							
Anti-PD-1 Ab (nivolumab)	Autologous DC loaded with CMV pp65 mRNA	Neoadjuvant + adjuvant DC vaccination with anti-PD-1 therapy	Without neoadjuvant DC vaccination	Recurrent brain tumors	I	7	NCT02529072	Active, not recruiting
	Autologous DC loaded with NY-ESO-1 peptide	Therapy cycles of cyclophosphamide, TCR-transduced PBMC, anti-PD-1 therapy, DC vaccination, and rhIL-2	Single group	NY-ESO-1^{+} solid tumors	I	12	NCT02775292	Recruiting
	Autologous DC loaded with autologous tumor lysate	Therapy cycles of i.d. DC vaccination with anti-PD-1 therapy	DC therapy alone	Recurrent glioblastoma	II	30	NCT03014804	Not yet recruiting
Anti-PD-1 Ab (pembrolizumab)	Autologous DC loaded with peptide	Anti-PD-1 SoC post-DC therapy	Single group	Advanced melanoma	I	12	NCT03092453	Recruiting
	Autologous DC loaded with autologous tumor antigens	Therapy cycles of anti-PD-1 and cryosurgery plus i.t. DC vaccination	Single group	Non-Hodgkin lymphoma	I/II	44	NCT03035331	Recruiting
	Autologous DC	Therapy cycles of i.n. DC vaccination with anti-PD-1 therapy, radiotherapy, GM-CSF and anti-TNF-alpha therapy	Single group	Follicular lymphoma	II	20	NCT02677155	Recruiting
	DC-CIK	Therapy cycles of i.v. DC vaccination with anti-PD-1 therapy	Anti-PD-1 Ab alone	Advanced solid tumors	I/II	100	NCT03190811	Recruiting
	DC-CIK	Therapy cycles of i.v. DC vaccination with anti-PD-1 therapy	Anti-PD-1 Ab alone	NSCLC	I/II	60	NCT03360630	Recruiting
Anti-PD-1 Ab	DC-CIK	i.v. anti-PD-1 Ab-treated DC vaccination	Single group	Refractory solid tumors	I/II	50	NCT02886897	Recruiting
Anti-PD-1 Ab (CT–011)	DC/tumor cell fusion vaccine	Therapy cycles of anti-PD-1 therapy with DC vaccination post-auto-SCT	Anti-PD-1 Ab alone	Multiple myeloma	II	35	NCT01067287	Active, not recruiting
SoC CPI therapy	Autologous TLPLDC vaccine	DC vaccination (tumor lysate + yeast cell wall particles + DC) following CPI monotherapy (*comparison based on response to CPI therapy*)	CPI non-responder, progressive disease following initial response to CPI, stable disease after CPI	Metastatic melanoma	I/II	45	NCT02678741	Recruiting
Anti-PD-L1 Ab (avelumab)	Autologous DC vaccine	Therapy cycles of DC vaccination with anti-PD-L1 therapy	Single group	Metastatic colorectal cancer	I/II	33	NCT03152565	Not yet recruiting
Anti-PD-L1 Ab (durvalumab)	DC/AML fusion vaccine	*Not specified*	DC therapy alone, traditional care	Acute myeloid leukemia	II	105	NCT03059485	Recruiting
PD-L siRNA lipofection of the DC vaccine	MiHa-loaded DC	Post-allo-HSCT	Single group	Hematological malignancies	I/II	10	NCT02528682	Recruiting

AML, acute myeloid leukemia; CPI, checkpoint inhibitor therapy; CIK, cytokine-induced killer cells; DC, dendritic cell; HSCT, hematopoietic stem cell transplantation; IL-2, interleukin 2; i.d., intradermal; i.n., intranodal; i.t., intratumoral; i.v., intravenous; MiHa, minor histocompatibility antigens; NSCLC, non-small-cell lung cancer; PBMC, peripheral blood mononuclear cells; PD-1, programmed death-1; PD-L1, programmed death ligand 1; siRNA, small interfering RNA; SoC, standard of care; TCR, T cell receptor; TLPLDC, tumor lysate particle-loaded dendritic cell.

demonstrated increased expression of activation markers on DC in mice treated with sPD-1. Kuipers et al. (102), however, demonstrated a decrease in the expression of maturation markers on DCs treated with sPD-1. These discrepancies might be ascribed to different experimental settings such as the use of other sPD-1 encoding vectors. Applying the sPD-1 approach in human moDCs, Pen et al. (127) transfected mRNA encoding for sPD-1 or sPD-L1 in DC for transient local expression, thereby limiting possible adverse effects seen with systemic PD-1/PD-L blockade. With this approach, they demonstrated an upregulation of CD80 on sPD-1- or sPD-L1-expressing DCs and an increase in both $CD4^+$ and $CD8^+$ T cell effector functions without influencing the induction of Treg cells. Today, clinical trials evaluating this approach have not been registered.

Chemo-Immunotherapy

Anticancer chemotherapeutics remain an important systemic treatment modality to arrest or eliminate rapidly growing cancer cells. Besides lowering the tumor burden, evidence is growing that these cytotoxic drugs also rely on several off-target immunological effects, including enhancement of the immunogenicity of malignant cells and, at least for some chemotherapeutics, suppression of inhibitory mechanisms (128, 129). Complementing conventional chemotherapy regimens with DC-targeted immunotherapy is therefore a promising strategy, actively investigated in clinical trials for a range of malignancies (>140 registered trials at Clinicaltrials.gov based on "DC and chemo" search). DC vaccine efficacy can avail from chemotherapy-induced immunogenic tumor cell death that facilitates an adaptive immune response specific for dead cell-derived antigens (130). In the context of immune checkpoint inhibition, the clinically established class of platinum-based chemotherapeutics has been designated to act *via* the PD-1 signaling pathway. In addition to DNA-interfering properties, oxaliplatin, cisplatin, and carboplatin were shown to inhibit the STAT6-pathway that is responsible for the upregulation of PD-1 ligands, leading to downregulation of PD-L1 and PD-L2 on both moDCs and tumor cells (131). The combination of platinum-based chemotherapeutics and DCs boosted *in vitro* T cell proliferation and enhanced T cell IFN-γ and IL-2 production (131). In other studies, however, platinum-based chemotherapeutics were reported to promote PD-L expression on blood DC subsets (132) and tumor cells (133). Enhanced PD-L expression on DCs resulted in impaired T cell activation, rationalizing that the chemotherapy effect likely depends on environmental cues, such as TLR expression on those DC subsets (132). In hepatocarcinoma cells, cisplatin promoted PD-L1 overexpression both *in vitro* and *in vivo*, suggesting a mechanism of chemotherapy resistance eventually leading to a suboptimal clinical effect of cisplatin treatment (133). The contradictory outcomes of these studies highlight the need for further research on the effect of platinum-based chemotherapeutics on the functionality of different immune cells, as well as on tumor cells of various origins. In addition, it will be interesting to extend research to the clinic to determine the optimal treatment schedule where chemotherapy and DC vaccination are combined. Such combination therapies are listed in **Table 2**. Although these studies are not yet completed, a pilot study on the immunogenicity of DC vaccination during adjuvant platinum-based chemotherapy in colon cancer patients demonstrated enhanced antigen-specific T cell responses after combinatorial treatment (134).

DC-Targeted PD-L RNA Interference (RNAi) Technology

Taking into account the orchestrating role of DCs, targeted downregulation of PD-L expression on DCs is expected to potentiate DC-mediated T cell and NK cell activation and prevent Treg cell stimulation. RNAi approaches targeting immunosuppressive factors in DCs have been applied to improve immunogenic functions of next-generation DC vaccines (13). This strategy aims at enhancing DC-mediated antigen-targeted T cell responses at the level of the DC/effector cell immunological synapse, irrespective of tumor PD-L expression. Analogous to DCs expressing sPD-1 or sPD-L1 (*vide supra*), this technique offers attractive safety considerations compared to systemic antibody administration. The targeted nature of this approach shifts the *in situ* balance between immune stimulatory and inhibitory signals in the DC/effector cell immunological synapse toward immune stimulation, which has been suggested to result in reversal of the PD-1-mediated T cell exhaustion status (135).

Various preclinical studies demonstrated feasibility and effectivity of introducing small interfering RNAs or short hairpin

TABLE 2 | Clinical trials combining DC vaccination strategies with PD-1-/PD-L1-modulating chemotherapeutics (clinicaltrials.gov, January 14, 2018).

DC-based therapy	PD-1-/PD-L-modulating chemotherapy	Indication
Autologous DC loaded with TAA-coding RNA(s)	Cisplatin	Melanoma (NCT02285413), malignant pleural mesothelioma (NCT02649829)
Autologous DC loaded with tumor lysate		Multiple myeloma (NCT00083538), ovarian cancer (NCT02432378)
Autologous DC-CIK		Esophageal cancer (NCT01691625, NCT02644863), NSCLC (NCT02651441)
DC-CTL		NSCLC (NCT02766348)
Autologous DC loaded with TAA(s)	Oxaliplatin (as part of FOLFIRINOX)	Pancreatic cancer (NCT02548169), colorectal neoplasms (NCT01413295, NCT02503150)
Autologous DC-CIK		Gastric cancer (NCT02504229, NCT02215837), colorectal cancer (NCT02202928, NCT02415699)
Autologous DC	Carboplatin	NSCLC (NCT02669719), breast cancer (NCT03387553)

CIK, cytokine-induced killer cell; DC, dendritic cell; DC-CTL, dendritic cytotoxic lymphocyte; NSCLC, non-small-cell lung cancer; TAA, tumor-associated antigen.

RNAs interfering with inhibitory immune-related pathways in DCs, such as suppressor of cytokine signaling (136), indoleamine 2,3-dioxygenase (137), and PD-L1/PD-L2 (138–142). Focusing on the PD-1/PD-L pathway, silencing of PD-L1 and/or PD-L2 in DCs has been evaluated with different RNAi introduction techniques, including viral transduction and non-integrating electrotransfection, lipid nanoparticle transfection, and the cGMP-compliant transfection reagent SAINT-RED (77, 138, 141, 143, 144). Preclinical data demonstrated that PD-L-silenced DCs could (1) increase expansion, promote pro-inflammatory cytokine secretion and degranulation, and augment antitumor function of antigen-specific $CD8^+$ T cells in human *in vitro* models (138, 140, 142) and (2) induce significant antitumor immunity *in vivo* in different malignant mouse models (139, 141). Alternatively, *in situ* PD-L silencing can also be achieved through the use of small molecules. Dorsomorphin, a small molecule inhibitor of the bone morphogenic protein signaling pathway, was shown to efficiently downregulate PD-L1 and PD-L2 expressions on treated DCs resulting in increased T cell proliferation and enhanced NK cell-mediated killing of target cells (145).

Today, few DC-associated RNAi approaches are currently being tested in early-phase clinical trials, including one trial evaluating PD-L1/2-silenced DC vaccines (NCT02528682). Results of this trial are awaited.

CLINICAL TRIALS

Based on the general appreciation that DC vaccination can be improved by blockade of the PD-1/PD-L pathway, as shown by both *in vitro* experiments and *in vivo* animal models, most of these combination approaches are embedded in various clinical trials (146). With the exception of sPD-1, autologous DC vaccines are combined with (i) systemic mABs targeting PD-1 or PD-L1, (ii) platinum-based chemotherapeutics, and (iii) *in situ* PD-L RNAi to treat patients with both hematological cancers [multiple myeloma, acute myeloid leukemia (AML)] and solid tumors (renal cell carcinoma, mesothelioma, lymphoma, colon cancer, melanoma, ovarian cancer, pancreatic cancer, nasopharyngeal cancer, and glioblastoma). Clinical trials combining DC vaccination with PD-1/PD-L interference, registered by January 2018, are listed in **Tables 1** and **2** and discussed in the corresponding paragraphs. The fast-growing number of clinical studies combining DC-based therapy with PD-1/PD-L blockade strategies emphasizes the potential of this combinatorial approach in the future treatment of cancer patients.

FUTURE PERSPECTIVES

Multimodality strategies striving to maximize the efficacy of DC-based cancer immunotherapy are emerging (16, 20, 107). Evidenced by a growing body of preclinical and clinical data, engineering next-generation DC vaccines and redirecting the tumor microenvironment are highly promising (7). The significant role of PD-1-signaling in DC-mediated antitumor immunity rationalizes its therapeutic combinatorial use in the rapidly evolving cancer immunotherapy landscape. The PD-1-/PD-L-blocking industry—and the immune checkpoint industry in general—has expanded drastically in the last years. Leading pharmaceutical companies are putting huge efforts in the development of systemic antibody therapies, with an estimated market value of $35 billion (147). The market for DC-based therapies is as big, with approximately 500 clinical trials registered evaluating DC vaccines, reflecting the immense scientific and pharmaceutical impact of such combinatorial therapy. The growing understanding of the immunological effects of some conventional chemotherapeutics, related to DC activation and PD-1 therapy sensitivity and resistance, provides rationale for the development of synergistic adjuvant combinations and carefully designed chemoimmunotherapy schedules that aim beyond the mere elimination of the suppressive tumor (20, 107). In addition to the pioneering CTLA-4 and PD-1 inhibitors, other immune checkpoints have been attributed to hamper DC-mediated immunity, including LAG-3 and TIM-3 (56, 119, 148). The LAG-3 mAB IMP321 was demonstrated to induce DC maturation (149–151) and is now further tested in clinical trials (NCT00351949, NCT00349934). TIM-3, present on, among others, DCs, was shown to induce T helper 1 cell death when interacting with its ligand galectin-9 on T cells (119, 152), whereas dual blockade of TIM-3 and PD-1 or CTLA-4 was able to suppress tumor growth with possibility of cure in a fibrosarcoma mouse model (153). Overall, targeting multiple immune checkpoints simultaneously with DC therapy is likely to result in synergistic efficacy (107).

Designed to potentiate the patient's own immune system, unsatisfactory DC-based therapy efficacy led to an era of meticulous vaccine and protocol optimization aiming to enhance vaccine immunogenicity (7, 20). With the approval of immune checkpoint inhibitors, the significance of simultaneously targeting the inhibitory immune mechanisms was clinically established. In search of a balanced treatment, combinatorial DC and PD-1 pathway-targeted immunotherapy has some implications. The lack of specificity of systemic immune checkpoint blockade is prone to eliciting indiscriminate immune activation, resulting in significant immune-mediated adverse reactions and immune-related adverse events. In addition to the frequently observed development of therapy resistance, vigilant immunomonitoring to elucidate these mechanisms and advance early detection is warranted (105, 154, 155). Recently, resistance to anti-PD-1 therapy has been related to disturbance of antigen presentation, DC migration, and DC maturation (156), underscoring the importance of combinatorial treatment schedules. More than 20 years of clinical testing affirms that tumor-specific DC therapy is well tolerated and safe, and overstimulation, autoimmunity, or therapy resistance has been described (11, 20). By robustly breaching PD-1-related inhibitory signaling and demasking immune evasion, DC therapy could get that extra push to prevail durable antitumor immunity while compensating for the lack of specificity of immune checkpoint blockade (107).

Taken apart, it can be concluded that DC therapy and PD-1 blocking approaches will prove best in a combinatorial setting subject to the malignancy and the disease status (157). In this perspective, the search for biomarkers predicting response

to DC therapy and to PD-1 pathway blockade is imperative (20, 155, 158). Although immune checkpoint inhibition can be strikingly effective in immunogenic cancers with high mutational burden like melanoma and lung cancer, tumors with a lower number of mutations and lower immunogenicity may be inherently resistant to this form of therapy (154, 155). Complementary, DC efficacy is high for at least some tumors with low mutational burden, like leukemia (159–162) and glioblastoma (20), further emphasizing the combinatorial use of DC vaccination with PD-1-targeted strategies to improve DC performance. Exemplifying a combinatorial approach with AML, DC vaccinations are typically administered as a consolidation therapy after conventional chemotherapy, to prevent relapse by eliminating residual leukemic cells and by generating durable antileukemic immunity (159, 161, 163). A role for PD-1 after conventional leukemia therapy has been demonstrated, supported by chemotherapy-induced upregulation of PD-1 on T cells and increased T cell PD-1 expression at relapse after hematopoietic stem cell transplantation (47, 164). Therapeutically, PD-1 checkpoint blockade in AML has been suggested to relieve Treg-mediated immunosuppression (47). Empowering adjuvant DC vaccination by blocking the inhibitory PD-1 axis could alleviate DC-mediated adaptive and innate antitumor immune responses, reflecting a promising combination as a follow-up therapy.

CONCLUSION

In this review, we highlighted the role of the PD-1 pathway in DC-mediated antitumor immunity. Aiming to improve DC therapy efficacy, different strategies to invigorate DC immunopotency by impeding PD-1-mediated immune regulation were discussed. From the most advanced research on therapeutic blocking antibodies, lessons learned from chemotherapy-induced immune regulation, and data from more recent developments with gene-silencing techniques, it can be concluded that combinatorial DC and PD-1 pathway-targeted therapy approaches could complement or even synergize under defined circumstances. Five years after the comprehensive review on combination therapy with DC vaccines and immune checkpoint blockade by Vasaturo et al. (107), touching upon the first few preclinical studies on PD-1 combination strategies in particular, we witness that preclinical research has expanded drastically and has been translated into a number of clinical trials. We are now awaiting the first clinical results that will substantially direct future anticancer treatment approaches.

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MV, JVDB, EM, and EL wrote the paper. ES, VVT, and WH critically revised the manuscript.

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Type I Interferon-Independent Dendritic Cell Priming and Antitumor T Cell Activation Induced by a *Mycoplasma fermentans* Lipopeptide

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Mycoplasma fermentans-derived diacylated lipoprotein M161Ag (MALP404) is recognized by human/mouse toll-like receptor (TLR) 2/TLR6. Short proteolytic products including macrophage-activating lipopeptide 2 (MALP2) have been utilized as antitumor immune-enhancing adjuvants. We have chemically synthesized a short form of MALP2 named MALP2s (*S*-[2,3-bis(palmitoyloxy)propyl]-CGNNDE). MALP2 and MALP2s provoke natural killer (NK) cell activation *in vitro* but only poorly induce tumor regression using *in vivo* mouse models loading NK-sensitive tumors. Here, we identified the functional mechanism of MALP2s on dendritic cell (DC)-priming and cytotoxic T lymphocyte (CTL)-dependent tumor eradication using CTL-sensitive tumor-implant models EG7 and B16-OVA. Programmed death ligand-1 (PD-L1) blockade therapy in combination with MALP2s + ovalbumin (OVA) showed a significant additive effect on tumor growth suppression. MALP2s increased co-stimulators CD80/86 and CD40, which were totally MyD88-dependent, with no participation of toll-IL-1R homology domain-containing adaptor molecule-1 or type I interferon signaling in DC priming. MALP2s + OVA consequently augmented proliferation of OVA-specific CTLs in the spleen and at tumor sites. Chemokines and cytolytic factors were upregulated in the tumor. Strikingly, longer duration and reinvigoration of CTLs in spleen and tumors were accomplished by the addition of MALP2s + OVA to α-PD-L1 antibody (Ab) therapy compared to α-PD-L1 Ab monotherapy. Then, tumors regressed better in the MALP2s/OVA combination than in the α-PD-L1 Ab monotherapy. Hence, MALP2s/tumor-associated antigens combined with α-PD-L1 Ab is a good therapeutic strategy in some mouse models. Unfortunately, numerous patients are still resistant to PD-1/PD-L1 blockade, and good DC-priming adjuvants are desired. Cytokine toxicity by MALP2s remains to be settled, which should be improved by chemical modification in future studies.

Keywords: antitumor adjuvant, cross-presentation, diacylated lipopeptide, programmed death ligand-1 blockade, toll-like receptor 2

Abbreviations: Ab, antibody; Ag, antigen; AP-1, activator protein-1; BMDC, bone marrow-derived dendritic cell; CFSE, carboxyfluorescein diacetate succinimidyl ester; CTLs, cytotoxic T lymphocytes; DAMPs, damage-associated molecular patterns; DC, dendritic cell; IFN, interferon; i.p., intraperitoneally; i.v., intravenously; MALP2, macrophage-activating lipopeptide 2; MHC, major histocompatibility complex; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor-kappa B; OVA, ovalbumin; PAMPs, pathogen-associated molecular patterns; PD-1, programmed cell death-1; PD-L1, programmed death ligand-1; poly(I:C), polyinosinic-polycytidylic acid; PRR, pattern-recognition receptors; s.c., subcutaneously; TAA, tumor-associated antigen; TICAM-1, toll-IL-1R homology domain-containing adaptor molecule-1; TLR, toll-like receptor; WT, wild type.

INTRODUCTION

Toll-like receptor (TLR) 2 is a pattern-recognition receptor (PRR) that recognizes microbial lipopeptides, lipoproteins, and peptidoglycans (1). We happened to identify the mycoplasma lipoprotein M161Ag, also called MALP404 (2), as a TLR2 agonist (3, 4). Notably, antigen-presenting dendritic cells (DCs) express TLR2 in addition to TLR3. TLR2, unlike TLR3, shows a broad expression spectrum including endothelial cells, epithelial cells, and immune cells (5, 6). Macrophage-activating lipopeptide 2 (MALP2) is a diacylated lipopeptide isolated from the outer membrane of *Mycoplasma fermentans* (7) and is known to be a proteolytic product of M161Ag (2–4). MALP2 is an agonistic ligand of the TLR2/6 heterodimer and induces inflammatory cytokine production from macrophages, monocytes, and DCs (8, 9). MALP2, as well as a short form of MALP2 named MALP2s, efficiently induces immune activation in mouse and human DCs (8, 10, 11). We have chemically synthesized MALP2s composed of the first six amino acids following Pam2 (*S*-[2,3-bis(palmitoyloxy) propyl]-CGNNDE), which lacks the last eight amino acids of full-length MALP2 (Pam2-CGNNDESNISFKEK) (8). MALP2s and MALP2 similarly induce cytokine production from DCs and upregulate major histocompatibility complex (MHC) class I and maturation marker CD86. Antigen (Ag)-specific cytotoxic T lymphocyte (CTL) expansion is primed by DCs, a process called cross-presentation (12). Generally, cross-presentation is augmented by DC maturation involving (i) upregulation of MHC class I molecules; (ii) upregulation of co-stimulatory molecules, including CD80, CD86, and CD40; (iii) increase in Ag peptide-loading on MHC class I; and (iv) production of cytokines enhancing CTL proliferation/activation (13, 14). Since enhancement of cross-presentation was reported with TLR2 ligands (15–17), we assessed T cell cross-priming activity of MALP2s in the present study. We also investigated antitumor activity of MALP2s in tumor-bearing mouse models.

Antitumor immunotherapy is an effective approach to refractory cancers inapplicable to other standard therapies. To evoke a potent antitumor response, an immunostimulatory adjuvant targeting PRRs would be an optimal agent. PRRs recognize pathogen-associated molecular patterns (PAMPs) commonly conserved in foreign microbes. PRRs also recognize damage-associated molecular patterns (DAMPs) released from dying host cells. PRR signaling initiates innate immunity involving DCs, macrophages, and natural killer (NK) cells, and subsequently activates adaptive immunity including T cells and B cells (18). Since a tumor is autologous and lacks endogenous immunostimulatory signals in most cases, an adjuvant targeting PRR is mandatory to invoke an efficient antitumor response. CTLs play a critical role in tumor eradication. Cross-presentation by DC is an essential process for Ag-specific CTL expansion (12). However, immature DCs have poor cross-presentation ability and must mature to induce potent CTL expansion (13). Thus, to develop an effective antitumor immunotherapy, we need to devise an adjuvant capable of inducing DC maturation.

Programmed cell death-1 (PD-1)/programmed death ligand-1 (PD-L1) blockade therapy has improved clinical outcomes in many types of malignant tumors. However, responders to blockade therapy are few, and more than 70–80% of patients still require relief for the unresponsiveness (19, 20). One of the factors influencing therapeutic efficacy is the pre-existence of tumor-specific CTLs (21). The lack of endogenous DC/CTL-priming stimuli may in part cause the unresponsiveness to PD-1/PD-L1 blockade. Determining a CTL-priming adjuvant to complement PD-1/PD-L1 therapy will thus be needed to improve therapeutic efficacy. Here, we investigated the effectiveness of a combination therapy employing MALP2s and PD-L1 blockade.

MATERIALS AND METHODS

Mice

Wild-type (WT) C57BL/6J mice were purchased from CLEA Japan. *Ticam1*$^{-/-}$ mice were made in our laboratory. *Ifnar*$^{-/-}$, *Myd88*$^{-/-}$, and OT-I mice were kindly provided by Dr. T. Taniguchi (Tokyo University, Tokyo, Japan), Dr. S. Akira (Osaka University, Osaka, Japan), and Dr. N. Ishii (Tohoku University, Sendai, Japan), respectively. All mice were backcrossed >8 times to C57BL/6 background and maintained under specific pathogen-free conditions in the animal faculty of the Hokkaido University Graduate School of Medicine. Animal experiments were performed according to the guidelines set by the animal safety center, Hokkaido University, Japan.

Cells

Mouse bone marrow-derived DCs (BMDCs) were prepared as described previously (22). CD8α^{+} DCs were isolated from mouse spleen by CD8^{+} DC isolation kit (Miltenyi Biotec, the catalog number: 130-091-169). Cells were cultured in RPMI 1640 (GIBCO, 11875-093) supplemented with 10% heat-inactivated FBS (Thermo Scientific, SH30910.03), 10 mM HEPES (GIBCO, 15630-080), and 50 IU penicillin/50 μg/ml streptomycin (GIBCO, 15070-063). EG7 (ATCC® CRL-2113™) cells were purchased from ATCC (Manassas, VA, USA) and cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 10 mM HEPES, 1 mM sodium pyruvate (GIBCO, 11360-070), 55 μM 2-mercaptoethanol (GIBCO, 21985-023), 50 IU penicillin/50 μg/ml streptomycin, and 0.5 mg/ml G418 (Roche, 04 727 894 001). MO5 (23) was kindly provided by Dr. H. Udono (Okayama University, Japan) and cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 50 IU penicillin/50 μg/ml streptomycin, and 0.1 mg/ml G418.

Reagents and Antibodies

MALP2s (Pam2CGNNDE) was synthesized by Synpeptide Co., Ltd. (Shanghai, China). Pam2CSK and Pam2CSK4 (Pam2CSKKKK) were synthesized by Biologica Co., Ltd. (Nagoya, Japan). Polyinosinic-polycytidylic acid [poly(I:C)] (27-4732-01) was purchased from GE healthcare Life Sciences. EndogGade® Ovalbumin (OVA) (321001) was purchased from Hyglos. OVA (H2Kb-SL8) Tetramer (TS-5001-P) was purchased from MBL. Mouse interferon (IFN) gamma ELISA KIT (88–7314) was purchased from eBioscience. Carboxyfluorescein diacetate succinimidyl ester (CFSE) (C1157) and Ovalbumin Alexa Fluor™ 647 Conjugate (O34784) were purchased from Molecular Probes.

α-PD-L1 antibody (Ab) (clone: 10F.9G2, the catalog number: BE0101) and rat IgG2b isotype control Ab (LTF-2, BE0090) were purchased from Bio X Cell. α-IFNAR-1 Ab (MAR1-5A3, 127304) and mouse IgG1κ isotype control Ab (MOPC-21, 400124) were purchased from BioLegend. Abs used for flow cytometry analysis are listed in Table S1 in Supplementary Material.

OT-I Proliferation Assay

OT-I T cells were isolated from spleens of OT-I mice by CD8- microbeads (Miltenyi Biotec, 130-049-401). OT-I cells were labeled with 1 μM of CFSE for 10 min at 37°C. In the coculture with OT-I cells and BMDCs, 5×10^5 BMDCs were seeded in a 24-well plate. PBS, 100 nM of Pam2CSK, Pam2CSK4, or MALP2s was added in the wells. After 18 h, 500 ng/ml of OVA was added. After 4 h, OVA was washed out and 1×10^5 BMDCs were re-seeded in a 96-well plate and were cocultured with 1×10^5 CFSE-labeled OT-I cells for 60 h. In the coculture with OT-I cells and CD8α$^+$ DCs, 3.5×10^4 CD8α$^+$ DCs were seeded in a 96-well plate. PBS, Pam2CSK4, or MALP2s was added in the presence or absence of 2.5 μg/ml of OVA. After 3 h, CD8α$^+$ DCs were cocultured with 3.5×10^4 CFSE-labeled OT-I cells for 60 h. After the coculture, cells were stained with anti-CD8α and anti-TCR Vβ5.1,5.2 Abs. Dead cells were excluded by 7-amino actinomycin D staining. OT-I proliferation was evaluated by the attenuation of CFSE with FACS calibur (BD Biosciences). The concentrations of IFN-γ in the culture media were measured by ELISA. For *in vivo* OT-I assay, 6×10^5 CFSE-labeled OT-I cells were intravenously (i.v.) injected to mice. After 24 h, PBS, 25 μg of OVA, or 50 nmol of MALP2s + OVA was subcutaneously (s.c.) injected, respectively. After 60 h, spleens were harvested and OT-I proliferation was evaluated with FACS AriaII (BD Biosciences).

Tumor Challenge and MALP2s Therapy

Mice were shaved at the back and s.c. injected with 200 μl of 2×10^6 EG7 cells or MO5 cells. Tumor volume was calculated by using the formula: tumor volume [mm^3] = 0.52 × (long diameter [mm]) × (short diameter [mm])^2. In the EG7 tumor-bearing model, PBS, 100 μg of OVA, 50 nmol of MALP2s, or MALP2s + OVA was s.c. injected around tumor when the tumor volume reached to 500–600 mm^3. These treatments were performed once or twice. The second treatment was performed 8 days after the first treatment. For the CD8β$^+$ cells or NK1.1$^+$ cells depletion, hybridoma ascites containing anti-CD8β or anti-NK1.1 monoclonal Ab was intraperitoneally (i.p.) injected into mice 1 day before MALP2s + OVA treatment. In the MO5 tumor-bearing model, PBS or MALP2s + OVA was s.c. injected around tumor 7 days after tumor implantation. 130 μg of isotype control Ab or α-PD-L1 Ab was i.p. injected into mice on days 7, 9, and 11. Mice were euthanized when a tumor volume reached to 2,500 mm^3.

Analysis of Tumor Microenvironment

For a gene expression analysis, a small piece of EG7 or MO5 tumor tissue was collected and total RNA was extracted using Trizol reagent (Thermo Fisher Scientific, 15596-018) as following the manufacturer's instructions. Real-time PCR was performed as described previously (24). Sequences of primers in this study are shown in Table S2 in Supplementary Material.

For analysis of intratumor CD8$^+$ T cells, tumor tissues were finely minced and treated with 0.05 mg/ml collagenase I (Sigma-Aldrich, C0130-100MG), 0.05 mg/ml collagenase IV (Sigma-Aldrich, C5138-1G), 0.025 mg/ml hyaluronidase (Sigma-Aldrich, H6254-500MG), and 0.01 mg/ml DNase I (Roche, 10 104 159 001) in Hank's Balanced Salt Solution (Sigma-Aldrich, H9269-500ML) at room temperature for 15 min. Tumor-infiltrating CD8$^+$ T cells were analyzed by FACS AriaII.

Statistical Analysis

p-Values were calculated by the following statistical analysis. For the multiple comparisons, one-way analysis of variance with Bonferroni's test or Kruskal–Wallis test with Dunn's multiple comparison was performed. For the comparison between two groups, Student's *t*-test was performed. Error bar represent the SD or SEM between samples.

RESULTS

MALP2s Induces Ag-Specific CTL Expansion by Augmenting Cross-Presentation of DCs

We previously showed that MALP2s upregulated MHC class I and co-stimulatory molecules on mouse BMDCs (8). These responses are the signatures of DC maturation and facilitate Ag-specific CTL priming (25). First, we assessed CTL-priming activity of MALP2s by OT-I proliferation assay. In cocultures of BMDCs and OT-I cells, MALP2s-stimulated BMDCs exhibited higher cross-presentation ability than PBS- or Pam2CSK-added BMDCs in the presence of OVA Ag. Pam2CSK has no TLR2 agonistic activity (26) and was set as a negative control lipopeptide. The degree of OT-I expansion by MALP2s was comparable to Pam2CSK4 (Pam2-CSKKKK), which is another TLR2/6 agonist that exerts DC maturational activity (26) (upper panels of **Figure 1A**). Since CD8α$^+$ DCs are the DC subset which largely contributes to TLR2-induced cross-presentation (15), OT-I proliferation by MALP2s-stimulated CD8α$^+$ DCs was also assessed. MALP2s enhanced cross-presentation ability of CD8α$^+$ DCs as well as BMDCs (lower panels of **Figure 1A**). OT-I cells primed by MALP2s- or Pam2CSK4-stimulated BMDCs and CD8α$^+$ DCs secreted IFN-γ (**Figure 1B**; Figure S1A in Supplementary Material). We then performed OT-I proliferation assays *in vivo*. WT mice were injected s.c. with PBS, OVA, or MALP2s + OVA after adoptive transfer of CFSE-labeled OT-I cells. OVA administration but not PBS induced moderate OT-I cell expansion/proliferation without MAPL2s. However, OT-I cell expansion/proliferation was strongly enhanced by co-administration of MALP2s (**Figure 1C**; upper panels of Figure S1B in Supplementary Material). Moreover, OT-I cells were proliferated in response to i.v. administration of MALP2 + OVA (lower panels of Figure S1B in Supplementary Material). These results suggest that MALP2s is a potent CTL-priming adjuvant.

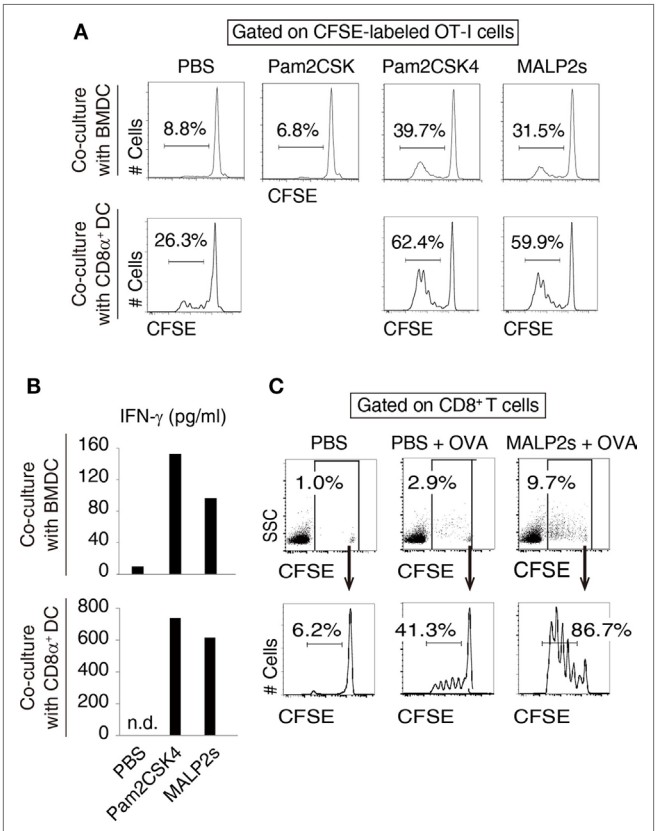

FIGURE 1 | MALP2s induces cross-presentation in dendritic cells (DCs). **(A,B)** Bone marrow-derived dendritic cells (BMDCs) or CD8α+ DCs was stimulated with Pam2CSK4 or MALP2s in the presence of ovalbumin (OVA) and cocultured with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled OT-I cells. PBS and Pam2CSK were negative controls. **(A)** The percentage of dividing cells among CFSE-labeled OT-I cells was analyzed by flow cytometry. The upper and lower panels show the result of coculture with BMDCs and CD8α+ DCs, respectively. **(B)** The concentrations of INF-γ in the culture medium were measured by ELISA. The upper and lower graphs show the results of coculture with BMDCs and CD8α+ DCs, respectively. n.d.: not detected. **(C)** CFSE-labeled OT-I cells-transferred WT mice were administered with PBS, OVA, or MALP2s + OVA. The percentage of CFSE-labeled OT-I cells among splenic CD8+ T cells (upper panels) and the percentage of dividing cells among CFSE-labeled OT-I cells (lower panels) were analyzed by flow cytometry. $n = 1$ per group. [**(A)** lower graph of **(B)** and **(C)**] The results are representative of more than two independent experiments.

The TICAM-1-Type I IFN Axis Does Not Influence MALP2s-Induced DC Maturation

Myeloid differentiation primary response 88 (MyD88) is an adaptor molecule of TLRs including TLR2 but not TLR3. Following ligand recognition by TLR2, nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) translocate to the nucleus (5). The activation of transcriptional factors is initiated by MyD88 signaling, which in turn forwards inflammatory responses. Although the MyD88-NF-κB/AP-1 axis does not induce type I IFN, endosomal TLR2 signaling may slightly promote type I IFN production (5, 27–29). Toll-IL-1R homology domain-containing adaptor molecule-1 (TICAM-1, also called TRIF) is the adaptor molecule for TLR3 and TLR4 (30, 31). Nilsen et al. showed that TICAM-1 participates in TLR2-dependent type I IFN production in mouse bone marrow-derived macrophages (29). To assess the contribution of TICAM-1 and type I IFN to TLR2-dependent DC maturation, we evaluated the expression level of maturation markers on WT, *Myd88*$^{-/-}$, *Ticam1*$^{-/-}$, and *Ifnar*$^{-/-}$ BMDCs after MALP2s stimulation. CD40, CD80, and CD86 expression was upregulated by Pam2CSK4 or MALP2s independent of TICAM-1 or IFNAR signaling. The upregulation was not induced at all in *Myd88*$^{-/-}$ BMDCs (**Figure 2A**). Since a decrease of endocytosis/phagocytosis is one of the signatures of DC maturation (32), endocytic activity in MALP2s-stimulated BMDCs was also evaluated. IFNAR signaling blockage by α-IFNAR Ab treatment did not affect the decrease in endocytic activity of DCs induced by Pam2CSK4 and MALP2s (**Figure 2B**). In this setting, α-IFNAR Ab treatment completely blocked induction of the IFN-inducible gene *Ifit1* by TLR2 ligands (**Figure 2C**). The endocytic activity of *Myd88*$^{-/-}$ BMDCs was also evaluated. The TLR3 agonist poly(I:C) was set as a positive control because TLR3-induced DC maturation is independent of MyD88. The endocytic activity of *Myd88*$^{-/-}$ BMDCs was decreased by poly(I:C) but not by TLR2 ligands (**Figure 2D**). These results indicate that TLR2-induced DC maturation completely depends on the MyD88 pathway: MyD88-derived DC priming exists independent of TICAM-1 and type I IFN signaling.

We further analyzed the contribution of type I IFN-IFNAR signaling in MALP2s-induced cross-presentation by tetramer assay. *Ifnar*$^{-/-}$ mice immunized with MALP2s and OVA showed the expansion of OVA-specific CD8+ T cells in the spleen at the same rate as observed in WT mice (**Figure 2E**). The result indicates that MALP2s induces Ag-specific CTLs irrespective of the type I IFN signal.

MALP2s/TAA Therapy Strongly Regresses CTL-Susceptible T Cell Lymphoma EG7

Tumor-associated antigen (TAA)-specific CTL plays an important role in effective cancer immunotherapy. We evaluated the potential of MALP2s as an antitumor adjuvant in a tumor-bearing mouse model. EG7 (OVA-positive EL4 T cell lymphoma)-implanted mice were locally administered with PBS, OVA, MALP2s, or MALP2s + OVA. Although OVA or MALP2s administration did not suppress tumor growth, the combination with MALP2s and OVA exerted potent tumor regressive effects (**Figure 3A**). The MALP2s/OVA-induced tumoricidal effect was completely dependent on CTL, while NK cells did not contribute to tumor regression (**Figure 3B**). On the sixth day after the MALP2s/OVA treatment, OVA-specific CD8+ T cells had expanded in the spleen and infiltrated the tumor tissue (**Figure 3C**). The OVA-specific CD8+ T cell induction was not observed in the PBS, OVA, and MALP2s groups (**Figure 3C**). Gene expression in tumor tissue was also analyzed simultaneously. The genes related to CTL cytotoxicity (*Gzmb*, *Prf1*, *Fasl*, and *Ifng*) and the chemokine genes related to CTL recruitment (*Ccl3*, *4*, and *5*; *Cxcl9*, *10*, and *11*) were elevated by MALP2s/OVA treatment (**Figure 3D**). The inflammatory cytokines (*Il6*, *Tnfa*, and *Il1b*) and immunosuppressive cytokine (*Il10*) were also analyzed. *Il1b* and *Il10* but not *Il6* and *Tnfa* were significantly elevated by MALP2s/OVA treatment (**Figure 3D**).

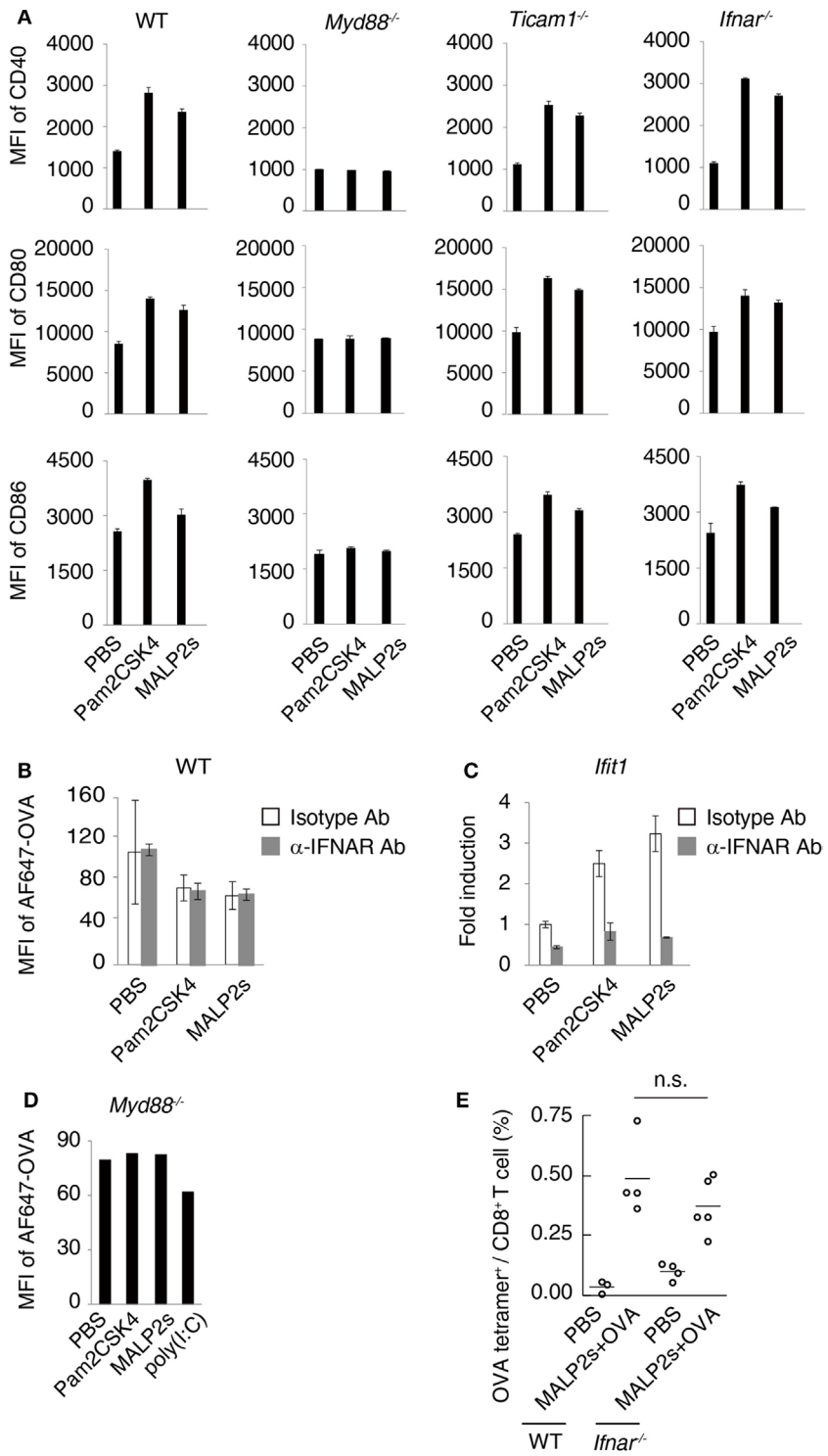

FIGURE 2 | Myeloid differentiation primary response 88 (MyD88) but not TICAM-1-Type I interferon (IFN) pathway is responsible for MALP2s-induced dendritic cell (DC) maturation. **(A)** PBS, 100 nM of Pam2CSK4, or MALP2s was added in bone marrow-derived dendritic cells (BMDCs) derived from various gene knockout mice. After 18 h, CD40, CD80, and CD86 expression levels on DCs were analyzed by flow cytometry. **(B,C)** WT-derived BMDCs were pretreated by 10 μg/ml of isotype antibody (Ab) or α-IFNAR Ab. After 1 h, PBS, Pam2sCSK4, or MALP2s was added. **(B)** After 18 h, 10 μg/ml of AF647-OVA was added and DCs were incubated for 20 min. After washing out OVA, an endocytic activity was assessed by MFI of endocytosed AF647-OVA on BMDCs. **(C)** After 6 h, *Ifit1* gene expression was analyzed by qPCR. **(D)** An endocytic activity of *Myd88*$^{-/-}$-derived BMDCs was assessed as in **(B)**. poly(I:C) was a positive control. **(E)** WT and *Ifnar*$^{-/-}$ mice were subcutaneously (s.c.) administered with PBS or MALP2s + OVA. After 7 days, the percentage of OVA-specific cells among splenic CD8^{+} T cells was analyzed by flow cytometry. **(A–C)** Error bars show ± SD. **(E)** n = 3 to 5 per group. Kruskal–Wallis test with Dunn's multiple comparison test was performed to analyze statistical significance. n.s., not significant.

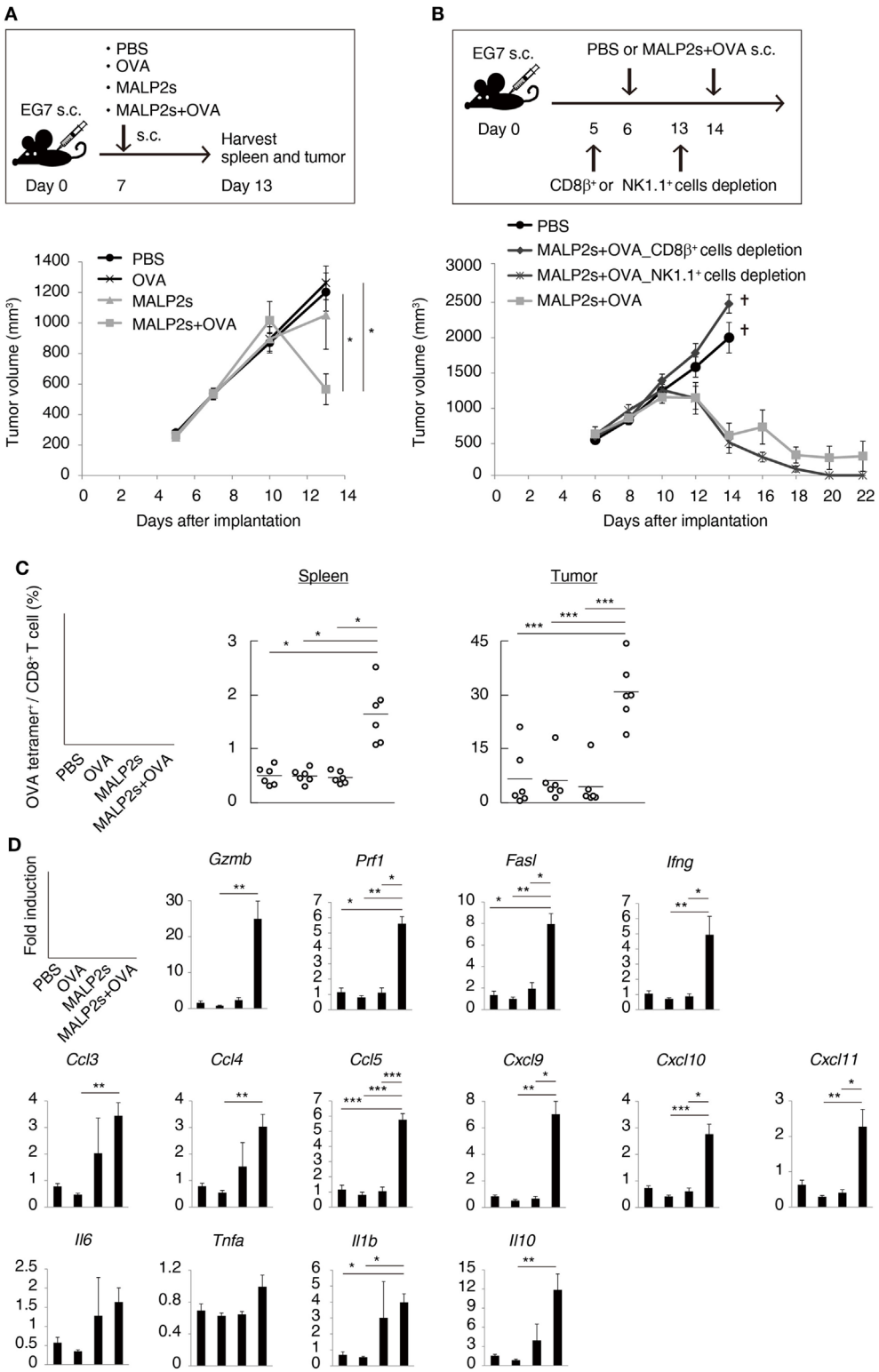

FIGURE 3 | MALP2s with tumor-associated antigen regresses EG7 tumor in a cytotoxic T lymphocytes-dependent manner. **(A,B)** EG7-bearing mice were treated as in the upper schemes. A tumor volume was measured in each group (lower Figures). PBS and CD8β$^+$ cells depletion groups of B were euthanized on day 14. **(C,D)** EG7-bearing mice of A were euthanized on day 13. **(C)** The percentages of OVA-specific cells among splenic and intratumor CD8$^+$ T cells were analyzed by flow cytometry. **(D)** Gene expressions in tumor tissue were analyzed by qPCR. Error bars show ± SEM; n = 4–6 per group. Kruskal–Wallis test with Dunn's multiple comparison test or One-way analysis of variance with Bonferroni's test was performed to analyze statistical significance (*$p < 0.05$, **$p < 0.01$ ***$p < 0.001$). **(A,C)** The results are representative of more than two independent experiments.

These results suggest that the combination therapy of MALP2s and TAA is an effective antitumor strategy in a CTL-susceptible tumor, though the cytokine problem still exists.

MALP2s and TAA Therapy Enhanced PD-L1 Blockade Efficacy

We previously reported that TLR3-specific CTL-priming adjuvant enhanced the therapeutic efficacy of PD-L1 blockade in some tumor-bearing mice models (33). Here, we also investigated the availability of MALP2s as an enhancer of PD-L1 blockade therapy in the MO5 (OVA-positive melanoma)-bearing mouse model. In tumor tissue from the MO5-bearing mouse, PD-L1 molecules expressed not only on the CD45$^-$ population including MO5 cells and mesenchymal cells but also on the CD45$^+$ population representing intratumor immune cells (**Figure 4**). The PD-L1 expression level was higher in intratumor immune cells than in MO5 cells. PD-L1 was also expressed in splenic immune cells (**Figure 4**). The PD-L1 distribution suggests that not only tumor cells but also intratumor and splenic immune cells are targets of PD-L1 blockade. MO5-implanted mice were locally administered with PBS or MALP2s + OVA and also treated with isotype Ab or α-PD-L1 Ab (**Figure 5A**). Both MALP2s + OVA and α-PD-L1 Ab therapy partially suppressed tumor growth, but the combination of MALP2s/OVA with α-PD-L1 Ab culminated in maximal tumor suppression (**Figure 5B**). On day 7 after MALP2s/OVA treatment, OVA-specific CD8$^+$ T cells had expanded in the spleen and infiltrated tumor tissue even without PD-L1 blockade (Figures S2A,B in Supplementary Material). However, on day 10 after MALP2s/OVA treatment without PD-L1 blockade, splenic OVA-specific CD8$^+$ T cells were decreased (**Figure 5C**). The proportion of OVA-specific cells in splenic CD8$^+$ T cells was also low in PBS and α-PD-L1 Ab monotherapy groups. On the other hand, the level of OVA-specific CD8$^+$ T cells was maintained high in spleens after combination therapy of MALP2s/OVA with α-PD-L1 Ab (**Figure 5C**). CD8$^+$ T cells infiltrated and remained longer in tumors by MALP2s/OVA therapy than α-PD-L1 Ab monotherapy. The infiltration increased most prominently by the combination therapy with MALP2s/OVA and α-PD-L1 Ab (**Figure 5C**). The ratio of OVA-specific cells among intratumor CD8$^+$ T cells was high in MALP2s/OVA groups with or without PD-L1 blockade, while α-PD-L1 Ab monotherapy did not induce TAA-specific CD8$^+$ T cell infiltration (**Figure 5C**). Gene expression in tumor tissue was also analyzed. Both α-PD-L1 Ab and MALP2s/OVA therapy modestly increased the expression levels of CTL cytotoxicity-related genes, but expression was increased most substantially after combination therapy. Combination therapy also significantly increased the expression of chemokine genes *Ccl3*, *4*, and *5* (**Figure 5D**). These results suggest that the MALP2s and TAA therapy enhance the efficacy of PD-L1 blockade by evoking TAA-specific CTL expansion in lymphoid tissue and facilitating TAA-specific CTL infiltration into the tumor site.

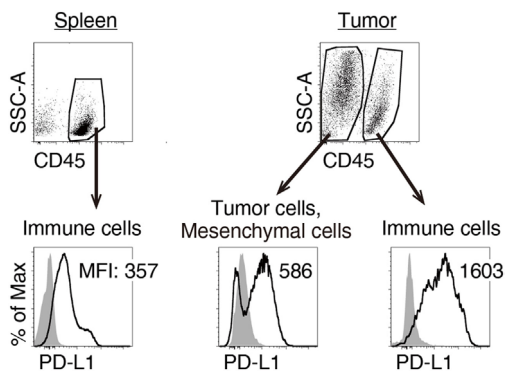

FIGURE 4 | Splenic and intratumor immune cells express programmed death ligand-1 (PD-L1). MO5-bearing mice were euthanized 16 days after tumor implantation. PD-L1 expression levels on splenic CD45$^+$ cells, intratumor CD45$^-$ and CD45$^+$ cells were analyzed by flow cytometry. Shaded and open histograms indicate isotype control and PD-L1 staining, respectively. The results are representative of more than three independent experiments.

DISCUSSION

The existence of TAA-specific CTLs is a key factor affecting clinical outcomes in many types of cancer. Many clinical trials have shown that intratumor CD8$^+$ T cell levels positively correlate with overall survival and the success of antitumor immunotherapy (34). For the expansion of TAA-specific CTLs, cross-presentation by mature DCs is essential. Thus, an immunostimulatory adjuvant like a TLR agonist evoking DC maturation and subsequent CTL priming may be a promising antitumor agent. Human conventional DCs only express TLR2/TLR1/TLR6 and TLR4, in addition to TLR3 (35). In particular, human CD141$^+$ DCs, which are a professional Ag-presenting DC subset, showed selective high expression of TLR3, TLR2/TLR1, and TLR 6 (36). TLR1 and TLR6 are co-receptors of TLR2 for recognition of Pam3 and Pam2 lipopeptides, respectively (9, 37). Hence, the application of a TLR2 agonist as a clinical antitumor adjuvant is rational.

We previously showed that the short length of three amino acids following Pam2Cys was sufficient for TLR2 stimulation. The first amino acid residue following Pam2Cys determined the agonistic activity and Ser/Thr or Gly/Ala were functional residues (26). The first amino acid residue following Pam2Cys of MALP2s is Gly. In this study, MALP2s promoted DC maturation and potent cross-priming activity (**Figures 1** and **2**). The sequence containing the last eight amino acids of full-length MALP2 was not essential for cross-presentation. Amino acid substitution studies support this idea (38): we have evidence that the peptide sequence of the Pam2 lipopeptide alters the TLR2 agonistic function in DC models (26). The MALP2-induced inflammatory cytokines and NO production fully depend on the MyD88 pathway (39). Recent findings revealed that TLR2 signaling induces not only inflammatory cytokines but also low amounts of type I IFN production (5, 27–29). TLR2 located on the endosomal compartment but not on the plasma membrane can induce type I IFN production. Although the intracellular signaling pathway is still unclear and there seems to be a difference among cell types, the MyD88–interferon regulatory factors axis may be

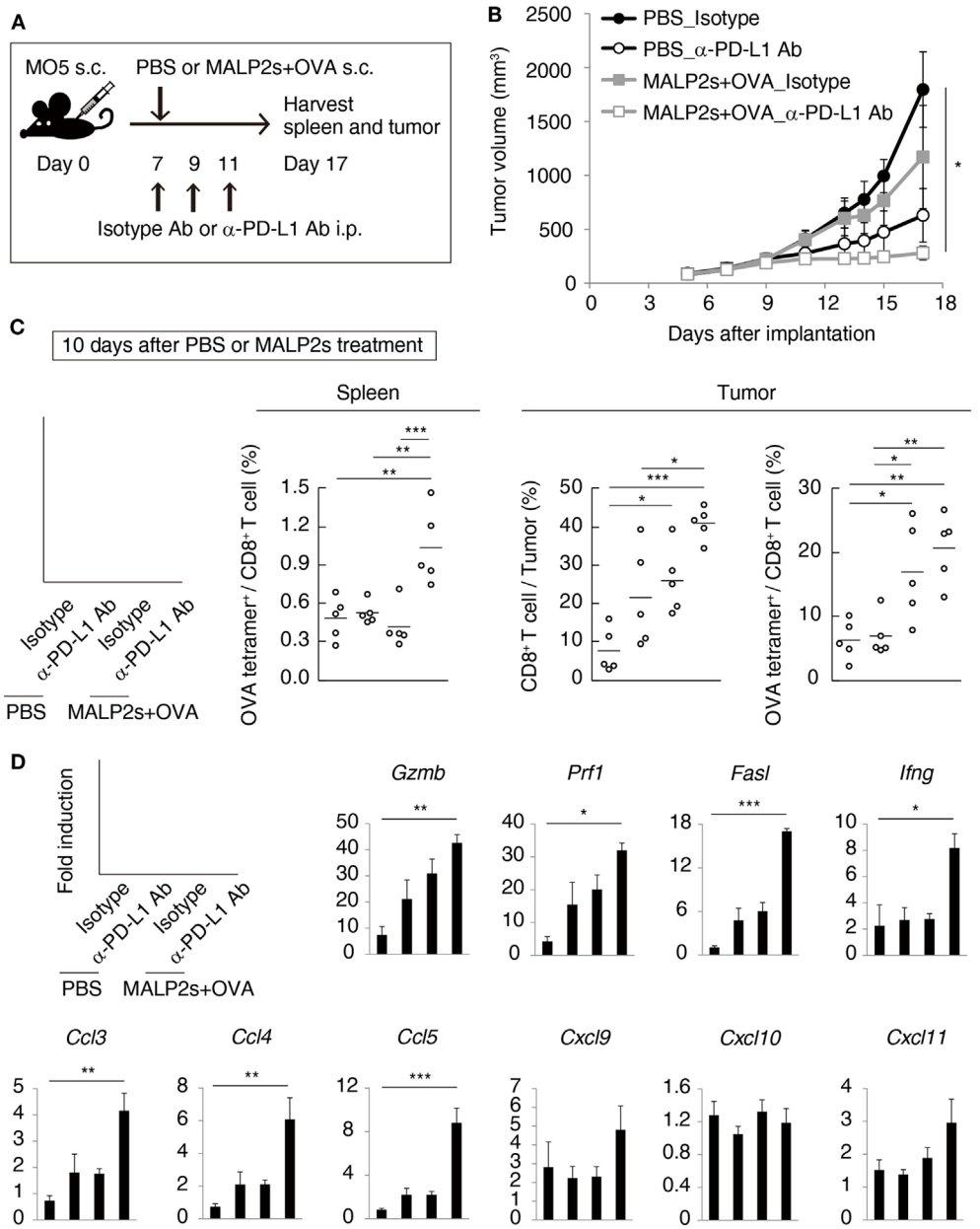

FIGURE 5 | MALP2s with tumor-associated antigen enhances antitumor response by programmed death ligand-1 (PD-L1) blockade. **(A)** The scheme of each treatment on MO5-bearing mice is shown. **(B)** A tumor volume was measured in each group. **(C,D)** Mice were euthanized on day 17. **(C)** The percentages of tumor-infiltrating $CD8^+$ T cells and splenic and intratumor OVA-specific $CD8^+$ T cells were analyzed by flow cytometry. **(D)** Gene expressions in tumor tissue were analyzed by qPCR. Error bars show ± SEM; $n = 5$ per group. Kruskal–Wallis test with Dunn's multiple comparison test or One-way analysis of variance with Bonferroni's test was performed to analyze statistical significance (*$p < 0.05$, **$p < 0.01$ ***$p < 0.001$).

dispensable for amplifying type I IFN production. TICAM-1 and/or TRAM molecules appeared to partially contribute to type I IFN production from bone marrow-derived macrophages (28, 29). Since type I IFN is an inducer of DC maturation and cross-presentation (40), we assessed the contribution of type I IFN to MALP2s-induced CTL priming. While TICAM-1 and type I IFN signaling were not involved in DC maturation, MyD88 was essential for MALP2s-mediated DC priming. Type I IFN signaling was also not required for MALP2s-induced cross-priming (**Figure 2**). The MyD88–NF-κB/AP-1 axis has been considered as the pathway responsible for CTL priming, but details of the intracellular molecular cascade have not been elucidated. Lymphocytes also utilize the MyD88 pathway (41), but their participation, if any, in antitumor CTL proliferation for tumor regression appears to be minimal, at least in this tumor model.

The antitumor activity of MALP2 has been shown in a pancreatic cancer-bearing mouse model (42). MALP2 administration prolonged survival and enhanced the effectiveness of combination therapy with gemcitabine. We show here that MALP2 induces splenic $CD8^+$ T cell expansion. Clinical research on MALP2 administration has been also performed in pancreatic carcinoma patients (43). In phase I/II trials, patients were treated with MALP2 and gemcitabine. Although the number of patients was small and clinical efficacy still remains to be demonstrated, the results suggested the potential of MALP2 using as a clinical antitumor adjuvant. One potential issue, however, was not the DC-priming activity but rather the cytokine toxicity of MALP2. We thus evaluated the antitumor activity of MALP2s in OVA-positive lymphoma (EG7)- and melanoma (MO5)-bearing mouse models. In the EG7-bearing model, MALP2s monotherapy did not show any tumor suppressive activity. However, combination with TAA dramatically induced CTL-dependent tumor regression, facilitated CTL priming in lymphoid tissue and fostered TAA-specific CTL migration to tumor sites (**Figure 3**). The result indicates that endogenous TAA is insufficient to evoke CTL induction in MALP2s therapy and that co-administration of exogenous TAA is needed. For future clinical application, appropriate selection and supply of exogenous TAA should be established.

The synthesis of a fusion peptide containing MALP2s and a TAA epitope might be a valid strategy. Shen et al. designed Pam2 lipopeptides containing the H-$2D^b$-restricted CTL epitope from HPV16 E7 protein and showed it had cross-priming activity (17). The short length of MALP2s may be suitable for the design of a fusion peptide. However, we have no rationale why the fusion of TAA with MALP2 is better than separate MALP2 and TAA administrations (44), since each acts upon different targets in DCs.

Immune checkpoint inhibition including CTL-associated Ag 4 and PD-1/PD-L1 blockade shows great clinical efficacy in refractory and metastatic cancers. However, the number of patients responsive to blockade therapy is small. A valid therapeutic strategy to overcome this clinical limitation is strongly desired. Neoantigen load may be a useful biomarker for assessing the sensitivity to immune checkpoint blockade which positively correlates with clinical benefit (45–48). However, nonresponders to blockade therapy with high neoantigen loads also exist (45, 47, 49). This fact suggests that even if TAA has immunogenicity, TAA peptide administration alone is insufficient and an additional factor may be necessary to evoke an antitumor response in some unresponsive patients. Roony et al. showed that not only neoantigen load but also the existence of an endogenous or exogenous virus was positively correlated with a cytolytic activity against tumors (50). DAMP might positively or negatively regulate the tumor microenvironment in PD-1/PD-L1 therapy. However, the exact constitution of DAMPs has not yet been identified. In contrast, the PAMPs which trigger cross-presentation with defined molecules are supported by protein–chemical database background. We previously showed that EG7, which expresses immunogenic OVA, was unresponsive to α-PD-L1 Ab monotherapy and that combination therapy with a TLR3 agonist relieved the unresponsiveness (33). Intratumor $CD8^+$ T cells were few in EG7-bearing mice with or without α-PD-L1 Ab monotherapy, and this observation suggests that EG7 lacks an endogenous immune stimulator like DAMPs. In addition to TLR3 agonists, TLR7 and TLR9 agonists also augmented the therapeutic efficacy of PD-1/PD-L1 blockade in preclinical mouse models (51, 52). These findings suggest that TLR adjuvant with DC-priming activity may overcome the clinical limitation of immune checkpoint blockade in patients lacking an endogenous immune stimulator. However, circumvention of cytokine toxicity still remains problematic. How we should design a less toxic derivative based on our current knowledge of the Pam2 lipopeptide (26) is an issue that will soon need to be addressed.

While TLR2 agonists have antitumor activity, tumor-supportive activities have also reported. TLR2 signaling promoted survival and proliferation of certain types of tumors (53). TLR2 signaling also promoted expansion of myeloid-derived suppressor cells and regulatory T cell expansion and enhanced their suppressive functions (54, 55). In order to eliminate or reduce these tumor-supportive effects, DC targeting by adjuvant, particularly DC-priming adjuvant, may be effective. Akazawa et al. designed a Pam2 lipopeptide containing a DC-targeting sequence which showed antitumor activity (56). MALP2s will likely be an appropriate lipopeptide for sequence modulation that can expect further development.

In conclusion, we showed the synergistic antitumor effect of MALP2s/TAA and α-PD-L1 Ab treatment (**Figure 5**). MALP2s/TAA contributed to TAA-specific CTL priming in lymphoid tissue while α-PD-L1 Ab helped to prevent CTL exhaustion and cell death in both lymphoid tissue and tumor tissue. Molecular modification of MALP2s will be a strategy for further development of an antitumor adjuvant that overcomes PD-1/PD-L1 blockade resistance in patients.

ETHICS STATEMENT

All animal research protocols for this work were reviewed and approved by the Animal Safety Center (#17-0096) in Hokkaido University, Japan.

AUTHOR CONTRIBUTIONS

YT, MA, KF, MM, and TS conceived and designed the experiments. YT, MA, and KF performed the experiments. YT, MA, KF, HS, MM, and TS analyzed the data. YT, MM, and TS wrote the paper. MM and TS supervised the research.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00496/full#supplementary-material.

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REVIEW
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Strategies to Genetically Modulate Dendritic Cells to Potentiate Anti-Tumor Responses in Hematologic Malignancies

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Dendritic cell (DC) vaccination has been investigated as a potential strategy to target hematologic malignancies, while generating sustained immunological responses to control potential future relapse. Nonetheless, few clinical trials have shown robust long-term efficacy. It has been suggested that a combination of surmountable shortcomings, such as selection of utilized DC subsets, DC loading and maturation strategies, as well as tumor-induced immunosuppression may be targeted to maximize anti-tumor responses of DC vaccines. Generation of DC from CD34+ hematopoietic stem and progenitor cells (HSPCs) may provide potential in patients undergoing allogeneic HSPC transplantations for hematologic malignancies. CD34+ HSPC from the graft can be genetically modified to optimize antigen presentation and to provide sufficient T cell stimulatory signals. We here describe beneficial (gene)-modifications that can be implemented in various processes in T cell activation by DC, among which major histocompatibility complex (MHC) class I and MHC class II presentation, DC maturation and migration, cross-presentation, co-stimulation, and immunosuppression to improve anti-tumor responses.

Keywords: dendritic cell, vaccination, genetic modification, hematopoietic cells, hematopoietic cell transplantation, cord blood

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INTRODUCTION

Although the overall survival rates of patients with hematologic malignancies have significantly increased in the past decades, the 5-year survival of certain acute leukemias, such as acute myeloid leukemia (AML) is still unsatisfactory due to high relapse risk (1–4). Currently, the only curative treatment consists of intense chemotherapy followed by hematopoietic cell transplantation (HCT), but only about 30% of candidates eligible for HCT transplantation have a human leukocyte antigen (HLA)-identical sibling as a donor for matched transplantation. Alternatively, bone marrow from unrelated volunteer donors could be used; however, this is limited by strict HLA-matching criteria, because of higher risks of graft-versus-host disease (GVHD), and donor availability.

Umbilical cord blood (UCB) transplantation has advantages because of its prompt availability from UCB banks, the possibility of HLA-mismatched transplantations, a lower risk of acute and chronic GVHD, and a potential higher graft-versus-leukemia effect (5–8).

Individualized dosing and timing of chemo and/or serotherapy improves overall survival of transplanted patients with hematologic malignancies after cord blood transplantation (9, 10). Cord blood T cells have shown the ability to rapidly reconstitute the immune system (9), and can

mediate enhanced anti-tumor effects when compared with adult peripheral T cells (11). In addition, cord blood CD8+ T cells have shown to exhibit stronger proliferation potential and function after antigen-specific stimulation (12). The relatively low survival rate of patients with hematologic malignancies underlines the relevance to investigate novel potential effective therapies in the context of UCB transplantation to treat AML or other hematologic malignancies.

Tumor-associated antigen (TAA)-specific immunotherapy to prime the TAA-specific T cells against the leukemia to consequently induce remission has been thoroughly investigated. Four decades of research revealed the central role of dendritic cells (DCs) as a link between innate and adaptive immunity, and thereby its essential role in the control of both immune tolerance and immunity (13). The antigen presentation machinery of DCs is exploited in cellular vaccination strategies to initiate an endogenous anti-tumor response (14). The rationale for this approach is the generation of TAA-specific cytotoxic T lymphocyte (CTL) responses to specifically eradicate tumor cells and to generate immunological memory to control potential future tumor relapse (15). However, DC vaccine trials have only sporadically shown clinical responses. Insufficient DC maturation, suboptimal antigen presentation, co-stimulation, migration, or impaired initiation of anti-TAA T cell responses could be inherent to the cultured DC subset, but may also be influenced by the inhibition of immune responses by the tumor microenvironment (14, 16). Hence, efficacy of DC vaccination strategies can be improved by state-of-the-art genetic modification tools, such as messenger RNA, adenoviral and lentiviral vectors, and gene-editing techniques to enhance processes in DC activation of T cells (15, 17) and consequently boost immune responses. In this review, we will address modification of phenotypes and function of DCs, including cord blood CD34-derived DCs, to optimize the anti-tumor response to protect for relapses after HCT.

DC SUBSETS ELIGIBLE FOR MODIFICATION

Although thoroughly investigated, there is still no consensus about the most optimal DC subset to use to induce optimal TAA-specific T cell responses (18). Circulating peripheral blood DCs are difficult to isolate, hence monocyte-derived DCs (moDCs), generated from peripheral blood mononuclear cells are the most commonly used. These cells are generated from monocytes by use of granulocyte macrophage colony stimulating factor and interleukin (IL)-4 (18). Although moDC-derived vaccines are reported to be safe, clinical responses have only sporadically been observed (15, 17).

Research investigating different DC subsets pointed to differential subsets (such as conventional and plasmacytoid DCs) and functionalities (19), which could be of importance to induce favorable immune responses. The advantage of using primary DCs is that they can be promptly isolated from blood, avoiding long differentiation incubation periods before administration to the patient, thereby making this strategy suitable for standardization for multicentre trials (20). However, the differentiated status of these cells is also a drawback, as this limits expansion of the cell population. As a result, large numbers of primary DC may be required to provide effective therapeutic dosing.

Another commonly used approach is to produce DCs from CD34+ hematopoietic stem and progenitor cells (HSPCs) (21, 22), which have an extensive proliferation capacity to generate antigen presenting cells (APCs) with a primary DC phenotype (23) and the capacity to induce robust anti-tumor T cell responses. These cells are distinct from moDCs (24–27), and more resemble conventional DC or Langerhans resembling cells (28) that induce stronger anti-tumor T cell responses compared with moDCs (29). In the setting of cord blood transplantation after chemotherapy in hematologic malignancies, CD34+ HSPCs can be extracted from 20% of the remaining unit that is not transplanted, and developed into an effective DC vaccine, that can be modified at different stages of the manufacturing process, which will be discussed below.

Vaccination with UCB CD34-derived DCs has been performed in clinical trials to treat patients with melanoma and showed TAA-specific responses in some patients (23, 30). The *ex vivo* culturing phase to generate CD34-derived DCs provides a unique opportunity to enhance efficacy through genetic modification. Principally, the expansion phase of the protocol could be extended to 2 weeks and this does this not affect DC maturation (26). This indicates that this two-step protocol allows opportunities to modify the CD34-derived DCs at the early stage as well as during the later stages of the protocol, as compared with DCs generated from other precursor subsets.

MODULATING TAA-LOADING AND MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)-I PRESENTATION TO ENHANCE DC EFFICIENCY

Tumor-associated antigens are ideally over expressed on malignant cells and are simultaneously not expressed on healthy tissues or contain mutations leading to neo-antigens recognizable to T cells. Hence, a commonly used TAA is the oncoprotein Wilms' tumor-1 (WT1), which has been ranked the number one cancer vaccine target antigen (31). WT1 is a zinc finger transcription factor with a well-established oncogenic role in WT1 overexpressing malignancies (32). WT1 overexpression is observed in the majority of acute leukemias (~90% of pediatric AML cases), as well as various solid tumors (33), making WT1 an obvious vaccine target. Despite its physiological expression in hematopoietic tissue–limited expression in the urogenital–and central nervous system (34), it has been shown that tumor overexpression of WT1 can be targeted without considerable safety concerns (35, 36). Several recent early-phase anti-WT1 DC vaccine clinical trials in multiple cancer types reported a correlation between anti-WT1 CTL responses and clinical response (35, 37, 38), showing its potential as a therapeutic strategy.

The most commonly used methods to present antigen are delivery of peptide pools or mRNA to express the tumor antigen-target, which result in the ability to transiently load DCs with antigen. An advantage to deliver mRNA is that it prevents HLA-restrictions and invasive tumor tissue isolation from patients. Alternatively, full-length WT1 mRNA can also

be combined with a WT1 peptide pool to enhance its potential (14, 39). Two main modification strategies have been reported to potentially optimize TAA-loading and MHC-I presentation of WT1 epitopes: increasing translational efficiency or increasing proteasome targeting of the TAA. Codon-optimization of nucleotide sequences is commonly used to enhance expression of a transgene to increase the amount of transgene product, which could be a limiting factor in vaccinations strategies. Algorithms include selection of more commonly used codons to improve translation, but can also include features addressing transcription, mRNA processing and stability as well as protein folding. For the delivery of mRNA, transcription can be excluded as a relevant parameter for improvement, but all others may be useful. It was reported that codon-optimization of the human papillomavirus (HPV) E7 oncoprotein sequence resulted in much higher protein translation and induced CD8+ T cell responses to cryptic epitopes not harbored by wildtype E7 (40). Codon-optimization could, therefore, confer additional advantages then using native mRNA sequences.

Benteyn et al. attempted to optimize translational efficiency of full-length WT1 mRNA (41), but there was no significant advantage of the codon-optimization detected. However, transgene expression was optimized using the pST1 RNA transcription plasmid to generate *in vitro* synthesized mRNA with enhanced translational properties (42). This modification resulted in doubling of the interferon-γ (IFN-γ) responses in a T cell clone. Another feature employed to improve antigen presentation in both MHC-I and MHC-II was the inclusion of endosomal or lysosomal targeting sequences fused to the antigen sequence (43, 44). In particular, the fusion of the C-terminus of LAMP/DC-LAMP to the WT1 mRNA enhanced the IFN-γ also in a T cell clone (41) by increasing both MHC-I presentation and cross-presentation of WT1 peptides. These modifications only require adaptation of the WT1 mRNA sequence, which makes it relatively easy and efficient to implement in a DC vaccine.

Hosoi et al. attempted to optimize proteasome targeting to increase protein degradation and enhance presentation of full-length TAA by triggering co-translational polyubiquitination (45). This triggering of co-translational ubiquitination of the TAA resulted in more efficient priming and expansion of TAA-specific CTLs (45).

To further improve DC vaccination multi-epitope delivery may be beneficial for enhanced CTL activation, e.g., WT1 for AML treatment can be combined with proteinase 3, preferentially expressed antigen in melanoma, telomerase reverse transcriptase, or FLT3-internal tandem duplication (46) for maximal responses. In a multi-epitope vaccine combining multiple myeloma special antigen-1 and Dickkopf-1 to treat multiple myeloma enhanced responses were observed (47).

Viral vectors can also be used to deliver antigen. DCs are highly amenable to lentiviral vector transduction (48). A study using mouse DCs comparing lentiviral vectors that stably integrate into the host genome and provide constant transgene antigen expression to mRNA electroporation showed that lentiviral vector delivery enhanced IFN-γ responses to MAGE-A3 epitopes (49). In the context of UCB-derived DCs, lentiviral vectors could potentially be very useful, since $<5 \times 10^6$ CD34+ progenitors can be used for the initial transduction and form the basis for expansion of large number of matured DCs ($>500 \times 10^6$). Another more recent approach uses lentiviral protein transfer vectors for targeting transfer directly into APCs and inducing cytotoxic T cell responses, which could also be used for *ex vivo* delivery (48).

More research is necessary to confirm that the above mentioned modifications could be generally applied to other TAAs or whether this enhances efficacy of CD34-derived DC vaccines.

MODULATING DC MATURATION TO IMPROVE DC EFFICIENCY

Although consensus is reached that DC vaccines should contain mature rather than immature DCs, there is no consensus about how to polarize and mature DCs to cause optimal anti-tumor responses (50). In 1997, Jonuleit et al. showed that incubation of immature DCs with a cocktail of IL-1β, TNFα, IL-6, and PGE_2, similar to the GMP-grade available CYTOMIX, resulted in induction of fully matured DCs that seemed to be optimal for generation of IFN-γ producing CD4/CD8+ T cells (51), but very limited efficacy was observed. It is questionable whether to include PGE_2 as it decreases the expression of IL-12p70 (50), a factor important in induction of tumor-specific Th1 T cells and CTLs facilitating tumor rejection in mouse models (52).

It is reported that DC maturation cocktails containing IFN-γ instead of PGE_2 [the α-type-1-polarized DC cocktail (αDC1)] increases IL-12p70 levels *in vitro* and *in vivo* boosting TAA-specific CTL levels 40-fold *in vitro* in melanoma (53, 54). Superiority of αDC1-induced maturation was also observed in chronic lymphocytic leukemia assays *in vitro* (55). Similarly, addition of IFN-γ to the CYTOMIX maturation cocktail can increase IL-12p70 production upon CD40 stimulation in WT1 expressing DCs (26).

Another strategy to mature DCs would be to introduce maturation agents with gene therapy. A major advantage of this approach is that DCs can be used within a few hours after delivery of maturation stimuli for vaccination, whereas culturing in maturation agents requires a 24-h incubation period (56). This incubation period *in vitro* potentially leads to DC exhaustion and dampening of the immune response, as shown by Bonehill et al. (56). Single introduction of constitutionally active toll-like receptor 4 (caTLR4) (56, 57) and CD40L (56–58) in immature DCs has shown to induce potent DC maturation, including IL-12p70 production, and both stimuli also act synergistically to superior DC maturation. Melan-A TAA-primed DCs co-electroporated with caTLR4, CD40L, and CD70 mRNA showed an even >200-fold increase in Melan-A specific CTL responses when compared with CYTOMIX matured DCs (56). To date, direct comparisons of this strategy with αDC1-induced maturation of DCs are lacking.

The combination of these three proteins is known as the TriMix strategy, which was developed at the Free University of Brussels, introducing the danger signal caTRL4, the co-stimulatory protein CD40L both to stimulate maturation, and a co-stimulatory protein involved in early T cell activation (CD70) (41, 56, 57, 59, 60). An interesting factor in this strategy is that the DCs mature after electroporation of these factors, eliminating the need

of DC incubation with maturation cocktails. A phase-II clinical trial in advanced melanoma showed that combining TriMix-matured moDCs presenting melanoma-associated antigens with ipilumab, an antagonistic CTLA4 antibody, resulted in a 6-month disease control rate of 51%, with an overall tumor response rate of 38% (59). This strategy nicely shows the potential of combining multiple modifications to improve tumor-immunity of DC vaccination.

In the TriMix DCs, maturation of DCs is maximized to improve activation, and polarization of T cells to increase tumor-immunity. However, for an optimal result, it is widely suggested that immunosuppression should be counteracted as well. This is partly established in the TriMix trial by combining the TriMix-matured DCs with ipilimumab, as it inhibits the co-inhibitory effect of the T cell membrane protein CTLA4 on CD80/CD86/CD28 co-stimulation (59).

MODULATING DC MIGRATION TO ENHANCE DC EFFICIENCY

There is no consensus about the most efficient administration route of DC vaccines to migrate to the draining lymph nodes (16). Administration of 111-indium labeled moDCs into patients revealed that less than 5% of the intradermally injected mature moDCs reach the draining lymph nodes (61). A major player in DC migration to the lymph nodes is the C-C motif chemokine receptor 7 (CCR7) (62). Migration to the lymph node is stimulated upon interaction with its ligand, the chemokine C-C motif ligand (CCL21) (63). Adenoviral transduction of DCs with CCR7 (64) and CCL21 (65, 66) showed an ~5.5-fold increase in DC lymph node accumulation, and enhanced tumor rejection and T cell priming in mice *in vivo,* respectively. This could not only increase the effectiveness of the vaccine, but may also reduce the required dose, hence, the efforts and costs associated with vaccine preparation (64). Based on these results, a GMP-grade CCL21 gene-modified monocyte-derived DC vaccine was developed (67), subsequently used in a phase-I clinical trial with non-TAA loaded CCL21 expressing DCs, which triggered TAA-specific T cell responses and enhanced CD8+ T cell tumor infiltration in a subset of patients with non-small cell lung carcinoma (68). Interestingly, CCL21 excretion attracts naive T cells and addition of TAA peptide pools and maturation of DCs may further increase the therapeutic effect. CCL21 could also be applied by mRNA delivery for transient expression similar to adenoviral vectors.

Alternatively, strategies to reduce DC tissue retention could be applied to increase DC migration by disrupting the homing factor E-cadherin (69) (or its positive regulator TGF-β) inducing upregulation of CCR7 (70). Downregulation of E-cadherin upon pro-inflammatory signaling (*via* TNFα, LPS, and IL-1β) further strengthens the hypothesis of involvement of E-cadherin in DC migration (69). The use of small interfering RNAs to downregulate E-cadherin expression on DCs and its effect on migratory function and immune stimulation may be an interesting option. TGF-β is also a known immunosuppressant of DCs, which makes interference of its expression a potential strategy to improve DC vaccination (71).

MHC-II CROSS-PRESENTATION TO ENHANCE DC FUNCTION

Major histocompatibility complex-II antigen presentation is required to establish long-term memory anti-tumor immunity through stimulation of CD8+ T cells by CD4+ T cells inducing strong clonal expansion, cytokine production, tumor cell lysis, and T cell memory (72–75). MHC-II knockout DCs were able to generate potent anti-tumor CTL responses *in vivo*, however, without subsequent establishment of a memory anti-tumor response. Therefore, a critical factor in the development of a successful DC vaccine is the ability to present the TAA in both MHC-I and MHC-II context (39).

Full-length TAA mRNA translates into proteins ensuring the presence of MHC-I and MHC-II TAA epitopes, without the requirement of algorithms to predict epitopes per HLA-subtype (14, 39). To further boost this response, a broad TAA peptide pool can be administered in addition to the mRNA electroporation or viral vector delivery. To improve MHC-II presentation of TAA, antigen has also been targeted to endolysosomal compartments to try to improve MHC-II antigen presentation, but this resulted in increased numbers of regulatory T cells (Tregs) and attenuation of tumor immunity (76).

Many studies exploited strategies that link small epitopes to proteins increasing their likelihood of MHC-II presentation (77), however, these epitopes are difficult to predict, are MHC-II restricted, and vary per HLA-subtype and antigen. Therefore, targeting of full-length antigens to the MHC-II pathway is more desirable. Two main MHC-II pathway targeting strategies can be distinguished. The first strategy links the TAA of interest to the cytoplasmic tail of residential endolysosomal proteins, which contains the information for transport to the endolysosomal compartment. Residential endolysosomal proteins tested for this strategy include DC-LAMP (41, 43), LAMP1 (43, 44), and LIMPII (78). The second strategy entails linking of the TAA of interest to the MHC-II associated invariant chain (Ii), a protein important in MHC-II conformational regulation, thereby targeting the TAA to the endolysosomal compartment (43, 79). All studies, irrespective of the endolysosomal protein used, concluded that the increased cross-presentation enhanced CD4+ and CD8+ T cell activation and increased anti-tumor immunity *in vitro* and *in vivo*. Direct comparison of strategies using DC-LAMP, LAMP1, and Ii showed that DC-LAMP and LAMP1 have more pronounced effects than using Ii (43). Interestingly, no clinical studies incorporated these cross-presentation tools into vaccines, even though some cited papers are over 20 years old.

MODULATION OF CO-STIMULATION TO BOOST DC FUNCTION

A T lymphocyte requires three signals to become fully activated (80), of which co-stimulation is provided by interaction between co-stimulatory molecules expressed on the DC and T lymphocyte. Lack of DC maturation and subsequent co-stimulation induces tolerance against the presented antigen, making these processes of vital importance in the generation of an anti-tumor response. Several co-stimulatory interactions between DCs and T cells have

been explored, including CD40/CD40L, 4-1BB/4-1BBL, OX40/OX40L, CD80/86/CD28, CD27/CD70, and GITR/GITRL.

The interaction between CD40 and CD40L, expressed on DCs and T cells respectively, is one of the most potent DC activating signals (56, 81). Modifications to this axis have, therefore, been widely studied to optimize DC vaccination (41, 56–60, 82). Introduction of CD40L into DCs provides autonomous maturation and co-stimulation of DCs (83). In this way, "licensing" of DCs through CD40L interaction with CD4+ Th1 T cells is not required for initiation of a TAA-specific CTL response, and these DCs elicited superior anti-tumor immunity and inhibition of pre-existing tumor growth *via* induction of a TAA-specific CD4+/CD8+ anti-tumor response *in vitro* (56–58, 83) and *in vivo* (41, 82). In addition, introduction of OX40 (84), 4-1BB (85, 86), GITRL (87), and CD70 (41, 56, 57, 59) in DCs is all reported to increase the anti-tumor effect *in vitro* and *in vivo*. All the approaches used mRNA to deliver the co-stimulatory signals.

Upon maturation, OX40L expression is induced in DCs, a ligand of the T-lymphocytic membrane protein OX40. Upregulation of OX40L is stimulated by PGE_2 (88), but PGE_2 also downregulates IL-12p70 (50, 52). Therefore, the observed positive effect of OX40/OX40L co-stimulation on tumor rejection (89, 90), through CD4+/CD8+ T cell proliferation, prevention of T cell death, and prevention of tolerance induction, is caused by an unknown mechanism independent of IL-12p70 upregulation (84, 88, 91). Dannull et al. showed that targeting OX40L as a downstream factor of PGE_2 potentially circumvents the PGE_2-mediated attenuation of DC function, while utilizing its IL-12p70 independent immunostimulatory capacity in DC vaccination (84).

Another co-stimulatory interaction, 4-1BB/4-1BBL, plays a key role in activation, proliferation, and memory development of CTLs (92). 4-1BB is exploited in second and third generation chimeric antigen receptors in CTLs to provide long-lasting activation potential. 4-1BBL mRNA introduction in HER2/neu TAA expressing DCs resulted in an increased TAA-specific CTL response *in vitro* (85), which was also supported by studies using agonistic anti-4-1BB antibodies *in vitro* and *in vivo* (86, 93). Similar results were observed in the context of HIV-specific T cell responses (86).

A less pronounced effect has been reported for GITR/GITRL co-stimulation, which enhances CD4+/CD8+ T cell responses, while inhibiting Treg-mediated immune suppression (87, 94). A new approach to introduce heavy and light chains of an agonistic anti-GITR antibody in DCs could stimulate this pathway (87). Combining vaccination of these anti-GITR-secreting DCs with TAA-presenting DCs resulted in an increased CTL response, and inhibition of sensitivity to Treg mediated immune suppression, thereby increasing anti-tumor immunity *in vitro* and *in vivo*. This approach may cause less systemic adverse effects, while maintaining the anti-tumor response (87).

Finally, CD27/CD70 interaction promotes clonal expansion of primed CD4+/CD8+ T cells, mostly *via* supporting survival of primed T cell clones (87). The constitutive expression of CD27 on T cells, by contrast to the other T-lymphocytic co-stimulatory molecules, indicates an important role during early T cell priming, making its ligand an interesting molecule to modify in DC vaccination. Keller et al. showed that constitutive expression of CD70 in steady-state immature DCs loaded with TAA can overcome peripheral resistance (95), and resulted in a robust effector and memory CTL response *in vitro* and *in vivo*, even in absence of CD4+ T cells (96).

Multiple papers reported the beneficial effects of combining autonomous DC maturation *via* CD40L introduction with factors enhancing T cell activation through 4-1BBL (86) and CD70, in combination with caTRL4 (41, 56, 57, 59, 60), respectively, on tumor immunity. Introduction of these co-stimulatory provide multiple opportunities to enhance tumor immunity through incorporation into DC vaccines.

INTERFERANCE WITH CO-INHIBITORY AND IMMUNOSUPPRESSIVE PATHWAYS TO ENHANCE DC FUNCTION

Dendritic cells should live long enough to generate a potent anti-tumor response, but have a physiological short lifespan (14). Moreover, remaining activatory DCs presenting TAA in MHC-I context are killed by activated TAA-specific CTLs, which probably also is a physiological mechanism to prevent exaggeration of immune responses (97–99). A major concern in DC vaccination is that DC injection in TAA-primed mice results in DC elimination before reaching the draining lymph node (100, 101). DC elimination by CTLs can even be used as a measure for effective cytotoxic response (100, 101). DC apoptosis is triggered physiologically, as well as by the tumor microenvironment. Inhibiting DC apoptosis can prolong the DC lifespan after siRNA-mediated silencing of the pro-apoptotic proteins BAK/BAX (97, 102), BIM (98), and PTEN (99) *in vitro* and *in vivo*, which all resulted in more efficient TAA-specific CTL responses. Disadvantages inherent to modifications of pro-apoptotic proteins are the potential oncogenicity, restricting its use to temporary silencing strategies, e.g., siRNAs.

A second strategy is to inhibit tolerogenic DC development to prevent induction of anergic T cells. Silencing of several factors has been proposed, including suppressor of cytokine signaling 1 (SOCS1), IL-10, IL-10R, and TGF-βR. SOCS1 is an inducible negative feedback inhibitor of the JAK/STAT pathway and thereby negatively regulates expression of multiple cytokines, including IFN-γ, IL-2, IL-6, IL-7, IL-12, and IL-15 (103). SOCS1 deficient DCs are reported to be extremely hyperresponsive to IL-4 and IFN-γ and cause abnormal accumulation of antigen-specific T cells (104). Vaccination with HPV16mE7 pulsed, shRNA-mediated SOCS1-silenced DCs showed significantly improved anti-tumor effects compared to non-SOCS1-silenced controls *in vitro* and *in vivo* (103).

The most well-known immunosuppressive cytokines are IL-10 and TGF-β, produced by Tregs, among others, to induce DC tolerance and anergic T cells (105). The fact that high serum levels of both IL-10 and TGF-β are correlated with poor prognosis in several types of cancer indicates an interesting role of inhibition of their expression or responsiveness to their presence (71, 106). As IL-10 can be produced by DCs (107), one way to decrease its effect is to silence IL-10 expression by DCs. However, as IL-10 is also produced by other cell sources, it is probably more effective

to knockout its receptor, IL-10R (106, 108), or a combination of both (109). Both studies evidently report benefits on DC maturation and anti-tumor effects *in vitro* and *in vivo* and suggest that clinical translation of this will greatly enhance DC vaccination potency. A similar effect was observed TGF-β receptor (TGF-βR) was silenced (71, 108). Ahn et al. tested the individual as well as the combined potency of siRNA-mediated silencing of IL-10R, TGF-βR, PTEN, and BIM (108). IL-10R silencing initiated the strongest individual CTL response, followed by TGF-βR. Furthermore, a cocktail combining IL-10R and TGF-βR siRNAs generated the strongest overall CTL response *in vitro* and *in vivo*.

A third strategy aims to decrease DC-mediated T cell apoptosis through co-inhibitory signals, e.g., programmed cell death 1 (PD-1) interaction with its ligand (PD-L1), which is widely described as one of the most potent immunoinhibitory interactions (110). PD-1/PD-L1 interaction is known to inhibit T cell proliferation, survival and effector function, induces apoptosis of tumor-specific T cells, and promotes Treg differentiation as well as resistance of tumor cells to CTL attacks (111). PD-1 expressing TAA-specific T cell function is inhibited by tumor PD-L1 expression, as well as by tumor-induced PD-L1 expression of DCs (111). Advanced clinical trials with PD-1 and PD-L1 antibodies show very promising results in preventing axis signaling in non-small cell lung cancer, indicating the potential that blocking this pathway enhances anti-tumor immunity (112). Silencing of PD-L1, on its own (113) or in combination with its phagocyte-restricted relative PD-L2 (114), shows augmented *ex vivo* TAA-specific CTL responses, which is also confirmed *in vivo* (115). Moreover, combined silencing of PD-L1 and IL-10 in DC vaccination showed even stronger induction of anti-tumor responses *in vitro* and *in vivo* (106) indicating the potential of combining DC modifications in maximizing anti-tumor responses.

Next to PD-1/PD-L1, indoleamine 2,3-dioxygenase (IDO) could have similar effects on T lymphocytes. IDO can be secreted by DCs and depletes the microenvironment from tryptophan, an essential amino acid required for T cell proliferation and survival (116). Furthermore, various tryptophan metabolites are directly immunosuppressive to T cells. Moreover, IDO-expressing cells are able to differentiate naive T cells into Treg cells, thereby further suppressing anti-tumor immunity. The ability to produce IDO depends on the DC subset, and signals present in the tumor microenvironment that can contribute to the amount of IDO produced (117). IDO upregulation was clearly shown in DCs used for vaccination 24 h after maturation in melanoma patients, indicating the potential relevance of IDO silencing (116). Several studies have indicated decreased tumor sizes, reduced CD4+/CD8+ T cell apoptosis, enhanced T cell proliferation and CTL activity, and decreased Treg cell numbers upon IDO silencing, which was confirmed *in vitro*, *in vivo* as well as in patient studies (116, 118).

Nonetheless, immunosuppression of the DCs by factors like IL-10, TGF-β, PD-1, and IDO is ignored in many studies, including the TriMix trial, and may cause substantial downregulation of the anti-tumor response. Especially IL-10 and PD-1 are widely reported to be important inhibitors of immune responses, making these proteins (or their ligands) interesting targets to silence. Silencing of PD-L1 in DCs is expected to cause T cell priming and activation. In the case of solid tumors, PD-L1 is often also expressed by the tumor itself, and may locally provide inhibitory signals affecting these primed T cells. In that way, effector T lymphocyte function can still be inhibited by PD-L1 binding to the T cell membrane protein PD-1. However, the remaining tumor burden in most treated AML patients is relatively low; hence, this might turn out to be less of an issue in AML therapy. The application of DC vaccine delivery during the early stages of immune reconstitution may significantly induce priming to eliminate residual AML blasts effectively. It is expected that the generation of DCs from a UCB will take approximately 4 weeks to generate. Thereafter, the initial DC vaccine can be infused into the patient, followed by multiple DC injections to further boost anti-tumor responses of *de novo* generated T cells. In the future, an interesting strategy might be to add TAA-specific PD-1 knockout effector T cells to the DC vaccine as well, thereby potentially stimulating and expanding these gene-modified T cells to boost anti-tumor responses. A head-to-head comparison of silencing strategies of these proteins in CD34-derived DCs is needed to select the most promising to overcome immunosuppression. However, the use of siRNA in this application may not be as effective as techniques to permanently eliminate expression, because these cells are heavily replicating.

An overview of the numerous modifications tested on DCs is summarized in **Table 1**.

NOVEL TECHNIQUES TO MODIFY CD34-DERIVED DCs TO POTENTIALLY IMPROVE POTENCY

Numerous phase-I DC vaccination-based clinical trials have confirmed the safety of using immature, mature, and TAA-expressing DC vaccines (15, 17). The use of mRNA is relatively safe, because of the temporary expression of the antigen, DC maturation signal or co-stimulatory domain. However, expression cannot be restricted to certain cell types if that is required. Integrating viral vectors may provide longer expression of molecules of interest, but has the risk to potentially cause upregulation of proto-oncogenes (122). Since DCs are generally short-lived this risk may be minimal if the genetic alterations are applied close to application into the patient. Risks of insertional oncogenesis may be increased if CD34+ progenitors are genetically altered before expansion, differentiation, and maturation, particularly because these cells are actively dividing.

The use of siRNAs in DC vaccines is promising, and has shown potential use to reduce expression of co-inhibitory signals in moDCs. Efficiency in UCB-derived DCs has not been shown yet, but may be hampered by the loss of inhibitory ability of siRNAs in cycling cells. This may require precise fine-tuning of delivery of the siRNAs to obtain effective reduction of genes of interest.

The CD34+ expansion phase of the two-step protocol (26) provides a unique environment to modify the DCs to enhance treatment efficiency. However, it is important to carefully select the factors to be removed or introduced in this phase, as this might induce differentiation or decreased proliferation of CD34+ HSPCs. Gene-editing tools to permanently eliminate expression have been

TABLE 1 | Dendritic cell (DC) modifications to enhance anti-tumor induced immunity.

Process	Modification	*In Vitro* studies	*In Vivo* studies	Clinical studies	Reference
Major histocompatibility complex (MHC)-I presentation	↑ Translational efficiency[a]	↑ IFN-γ production	↑ Tumor-associated antigen (TAA)-specific cytotoxic T lymphocyte (CTL) response ↑ Anti-tumor response	N.A.	(41)
	Ubiquitin addition to mRNA	↑ CTL expansion ↑ Proteosome targeting ↑ IFN-γ production	↑ TAA-specific CTL response ↑ IFN-γ production	N.A.	(45)
DC maturation	caTRL4 introduction	↑ Interleukin (IL)-12p70 ↑ CD4+ and CTL expansion ↑ IFN-γ and TNF-α production ↑ CTL cytolytic activity	N.A.	X Objective responses + IFN-α-2β: partial response and stable disease + ipilimumab: 51% 6-month disease control rate[b]	(56, 57, 60)
	CD40L introduction	IL-12p70 ↑ CD4+ and CTL expansion ↑ IFN-γ and TNF-α production ↑ CTL cytolytic Activity	↑ Anti-tumor response ↑ CD4+ and CTL tumor Infiltration	X Objective responses + IFN-α-2β: partial response and stable disease + ipilimumab: 51% 6-month disease control rate[b]	(41, 56–60)
DC migration	C-C motif chemokine receptor 7 introduction	↑ Chemotactic activity ↑ CD40 and CD86 expression ↑ Anti-tumor response	↑ Chemotactic activity ↑ Anti-tumor response	N.A.	(64)
	chemokine C-C motif ligand introduction	↑ Chemotactic activity	↑ DC and T cell at tumor site ↑ Anti-tumor response ↑ IFN-γ and IL-12 production	↑ CTL tumor infiltration Induction of TAA-specific responses in a subset of patients in NSCLC	(65, 66)
	E-cadherin downregulation[c]	N.A.	N.A.	N.A.	N.A.
Cross-presentation	C-terminal tail addition of DC-LAMP/LAMP1/LIMPII	↑ CD4+ and CTL expansion ↑ IFN-γ production	↑ Anti-tumor immunity	N.A.	DC-LAMP: (41, 43, 119) LAMP1: (43, 44) LIMPII: (78)
	Linking to MHC-II associated invariant chain	↑ CD4+ and CTL expansion ↑ IFN-γ production	↑ Anti-tumor immunity	N.A.	(43, 79)
Co-stimulation	CD40L introduction	*See DC maturation*	*See DC maturation*	*See DC maturation*	*See DC maturation*
	OX40L introduction	↑ CD4+ and CTL expansion ↑ DC migration =IL-12p70 Th1 T cell polarization	↑ Anti-tumor immunity	N.A.	(84)
	4-1BBL introduction	↑ CD40 and CD86 expression ↑ CTL expansion and activity ↓ Treg activity	N.A.	N.A.	(85, 86)
	Anti-GITR introduction	↓ Treg activity ↑ Treg suppression	↑ Anti-tumor immunity Long-term memory responses ↑ CD4+ and CTL expansion ↑ Treg expansion	N.A.	(87)
	CD70	↑ CTL expansion ↑ CTL memory ↑ IFN-γ production	↑ CTL expansion ↑ CTL memory ↑ Anti-tumor response	X Objective responses + IFN-α-2β: partial response and stable disease in melanoma + ipilimumab: 51% 6-month disease control rate in melanoma[b]	(41, 56, 57, 59, 60, 96)
Immunosuppression	↓ Apoptosis[d]	↑ Resistance to CTL killing ↑ CTL expansion ↑ IFN-γ production	↑ Anti-tumor response ↑ DC survival	N.A.	(97–99, 102)

(*Continued*)

TABLE 1 | Continued

Process	Modification	*In Vitro* studies	*In Vivo* studies	Clinical studies	Reference
	Suppressor of cytokine signaling 1 downregulation	↑ DC maturation ↑ IL-12p70, TNF-α	↑ CTL cytolytic Activity ↑ Anti-tumor response	N.A.	(103)
	IL-10(R) downregulation	↑ MHC-II and CD40 expression ↑ IL-12p70 ↑ Anti-tumor response ↑ CTL expansion	↑ Anti-tumor response ↑ CTL expansion	N.A.	(106–109)
	TGF-βR downregulation	↑ CD80/86 expression	↑ Anti-tumor response ↑ IFN-γ and IL-12p70 ↑ CTL expansion	N.A.	(71, 108)
	PD-L1 downregulation	↑ IL-12p70 ↓ IL-10 secretion ↑ Anti-tumor response ↑ CTL expansion	↑ CTL expansion	N.A.	(113, 115)
	Indoleamine 2,3-dioxygenase downregulation	=DC maturation ↑ CD4+ and CTL expansion	↓ T cell apoptosis ↑ CTL expansion ↑ CTL cytolytic activity ↓ Treg expansion and activity	+Ipilimumab: ↓ size of melanoma metastases in subset of patients	(116, 118, 120, 121)

[a]*Removal nuclear localization signal, in silico mRNA optimization for optimal codon usage and G/C content, removal of splice sites, and subcloning in in vitro transcription pST1 vector.*

[b]*Only tested in the Trimix combination: caTRL4, CD40L, and CD70.*

[c]*Hypothesis, not supported by clinical DC vaccine studies yet.*

[d]*Through introduction of BAK/BAX, BIM, or PTEN.*

used for more than a decade. Initially, zinc finger nucleases were genome sequence specific with relatively low efficiency and toxicities in hematopoietic cells. Gene-editing tools that cause insertions and deletions on a genomic level have not been applied to DCs. These techniques have been mainly used on T cells. In particular, Transcription activator-like effector nucleases mediated gene-editing and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system are used to eliminate expression of the T cell receptor or co-inhibitory signal PD-1 (123).

More recently, Gundry et al. showed efficient knockout of genes in CD34+ HSPCs (~75%) by CRISPR/Cas9 (46). They show that their strategy to electroporate CD34+ HSPCs with Cas9/sgRNA ribonucleoprotein (RNP) complexes causes efficient CRISPR/Cas9 targeting of up to four sgRNAs. No major effects of gene-editing were observed on viability and proliferation capacity. On the other hand, CRISPR/Cas9-mediated targeting of multiple targets at the same time might cause genomic translocations (124), which are potentially cytotoxic.

This strategy permits for efficient knockout of one, two, or more factors involved in immunosuppression (e.g., PD-L1, IL-10, IL-10R, TGF-β, TGF-βR, and IDO). Strikingly, Gundry et al. also reported that this method allows for efficient homology-directed repair gene-editing (125). In this way, an expression cassette containing WT1 cDNA could even be integrated at one of the gene-editing target sites, allowing for constitutive WT1 expression. Combining the TriMix strategy with gene-edited DCs could potentially be a very potent combination.

The recent advances of CRISPR/Cas9 gene-editing in CD34+ HSPCs make this strategy more efficient, commonly applicable, and technically feasible to include in DC vaccines. If the co-inhibitory genes do not affect cell-cycling or viability, gene-editing tools could be very valuable in creating more potent off-the-shelf CB-derived DC vaccines.

The major risks of CRISPR/Cas9 gene-editing are the potential off-target genome cleavage sites it can create (124). Fu et al. showed that off-target effects can be observed at sites that differ by five nucleotides from on-target sequences, indicating that this might cause efficient gene-editing of off-target sites in CRISPR/Cas9 modified cells (124). CD34+ HSPCs are highly proliferative cells and off-target cleavage might promote tumorigenesis. This risk can be reduced by using Cas9-gRNA RNP complexes rather than using mRNA or plasmids to deliver Cas9, thereby limiting their time-frame of action. Kleinstiver et al. showed that mutating four Cas9 amino acids important in DNA binding energy almost completely diminishes the off-target risk of CRISPR/Cas9, while maintaining its on-target effect (126). This indicates that switching to this mutated version of Cas9 (spCas9-HF1) will potentially further increase CRISPR/Cas9 safety.

It is important to note that, even though studies report extremely low incidences of off-target mutations with wildtype Cas9 in CD34+ HSPCs (127), more research is required to develop accurate off-target site prediction tools. Many studies report low off-target effects, but based on this *in silico* predicted off-target sites rather than on whole-genome sequencing. Hence, whole-genome sequencing of gene-edited cells should be performed to improve the off-target prediction algorithms.

It is also reported that multiplexing CRISPR/Cas9-mediated gene-editing targeting more than one target may result in genomic translocations (128). Poirot et al. performed CRISPR/Cas9 gene-editing in duplex and reported a translocation frequency ranging from 10^{-4} to 2×10^{-2} (128). After 38 days of culturing translocation frequencies remained stable or reduced, indicating that these translocations are safe and did not cause proliferative advantages. It is very important to assess the translocation frequency and the consequence of these translocations per specific gRNA sequence and the downstream effects on highly proliferative CD34+ HSPCs,

and whether this may cause gene-edited related tumorigenicity is yet unknown.

When evaluating the *in vivo* mouse studies performed to assess DC vaccination, two main strategies could be distinguished. Most studies test the DC vaccines on a complete murine background (wildtype mice, with murine tumors and murine DCs) (87, 96, 120). Inherent to these mouse studies is that translation can be difficult due to interspecies differences. Another option is the use of humanized mouse models, e.g., NOD/SCID or more severe immune compromised NOD/SCID gamma mice that allow introduction of human DCs, TAA-specific CD8+ T cells, and human tumor cells (115). However, this also has its limitations, as these models lack the presence of interaction with human immune cells that could contribute to tumor immunity. The translation of gene-modified DCs to clinical application could be improved by the use suitable mouse models with the humanized immune systems (129).

To summarize, DC vaccination has a proven track-record of safety, but addition of genetic modifications could introduce some safety concerns that need to be addressed. The short lifespan of DCs to generate tumor immunity should improve safety of using these cells, which reduces the likelihood that DCs acquire the ability to divide uncontrollably.

AN OFF-THE-SHELF DC VACCINATION APPROACH

CD34-derived DC can be used as a basis to develop personalized cellular vaccines. This strategy is very promising in combination with UCB transplantation. By using the same UCB-unit the risk of adverse effects is significantly decreased by preventing mismatching. Nonetheless, the personalized nature makes this strategy laborious, relatively expensive and requires automated systems to obtain consistent high quality products. The generation of an off-the-shelf product could make this approach more cost-effective and potentially more suitable for standardization for multicentre trials.

Off-the-shelf DC vaccination products are still in their infancy, and more research and technical advances are needed to be able to generate more effective gene therapy products that have a proven quality ready for infusion into cancer patients.

CONCLUSION AND OUTLOOK

The sporadically observed clinical responses indicate the necessity to improve DC vaccinations. Literature suggests that intervening in early DC maturation and activation can cause a cascade-like reaction that eventually also improves downstream activatory processes. It is also widely reported that the immunosuppressive tumor microenvironment is still able to downregulate the most potently activated DCs. Hence, combining modifications of early DC activation processes, such as caTRL4, CD40L, CD70 with elimination of immunosuppressive signaling, such as IL-10R and PD-L1, may drive optimal anti-tumor T cell responses by maximizing both co-stimulatory/co-inhibitory ends of the spectrum.

Tumor-associated antigens can be delivered by optimized mRNA sequences for efficient processing and MHC-I and MHC-II presentation, which could incorporate DC-LAMP C-terminal sequences, ubiquitination or mRNA transcription from optimized transcription vectors to be electroporated in CD34+ derived DCs.

The use of CD34+ HSPCs to generate UCB-derived DCs provides an opportunity during the expansion/differentiation phase to manufacture gene-modified cellular products. Recent progress using state-of-the-art gene therapy vectors, such as self-inactivating third generation lentiviral vectors, that are used in clinical trials to treat inherited diseases and in T cell immunotherapies to treat cancer, have shown the ability to transduce hematopoietic stem cell progenitors effectively, as well as provided evidence for long-term safety. Application to DC vaccines may provide advantageous effects compared with using mRNA. Together with the recent progress in CRISPR/Cas9-mediated gene-editing efficiency of CD34+ HSPCs, this provides a unique cell pool to knockout immunosuppressive factors. The small number of CD34+ HSPCs may aid to reduce the viral vector batches and gene-editing tools required before expansion, differentiation, and maturation. It is important to investigate any negative effects on these phases during DC development.

There is a clear need for consistent comparative studies to compare DC subsets, maturation strategies, and modifications. Although many modifications have been tested in laboratory/preclinical studies and resulted in improved efficiency *in vitro* and *in vivo* models, very few of these modifications have translated into clinical applications. The use of state-of-the-art gene therapy vectors and gene-editing tools may create opportunities for next generation therapies with high efficacy for treating hematologic cancers and solid tumors.

AUTHOR CONTRIBUTIONS

AC and NT wrote the manuscript. JB and SN made substantial and intellectual contributions to the work and approved it for publication.

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Neoantigen Vaccine Delivery for Personalized Anticancer Immunotherapy

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Cancer neoantigens derived from random somatic mutations in tumor tissue represent an attractive type of targets for the cancer immunotherapies including cancer vaccine. Vaccination against the tumor-specific neoantigens minimizes the potential induction of central and peripheral tolerance as well as the risk of autoimmunity. Neoantigen-based cancer vaccines have recently showed marked therapeutic potential in both preclinical and early-phase clinical studies. However, significant challenges remain in the effective and faithful identification of immunogenic neoepitopes and the efficient and safe delivery of the subunit vaccine components for eliciting potent and robust anticancer T cell responses. In this mini review, we provide a brief overview of the recent advances in the development of neoantigen-based cancer vaccines focusing on various vaccine delivery strategies for targeting and modulating antigen-presenting cells. We discuss current delivery approaches, including direct injection, *ex vivo*-pulsed dendritic cell vaccination, and biomaterial-assisted vaccination for enhancing the efficiency of neoantigen vaccines and present a perspective on future directions.

Keywords: neoantigen, cancer vaccine, cancer immunotherapy, vaccine delivery, *in vitro* transcribed mRNA, synthetic long peptide, dendritic cell, nanoparticle

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INTRODUCTION

Vaccines activating the immune system for prevention and treatment of infections and other diseases have made major impact in human healthcare. Cancer vaccines have been actively pursued and studied for decades with several successful examples that are now in the market (1). However, prophylactic cancer vaccines so far have been effective only for virus-related cancers, such as human papillomavirus-induced cervical cancers (2). Provenge (Sipuleucel-T), the only U.S. Food and Drug Administration-approved therapeutic cancer vaccine to date, has only had modest clinical effect for the treatment of prostate cancer (2, 3). Compared to other immunotherapies, such as checkpoint blockade and adoptive T cell therapy (ACT), most cancer vaccines fail to demonstrate notable clinical efficacy. One of the key obstacles to the development of an effective cancer vaccine is the difficulty in antigen selection (4). Traditionally, cancer vaccines are designed to target tumor-associated antigens (TAAs) as they are overexpressed in cancers and could be universal targets among patients of the same malignancy (4). However, TAAs are also present in normal tissues and vaccines against TAAs can potentially initiate central and peripheral tolerance responses leading to low vaccination efficiency or autoimmunity against normal tissues (1, 5).

Tumor-specific antigens, also termed as neoantigens, are derived from random somatic mutations in tumor cells and not present in normal cells (6, 7). Compared to those non-mutated self-antigens,

neoantigens could be recognized as non-self by the host immune system and are thus attractive targets for immunotherapies with potentially increased specificity, efficacy, and safety (4). The immunogenicity of neoantigens leading to T cell response has long been demonstrated in human (8). In fact, a number of preclinical and clinical studies have shown that neoantigen-specific cytotoxic T lymphocytes (CTLs) represent the most potent tumor-rejection T cell populations (9–12). However, naturally occurring neoantigen-specific CTLs in patients are typically rare likely because of low clonal frequency and inefficient presentation of neoantigens (13, 14). Therefore, cancer vaccine or ACT is necessary to potentiate potent immunity against neoantigens for cancer immunotherapy.

Recently, three independent clinical studies provided solid evidence that neoantigen-based cancer vaccines could be developed to elicit potent neoantigen-specific T cell responses against late stage melanoma with remarkable safety and efficacy (15–17). These and other recent advances (listed in **Table 1**) have triggered the enthusiasm in pursuing cancer vaccines against neoantigens. Many efforts are currently focused on addressing two key challenges in the development of neoantigen-based cancer vaccines for wide clinical applications. First, immunogenic neoantigens are rare and difficult to predict. Current predictive algorithm and validation tools need to be optimized for accurate prediction of major histocompatibility complex (MHC)-binding peptides and reliable selection of highly immunogenic neoepitopes (18). Second, it remains challenging to develop an universal and effective delivery strategy to target neoantigen-based vaccines to professional antigen-presenting cells (APCs) for eliciting robust and potent T cell responses against cancer (14). In this mini review, we summarize and discuss the recent progress in addressing these issues for the development of neoantigen-based cancer vaccines with an emphasis on various delivery strategies.

IDENTIFICATION AND SELECTION OF NEOANTIGENS

Neoantigen-related immunotherapy is a truly personalized therapy because most neoantigens are derived from unique mutations in each tumor genome (2). Therefore, identification of patient-specific immunogenic neoantigens is the first step in developing such personalized vaccines (**Figure 1**) (5). With the recent advances in genome sequencing technology as well as the MHC epitope database and predictive algorithms, it now becomes possible to identify and screen cancer neoantigens for individual patients (4, 5). In general, tumor or tumor-related samples are subjected to whole exome or transcriptome sequencing (2, 30). Non-synonymous somatic mutations in cancers, such as point mutations and insertion–deletions, could be identified by comparing the sequences of tumor and matched healthy tissues. Next, the discovered mutations are screened using predictive algorithms for MHC peptide binding affinity in order to identify the most immunogenic antigen candidates for manufacturing personalized cancer vaccines (5, 31). Currently, there are many predictive algorithms available for the identification of potential high-affinity binders of MHC class I molecule. However, the reliability of these predictive algorithms still needs to be improved (32). Most of the existing programs are not able to take into account every factor that impacts immunogenicity, for example, peptide processing by the proteasome, MHC binding stability, genetic insertion–deletions, or fusions, and so on (5). In addition, there are far less data available for predicting MHC class II restricted antigenic peptides (4, 30).

Other methods are also exploited currently to identify the cancer neoantigens besides sequencing of tumor samples. For example, mass spectrometry analyses of peptides from the peptide–human leukocyte antigen (HLA) complex have enabled the discovery of HLA ligandome tumor antigens for personalized vaccines (33–35). New strategies based on the functional analyses of peripheral blood mononuclear cells or tumor filtrating lymphocytes are being developed to identify neoantigen-reactive T cells (12, 36). These assays aiming to identify the pre-existing neoantigen-reactive T cells may fail to detect the subdominant and/or dormant neoantigens that do not elicit naturally occurring immune responses but are potentially important therapeutic targets.

DELIVERY STRATEGIES FOR NEOANTIGEN VACCINES

A large number of approaches have been developed for the preparation, formulation, and delivery of different cancer vaccines, for example, whole tumor cell lysate-, nucleotide (mRNA/DNA)-, protein or peptides-based vaccines, dendritic cell (DC)-based vaccines, viral vectors, biomaterial-assisted vaccines, and so on (1, 2). In the context of neoantigen-based cancer vaccines, mRNA/DNA or synthetic long peptides (SLPs) are typically employed (**Figure 1**) (32). However, it remains challenging to develop a general method for the efficient delivery of these subunit vaccines for stimulating potent antitumor T cell responses (1, 14).

In general, parenterally injected soluble subunit antigens or molecular adjuvants rapidly disseminate into systemic circulation due to their small molecular sizes and show very poor targeting and accumulation in draining lymph nodes (LNs) resulting in limited immune response (37–39). Moreover, soluble molecular adjuvants administered subcutaneously often cause significant systemic inflammatory toxicities (39–41). To solve this problem, vaccines were administered in "depot"-based adjuvants, such as incomplete Freund's adjuvant. However, these passive depots of antigens likely lead to tolerogenicity rather than immunogenicity (42). In addition, the therapeutic cancer vaccine is expected to elicit robust $CD8^+$ T cell responses, which is essential to act synergistically with $CD4^+$ T cell responses to destroy tumors (43). This presents another significant challenge for neoantigen vaccine delivery as typically only live infections induce potent $CD8^+$ T cell priming (44). Soluble tumor antigens acquired by DCs are trapped in endolysosomal compartments and digested into peptides, which are subsequently loaded almost entirely

TABLE 1 | Recent examples of neoantigen vaccine delivery.

Status	Indication	Antigen	Adjuvant	Route	T cell responses		Reference
					$CD4^+$	$CD8^+$	
1. Direct injection of unformulated neoantigen vaccines							
Phase I	Melanoma (stage III and IV)	mRNA	None	i.n.	0.1–2.0%[a]	0.02–0.55%[a] 0.03–1.9%[b]	(16)
Phase I	Melanoma (stage IIIB/C and IVM1a/b)	SLP	Poly-ICLC	s.c.	0.03–0.06%[a] 0.001–0.05%[b]	0.2–1.2%[c]	(17)
Preclinical study	MC-38 colon cancer	SLP	CD40 antibody and poly (I:C)	i.p.	NM	0.18–1.4%[a] 0.48–1.33%[b]	(19)
Preclinical study	B16F10 melanoma	SLP	Poly(I:C)	s.c.	1.54%[c]	3.61%[c]	(20)
Preclinical study	d42m1-T3 sarcoma	SLP	Poly(I:C)	s.c.	NM	2.8–17.5%[b]	(21)
Preclinical study	A2.DR1 sarcoma	SLP	CFA, montanide-ISA51, and imiquimod	s.c.	1.91%[b]	NM	(22)
Preclinical study	B16F10 melanoma	SLP	Poly(I:C)	s.c.	NM	NM	(23)
***2. Ex vivo*-pulsed dendritic cell (DC) vaccine**							
Phase I	Melanoma (stage III)	*Ex vivo* SLP pulsed DCs	Poly(I:C), R848	i.v.	NM	0.06–0.9%[a]	(15)
3. Biomaterials-assisted neoantigen vaccines							
Preclinical study	B16F10 melanoma, 4T1 breast cancer, and CT26 colon cancer	mRNA-lipoplex	None	i.v.	1.36%[c]	1.67%[c]	(20)
Preclinical and phase I study	CT26 colon cancer, TC-1, and melanoma	mRNA-lipoplex	None	i.v.	NM	30–60%[a], 0.62%[a]	(24)
Preclinical study	MC-38 colon cancer and E6/7-TC-1 lung cancer	SLP/PC7A nanoparticles	None	s.c.	NM	NM	(25)
Preclinical study	MC-38 colon cancer and B16F10 melanoma	SLP/nanodiscs	CpG	s.c.	~14.0%[c]	~30%[a]	(26)
Preclinical study	B16F10 melanoma	Endogenous neoantigen-containing proteins	None	s.c.	1.0–3.0%[c]	1.5–12%[c]	(27)
Preclinical study	E7-TC-1 lung cancer, B16F10 melanoma, and CT26 colon cancer	SLP/mesoporous silica microrod with PEI	CpG, PEI	s.c.	~0.6%[c]	~2.0%[a] 1.5%[c]	(28)
Preclinical study	MC-38 colon cancer	SLP/DNA-RNA nanocapsule	CpG	s.c.	NM	9.5%[a]	(29)

[a]*Percentage of neoantigen-specific $CD4^+$ (or $CD8^+$) T cells among total $CD4^+$ (or $CD8^+$) T cells in peripheral blood or spleen detected by multimer staining or the Enzyme-Linked ImmunoSpot (ELISPOT) assay.*

[b]*Percentage of neoantigen-specific $CD4^+$ (or $CD8^+$) T cells among total $CD4^+$ (or $CD8^+$) T cells in tumor detected by multimer staining.*

[c]*Percentage of neoantigen-specific $CD4^+$ (or $CD8^+$) T cells among total $CD4^+$ (or $CD8^+$) T cells in peripheral blood or spleen detected by intracellular interferon-γ (IFN-γ) staining.*

i.n., intranodal injection; s.c., subcutaneous injection; i.v., intravenous injection; i.p., intraperitoneal injection; NM, not measured; poly-ICLC, polyriboinosinic-polyribocytidylic acid-polylysine carboxymethylcellulose; poly(I:C), polyinosinic:polycytidylic acid; SLP, synthetic long peptide; CFA, complete Freund's adjuvant; CpG, unmethylated cytosine-phosphate-guanine oligodeoxynucleotides; PEI, polyethyleneimine.

onto MHC class II molecules for presentation to $CD4^+$ helper T cells. In contrast, only the antigen peptides in cytosol are processed and loaded onto MHC class I molecules for the presentation to $CD8^+$ killer T cells (44). Thus, it is also critical in neoantigen vaccine design to achieve cytosol delivery of those antigens for effective cross-priming of CTL responses (45).

To date, several different delivery strategies have been developed for neoantigen vaccines in preclinical and clinical studies, including direct injection of unformulated antigens, DC-based delivery strategy, and biomaterial-based delivery systems (**Table 1**). Here, we give a brief overview of various strategies and discuss their pros and cons.

Direct Injection of Unformulated mRNA Vaccines Encoding Neoepitopes

In vitro transcribed (IVT) mRNA has undergone many preclinical and clinical investigations for therapeutic cancer vaccination with the advantages of self-adjuvanting activity, direct translation into the cytoplasm, low risk of insertional mutagenesis, as well as simple and inexpensive manufacturing procedure (46). However, controlling the translational efficiency of IVT mRNAs remains challenging. Unformulated mRNA could be spontaneously taken up by many kinds of cells through scavenger receptor-mediated endocytosis. As a result, only a small part of administered mRNA could be captured by APCs and reach cytoplasm for subsequent translation and antigen presentation.

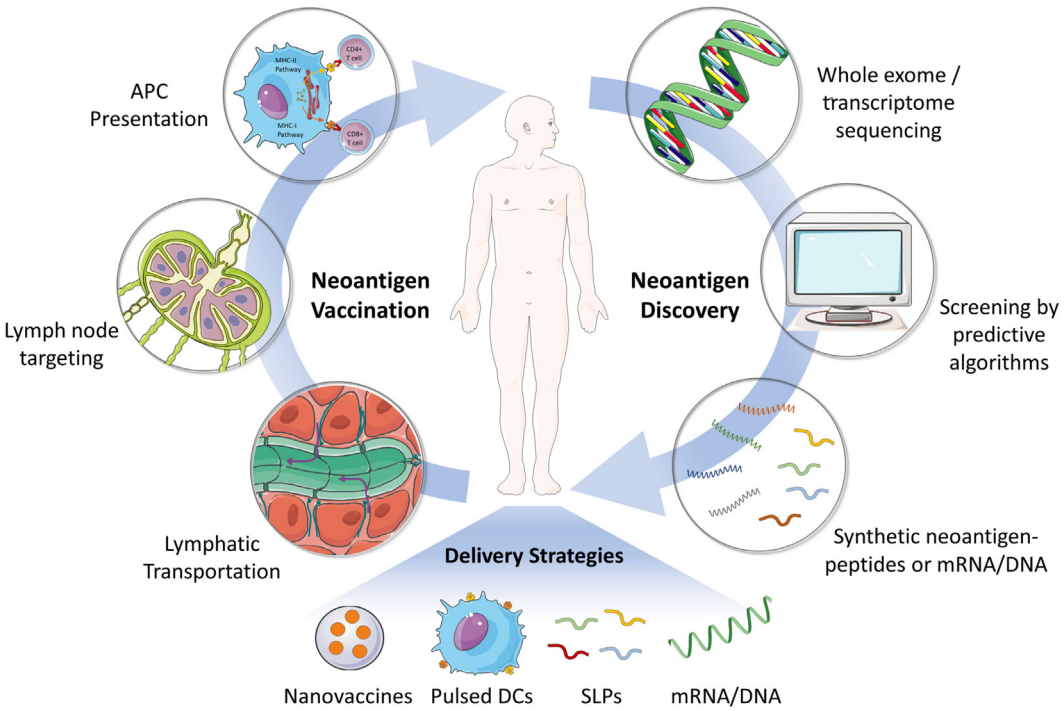

FIGURE 1 | Schematic illustration of the process of neoantigen discovery, vaccine manufacturing and formulation, and vaccination in patients. The first step for developing neoantigen cancer vaccine involves the identification of mutated tumor specific antigens by whole exome/transcriptome sequencing and prediction of immunogenic MHC epitopes. Next, neoantigen vaccines (e.g., SLP and mRNA) are manufactured and formulated for efficient delivery to secondary lymphoid organs (e.g., lymph node), where neoantigen vaccines are captured by APCs and presented to effector immune cells including $CD8^+$ or $CD4^+$ T cells. Various delivery strategies have been developed to achieve an effective and safe neoantigen-based cancer vaccine. Abbreviations: SLP, synthetic long peptide; DC, dendritic cell; APC, antigen-presenting cell; MHC, major histocompatibility complex.

In order to maximize the capture of antigens by APCs, unformulated IVT mRNA can be administered directly into LNs through ultrasound-guided percutaneous injection [noted as intranodal (i.n.) injection], a clinically applicable administration route for the direct access to inner organs or tissues through needle-puncture of the skin (47).

Most recently, Sahin and his group demonstrated an elegant example of immunizing advanced melanoma patients in a clinical study using vaccines based on synthetic mRNAs encoding poly-neoepitopes through i.n. injection (**Table 1**) (16). This administration route improved the stability and translation efficiency of the IVT mRNAs, and enhanced the presentation of the neoantigens with MHC class I and II molecules on DCs. These IVT mRNAs also promoted DCs maturation *via* TLR7 signaling pathway due to intrinsic adjuvant capability. Potent T cell responses against multiple neoantigens were successfully induced in all the patients after immunization. It is noticeable that the majority of neoantigen-elicited T cell responses were HLA class II restricted $CD4^+$ T cell responses even though they were predicted as high-affinity HLA class I binders. Although promising, the i.n. administration method may limit its wide application in many vaccination settings as the extensively repeated percutaneous injection (up to 20 vaccinations used in this study) may not always be practical.

Direct Injection of Unformulated SLP Neoantigens

Antigenic peptide has been extensively exploited for cancer vaccines as it presents several advantages including direct function as pivotal T cell epitope, low toxicity, low cost, and ease of synthesis (48, 49). In a pioneered phase I clinical study evaluating SLP-based neoantigen cancer vaccines, a selected pool of twenty SLPs (15–30 mers for each peptide) together with adjuvant (poly ICLC) were used to immunize 6 patients with advanced cutaneous melanoma (**Table 1**) (17). During the treatment, seven vaccine doses were administrated through subcutaneous (s.c.) injection within 20 weeks. These peptide-based neoantigen vaccines induced polyfunctional MHC class II restricted $CD4^+$ T responses targeting ~60% of neoantigens used across patients, while the induced MHC class I restricted $CD8^+$ T cells targeted ~16% of those neoantigens. Encouragingly, four of six vaccinated patients were cancer-free 25 months post treatment. Similarly, in a recent preclinical study, mice immunized intraperitoneally (i.p.) with three neoantigen SLPs together with adjuvant showed potent therapeutical $CD8^+$ T cell responses against MC-38 tumor with complete inhibition of tumor growth in 11 of 15 vaccinated mice (19).

However, subcutaneously administered peptide-based vaccines could rapidly diffuse into the peripheral blood vessels

leading to systemic dissemination due to the relatively small molecular sizes (14, 49, 50). The ultimate therapeutic efficacy of these peptide vaccines is limited by inefficient delivery to desired lymphoid organs. Increasing dose or dosing frequency could partly solve this problem but in turn increases the risk of systemic toxicity. Intradermal vaccination strategies for SLPs have been tested in some clinical trials to successfully stimulate antigen-specific T cell responses with a low dose of SLPs (51), and thus could potentially serve as an alternative.

Ex Vivo-Pulsed DC Vaccine

Neoantigens could also be delivered by DCs, which play a key role in antigen presentation in the immune system. Similar as Sipuleucel-T, DC vaccines targeting neoantigens have been developed and evaluated in a small-scale clinical trial (15). Patients' monocyte-derived immature DCs were first matured through co-culturing with irradiated feeder cells in the presence of adjuvants and then separately pulsed with different SLPs for loading on the HLA class I or II molecules. Three patients with advanced melanoma received the adoptive transfer of peptide-pulsed mature DCs *via* intravenous (i.v.) infusion (**Table 1**) (15). It was found that this vaccine increased the preexisting neoantigen-specific immune response and promoted a diverse patient-specific TCR repertoire against previously undetected HLA class I restricted neoantigens.

In addition to peptides, IVT mRNAs have been utilized to transfect DCs for the generation of DC-based vaccines in many preclinical and clinical studies (51, 52), and are potentially useful for preparing DC-based neoantigen vaccines (53). In general, although proven to be effective and safe in clinical trials, the approach of *ex vivo*-pulsed DC vaccine is costly, labor-intensive, and requires highly skilled technicians for manufacturing, which greatly limits its wide clinical applications in a large scale (54).

Biomaterial-Assisted Neoantigen Vaccines

Biomaterials have been extensively investigated for vaccine delivery as they could protect antigen and adjuvant molecules from degradation, enhance lymphoid organ targeting, and modulate APCs' functions. Biomaterial-assisted cancer vaccines have shown great potential in both preclinical and clinical development (55–57). For example, a scaffold-based vaccine is being evaluated in phase I clinical trial (NCT01753089) for preventing melanoma. Neoantigen-based cancer vaccine delivery with biomaterials is a nascent area (**Table 1**) (14). Rapid progress has been made in designing novel biomaterials to deliver mRNA- or SLP-based neoantigens in tandem with adjuvants for enhanced cancer vaccines (20, 24).

Biomaterial delivery systems have been employed to improve the efficacy of peptide- or mRNA-based neoantigen vaccines. For example, a responsive nanovaccine was developed by self-assembling peptide neoantigens with ultra-pH-sensitive polymers (25). Such nanovaccines could achieve efficient cytosolic delivery of antigens in response to the acidic pH in endosomes leading to enhanced cross-presentation. Interestingly, this nanovaccine is adjuvant-free and the carrier polymer itself serves as an adjuvant *via* the stimulation of STING pathway (25). By tuning the chemical structure of the side chains of the polymers for the optimized transition pH, the nanovaccines could induce robust antigen-specific CTL responses with comparable or better efficacy than several established adjuvants [e.g., alum and unmethylated cytosine-phosphate-guanine (CpG) oligodeoxynucleotides]. Also, the CTL responses were type I interferon (IFN) pathway dependent as the majority of CTL responses were abolished in IFN receptor knockout (IFN-α/βR$^{-/-}$) mice. As the micelle-based nanovaccine does not require any chemical modification of the peptide antigens, it could be easily adapted for different peptide antigens. In another elegant example, synthetic high-density lipoprotein nanodisc, a highly clinically safe and scalable material, was used to promote the co-delivery of peptide neoantigen through disulfide conjugation and cholesteryl-modified adjuvant to draining LN for prolonged antigen-presentation (26). The nanodisc elicited extremely high level (~30%) of antigen-specific CTL responses leading to eradication of established tumors when combined with checkpoint blockade antibody treatment.

Despite the technical challenges of systemic delivery of subunit vaccines (58), a recent study has been able to demonstrate a remarkably high delivery efficiency of IVT mRNA neoantigen vaccines into systemic DCs using lipid complex (**Table 1**) (24). Net charge of the RNA-lipoplexes (RNA-LPX) was found essential for the spleen targeting. When the charge ratio was optimized (+/− = 1.7/2–1.3/2), the model antigen was almost exclusively delivered and expressed in splenic cell populations. It is also noticeable that no molecular targeting ligands were used to modify the RNA-LPX surface. CD11c$^+$ conventional DCs in the marginal zone, and plasmacytoid DCs and macrophages in the spleen were found to internalize the most RNA-LPX; those DCs were also found effectively translate the delivered mRNAs. Such RNA-LPXs encoding neoepitopes induced unusually high level of antigen-specific CTL responses (up to 30–60% among the total CD8$^+$ T cell population). The potent effector and memory T cell responses together with IFN-α-mediated innate immune response effectively eradicated murine CT26 lung tumors (i.v. inoculated). The remarkably high efficiency of systemic APC targeting mediated by the negatively charged lipid complex is likely the reason for such potent elicitation of antigen-specific CTL responses.

In addition to actively targeting vaccines to LNs, biomaterials have also been designed for passive delivery *via* antigen capture *in vivo* (27). To prove this concept, poly(lactic-co-glycolic acid) nanoparticles with various surface modifications were developed to capture the tumor-derived antigens *in situ* post radiation therapy that induced immunogenic cell death. The capture efficiency could be fine-tuned by varying the surface chemistry of nanoparticles. Intratumorally injected nanoparticles captured released tumor antigens including neoantigens, and facilitated the internalization and presentation of tumor antigens by APCs. Such antigen-capturing nanoparticles substantially increased the ratio of tumor-infiltrating effector CD8$^+$ T cells to regulatory CD4$^+$ T cells. This *in situ* local vaccination strategy is facile and intrinsically personal. It also showed enhanced abscopal antitumor effect by inducing systemic immunity in mouse models.

Besides nanosized biomaterials, bulk biomaterials can also be utilized for enhancing cancer vaccines through constructing artificial antigen-presenting niche *in vivo* (59). Such artificial niche is designed to recruit DCs for antigen capture and presentation and

activate DCs *in situ* (60). One very recent example is a scaffold-like neoantigen vaccine made from mesoporous silica microrods (MSRs) (28). A cationic polymer, polyethyleneimine (PEI), was coated onto MSRs for the adsorption of neoantigens on the scaffold. Interestingly, PEI itself could stimulate DCs with increased expression of CD86, and production of interleukin-1β (IL-1β) and tumor necrosis factor-α. CpG and granulocyte–macrophage colony-stimulating factor were loaded on the scaffold surface to serve as vaccine adjuvant and DC-recruiting factor, respectively. Impressively, when loaded with a pool of B16F10 or MT26 neoantigens, this scaffold vaccine eradicated the lung metastases and synergized with anti-CTLA4 antibody inducing regression of subcutaneous tumors in mice. This simple and modular strategy without chemical modification of the peptide neoantigens has great potential to enable robust personalized vaccination.

FUTURE OUTLOOK

Vaccination against neoantigens has already demonstrated tremendous potential in both preclinical and clinical studies. As illustrated by diverse examples in this review, various vaccine delivery strategies, in particular, novel biomaterial-assisted vaccines, have shown great promise to elicit potent T cell responses for cancer treatment. Despite the rapid advances, enormous challenges remain for the future development of neoantigen-based cancer vaccines for wide clinical applications. So far, most clinical and preclinical studies using neoantigen vaccines have been focused on cancers with high mutation load; the feasibility of applying this approach to cancers with relatively low mutation rate is to be demonstrated (61). It also remains challenging to identify and select the immunogenic neoantigens from an individual's tumor for enhanced therapeutic efficacy.

A general efficient and safe delivery strategy for neoantigen vaccines is still lacking. Innovative delivery strategies are continually being pursued by scientists to address this issue. *Ex vivo*-pulsed DC vaccines are promising but suffer from several limitations including the difficulty in preparation and expansion. Alternative cells are currently under development, such as B cells, which are promising APCs with much higher abundance than DCs, improved proliferation capability, and increased lymphoid organ targeting properties (62). Another promising strategy is using synthetic APCs that mimic the functions of natural APCs and are much easier to manufacture (63).

Rationally designed biomaterials are of particular interest to boost the development of neoantigen vaccines as they could be engineered exquisitely to fulfill all the delivery requirements. These biomaterials should be highly biocompatible, facile in preparation requiring minimum modification of the antigen itself, and highly modular for various neoantigens. Biomaterials based carriers are expected to achieve the co-delivery of several to tens of exogenous neoantigens together with adjuvants to target APCs, which are necessary for eliciting potent and broad T cell responses to prevent tumor escape in the clinic (16, 17, 20). Biomaterials are particularly useful to modulate intracellular delivery and antigen processing in APCs. Intelligent biomaterials are also expected to achieve precise control of balanced MHC class I and II loading of antigens for eliciting the most potent antitumor immunity.

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The Protein Corona as a Confounding Variable of Nanoparticle-Mediated Targeted Vaccine Delivery

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Nanocarriers (NC) are very promising tools for cancer immunotherapy. Whereas conventional vaccines are based on the administration of an antigen and an adjuvant in an independent fashion, nanovaccines can facilitate cell-specific co-delivery of antigen and adjuvant. Furthermore, nanovaccines can be decorated on their surface with molecules that facilitate target-specific antigen delivery to certain antigen-presenting cell types or tumor cells. However, the target cell-specific uptake of nanovaccines is highly dependent on the modifications of the nanocarrier itself. One of these is the formation of a protein corona around NC after *in vivo* administration, which may potently affect cell-specific targeting and uptake of the NC. Understanding the formation and composition of the protein corona is, therefore, of major importance for the use of nanocarriers in vaccine approaches. This Mini Review will give a short overview of potential non-specific interactions of NC with body fluids or cell surfaces that need to be considered for the design of NC vaccines for immunotherapy of cancer.

Keywords: nanocarriers, cancer vaccines, immunotherapy, protein corona, cell-specific targeting

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INTRODUCTION

Immunotherapy of tumors has hit every day clinical practice in formerly hard-to-treat cancers due to the introduction of immune checkpoint modulators that block inhibitory, surface expressed molecules by antibodies (1). However, the use of antibodies against checkpoint inhibitors is not specific for a tumor antigen, since it reactivates pre-existing tumor immunity rather than priming novel T cell responses. This may result in insufficient clinical responses and in immune-related side effects due to unwanted autoimmunity in a substantial number of patients (2). The induction of tumor antigen-specific immunity remains a major goal of cancer therapy, targeting either overexpressed proteins or neoantigens that are unique to the individual tumor (3).

Tumor antigen-specific immunotherapy requires the delivery of the antigen—either as peptide, protein, DNA, or mRNA—to the correct cell type (4). Thus, targeting of antigen-presenting cells (APC), and concomitant induction of an appropriate APC activation status that enables immunogenic antigen presentation, is crucial for the success of therapeutic vaccination approaches (5). Nanotechnology holds great promise to transfer a packaged, protected cargo (antigen plus adjuvant) in high concentrations into the desired cell type by using appropriate nanocarriers (NC) (6). Indeed,

vaccination studies using NC have demonstrated their great potential as universal vaccine platforms (7). Numerous strategies for specific targeting of NC to APC have been pursued, including the use of antibodies or their derivatives, natural ligands for receptors on the APC surface, aptamers, cystine knot proteins, or by modifying biophysical characteristics of the NC such as size and surface charge.

However, appropriate targeting of systemically applied NC to APC can be affected by unintended interactions of the NC surface with components of blood plasma (8) and/or with cell surface structures (9) that are unrelated to the specific targeting structure. The "protein corona" around NC may affect their organ-specific or cell type-specific trafficking as well as endocytosis and/or functional properties of the NC (10). Most importantly, the protein corona has been shown to interfere with targeting moieties used to induce receptor-mediated uptake of the NC, both inhibiting (11) and enhancing (12) internalization by specific cell types. Moreover, the protein corona is taken up by the target cell, which may alter their function. In this review, we will address various properties of the NC cargo and of the protein corona for targeted delivery of nanovaccines (**Figure 1**; Table S1 in Supplementary Material).

NANOVACCINES

Conventional vaccines that include a tumor antigen and an adjuvant do not specifically address specific types of APC. In addition, both components may dissociate and cause unwanted side effects. On the one hand, uptake of an antigen in the absence of an adjuvant by endocytic/phagocytic APC, but also by tumor-promoted myeloid-derived suppressor cells and tumor-associated macrophages (13) may cause tumor immune tolerance. On the other hand, stimulation of APC by an adjuvant alone may promote autoimmune reactions (14).

In general, nanovaccines can facilitate co-delivery of antigen and adjuvant. Earlier studies have shown that the stimulatory

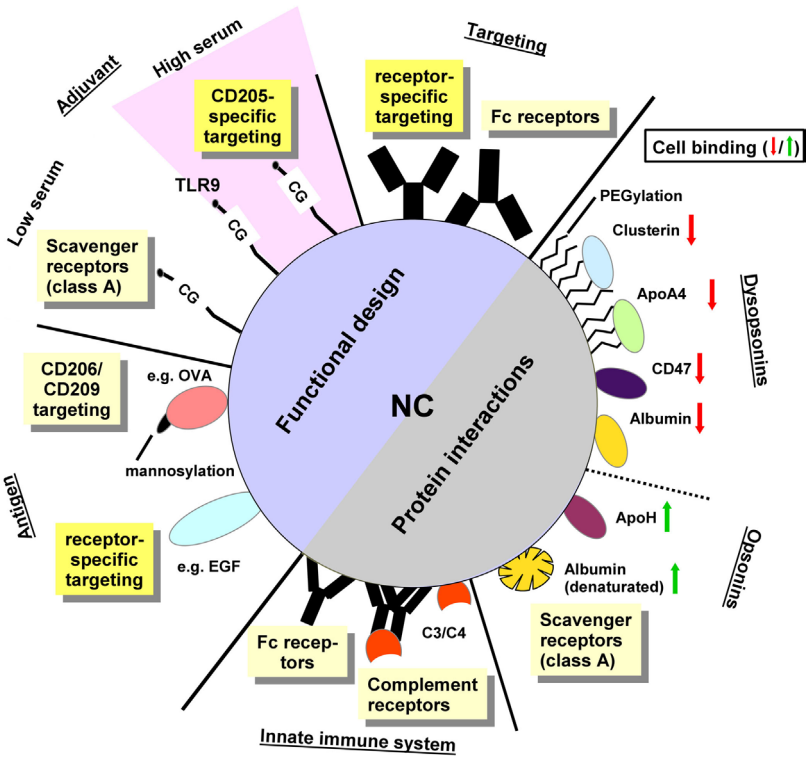

FIGURE 1 | The functional design of NC and their protein interactions determine the character of cellular binding. Functional design: antibodies specific for receptors expressed by the target cell type are supposed to mediate cell type-specific targeting. In case of non-directed conjugation, the exposed Fc portion may result in binding to Fc receptor expressing cells. The adjuvant itself may mediate receptor specific binding as reported for toll-like receptor 9-activating CpG-rich oligonucleotides which target CD205 *in vivo*. Under standard culture conditions *in vitro*, however, oligonucleotides engage class A scavenger receptors (SR). The antigen may exert receptor-specific targeting, e.g., when mannosylated or in case of using a protein which constitutes a genuine receptor ligand. Protein interactions: recognition of the NC surface by components of the innate immune system like natural antibodies may yield Fc receptor binding and classical complement pathway activation. Direct recognition of the NC surface may trigger lectin-dependent/alternative complement pathways. Surface-deposited active C3 and C4 fragments mediate binding of complement receptors. Moreover, parameters of the NC surface like charge and hydrophobic/hydrophilic state determine the composition of the protein corona as well which in turn regulate subsequent cellular binding: albumin when adsorbed onto the NC surface in a denatured state enhances NC binding to SR. NC-adsorbed ApoH also elevates cellular binding. In contrast to these "opsonins" other factors like ApoA4, and (native) albumin as well as the "don't eat me" signal protein CD47 and proteins found accumulated in the protein corona of PEGylated NC (e.g., clusterin) serve to reduce cellular interactions, and therefore were termed dysopsonins. The role of nanocarrier functionalizations and protein corona constituents for cellular binding is explained in more detail in Table S1 in Supplementary Material.

activity of a given adjuvant was enhanced when applied as a particulate formulation (15). For example, CpG-rich oligonucleotides, which engage endo/lysosomal Toll-like receptor 9 (TLR9) and are employed in clinical trials to boost anti-tumor responses (16), on an equimolar base exerted much stronger APC stimulation when coupled to a NC (15). Moreover, more recently small molecular TLR7/8 imidazoquinoline agonists were shown to be effective vaccine adjuvants when coupled to nanogels that drain lymph nodes, whereas they failed to trigger an immune response against co-injected antigen when applied in soluble form (17, 18).

For a number of nanovaccines containing antigen plus adjuvant, endocytic uptake by myeloid immune cell types has been reported, termed "passive targeting" (19). However, to prevent competitive uptake by unwanted phagocytically active myeloid cells (20), a specific targeting of APC which is capable to induce a (primary) anti-tumor response is essential. In this regard, dendritic cells (DC) are in the focus of nanovaccine development (21) since they are potently capable of priming naïve T cells (22). Most DC subpopulations present exogenous antigens rather exclusively *via* MHC-II. In order to obtain a profound antigen-specific $CD8^+$ T cell response to directly kill malignant (or infected) cells, current approaches aim to target cross-presenting DC subpopulations (23, 24) which in mouse express surface receptors like CD205 (25), CLEC9a, and XCR1 at high levels (26). To this end, natural ligands of these surface receptors including mannose (27) and XCL1 (28) were successfully tested for DC targeting. As an alternative, receptor-targeting antibodies have been used (25, 29).

The surface marker which is used to target specific cell populations can also trigger uptake and may determine the intracellular route and ultimately the effectiveness of immune activation (30, 31). For example, using CD205 as a targeted surface marker seems to be favorable (32) as it enhances cross-presentation on MHC-I but also has a high amount of antigen peptides presented on MHC-II (25). We could recently show that a nanocarrier which co-delivered the model antigen ovalbumin (OVA) as well as an adjuvant (CpG-rich oligo) and was further decorated with a CD205-targeting antibody, yielded profound therapeutic activity in a mouse B16-OVA tumor model (12). In contrast, treatment of tumor-burdened mice with a nanovaccine formulation that lacked the DC-targeting antibody had no therapeutic effect. Interestingly, CD205-targeted delivery does not always accumulate antigens to DC when compared to mannose targeting (33). Thus, intracellular processing is as important as the vaccine dose that is taken up. The speed of internalization has also been suggested to play a role as in some studies slower internalization may favor better the preservation of MHC-I epitopes (33). It has been hypothesized that early endosomes that are involved in slow uptake processes have a lower concentration of proteases and thus avoidance of late endosomes seems to be favorable in this context. Certainly, lysosomal degradation occurs later with slower transport processes and the amount of peptides not totally degraded should be higher if the transport toward the lysosome is reduced. Other interesting and promising surface targets are CD40, Clec9a, and Clec12a since they have been shown to change intracellular trafficking (24). What we lack at this stage is a well-coordinated comparative study demonstrating the effectiveness of these different targeting vaccines in one animal model as most studies only imply none versus targeted antigens or compare two targeting pathways.

Altogether, these findings support the rationale to design multifunctional nanovaccines. However, we and others also observed that the largest fraction of systemically applied nanovaccine accumulated in the liver, irrespective of its formulation (34), which suggests general involvement of yet unknown factors that interfere with cell type-specific targeting.

THE PROTEIN CORONA AROUND NC AS A CONFOUNDING VARIABLE FOR EFFECTIVE VACCINE DELIVERY

Despite their many advantages, NC are complex molecules that may interact with serum proteins and other components of body fluids in an unexpected manner, which may significantly alter their efficacy as vaccine carriers. One of these non-intended interactions is the spontaneous (ir-)reversible deposition of proteins onto the NC surface in complex fluids, which is modified by multiple parameters, either related to the NC source or the composition of the protein environment. Some basic principles of this process were elucidated by mimicking *in vivo* NC protein interactions *via in vitro* incubation with biological fluids (35). However, additional physiologically relevant factors (e.g., stability and dynamics of protein coronae under shear stress during passage through the blood) are still poorly understood (36). Nevertheless, some relevant determinants of protein corona formation around NC have been defined and verified in murine models *in vivo*.

Physico-Chemical Properties and Surface Functionalization of NC as Determinants of Protein Corona Formation

Hydrophilic Versus Hydrophobic Surfaces

Due to their large surface areas in relation to their volume, nano-sized materials are highly affected by surface interactions with body fluids such as plasma or lymph (37). The chemical composition of the NC at the surface enables protein binding through hydrogen bonds, hydrophobic interactions, electrostatic interactions, and π-π stacking (38). Therefore, NC surface chemistry needs to be optimized to prevent particle aggregation under biologically relevant conditions. For instance, Lundqvist et al. compared plain polystyrene NC with surface carboxy- or amine-modified ones and identified both proteins that were common on all types of NC as well as some that were specifically enriched on each of the surfaces (39). In general, hydrophobic particles including non-functionalized polystyrene NC are not well-dispersible and stable in water or even protein-rich solutions over time, as they require surface active agents (surfactants) to reduce the large surface tension between the two phases (40). Usually, these surfactants are small molecular detergents or amphiphilic (co-)polymers that stabilize the interface molecularly. However, proteins which usually provide a hydrophilic surface and a hydrophobic core can also partially expose some of their hydrophobic residues which might compete with the surfactants and replace

them irreversibly. Therefore, a well-defined and stable interface with immobilized surfactants on a hydrophobic carrier surface would be advantageous to prevent NC protein aggregation (41). On the other hand, hydrophobicity can also be utilized to control protein adsorption on the nano-bio-interface specifically, as demonstrated by Zhang et al. who covalently deposited (co-)polymers of varying amphiphilicity on gold NC and observed a variation in protein adsorption affording tailored cellular NC uptake (42).

Surface Charge

Besides hydrophobic interactions, proteins can undergo also charge-driven binding to the NC interface (as most protein surfaces are composed of charged amino acids) (43). Albumin as one of the most abundant proteins in blood plasma has a slightly negative net charge (44) and, therefore, instantaneously interacts with positively charged NC (8, 45). For instance, multi-angle dynamic light scattering in human blood plasma was applied as a highly sensitive method to monitor the binding of albumin on cationic nanohydrogel particles (46). Deposition of albumin onto nanogels was prevented by loading the nanogels with negatively charged siRNA oligonucleotides for RNA interference therapy and, thus, neutralizing the net charge of the nanogels and enhancing their circulation properties in the blood stream.

Yet, charge-neutral polymers can still adsorb proteins and influence the carriers' *in vivo* performance. As an example, iron oxide NC coated with dextran yielded deposition of activated complement C3 and triggered complement receptors (CR)1/2-mediated B cell targeting which was further exploited for the treatment of allergic immune responses (12).

PEGylation of NC

To minimize protein interaction with polymer coatings and biomaterials, Whitesides and co-workers investigated different chemical structures on self-assembled monolayer interfaces and identified four basic principles [so-called "four Whiteside's rules"] (47) that efficiently suppress protein adsorption (48, 49): (1) hydrophilicity, (2) no charges, (3) no hydrogen bond donors, and (4) only hydrogen bond acceptors. All these characteristics are fulfilled by poly(ethylene glycol) (PEG), one of the most frequently used polymer NC coatings to minimize—but not always completely abolish (see Composition of the Biological Fluids as Determinants of the NC Protein Corona)—protein adsorption but guaranteeing a stealth-like behavior for enhanced circulation properties after systemic administration (50–52). To that respect, we have recently shown that for PEGylated polystyrene NC the stealth effect is not due to the avoidance of protein adsorption, but rather the adsorption of specific proteins like clusterin or apolipoprotein A4 (ApoA4) (53). Still, the degree of PEGylation on the nano-biointerface as well as PEG density can modify the protein corona and its NC performance under biological conditions. For instance, Kataoka and co-workers recently showed that tethered PEG density with highly squeezed PEG chains on the interface of pDNA-polyplexes assured higher circulation properties to improve pDNA delivery (54, 55). For site-specific targeting of NC with ligands to manage selective interaction with the ligand-corresponding receptor, PEGylation is often the only way to reduce additional uncontrolled protein corona formation, which would counteract with the targeting groups (56). However, in some cases too dense PEGylation can also entrap a targeting ligand inside the PEG interface and suppress its interaction with its target receptor (57). Yet, in these cases PEG backfilling with shorter PEG chains can help to both reduce massive protein corona formation as well as assuring ligand accessibility to their receptors (58). While a better understanding of the PEGylation process on the NC surface has become increasingly evident, controversial concern of use of PEG for biomedical purposes (59) has motivated the development of alternative stealth-like polymers (60) which might result in a better controllable protein corona formation on NC after administration into a biologically relevant environment.

Endotoxin Contamination

The formation of a NC protein corona was found to be further modulated by prior adsorption of the Gram-negative bacteria cell wall component lipopolysaccharide (LPS) (61). LPS is a frequent contaminant of proteins used for nanovaccine generation (e.g., antigen and targeting antibody), and of non-sterile lab environments. LPS was reported to bind various types of NC both *via* charge-driven interactions (negatively charged phosphate head groups interact with cationic nanoparticles) and by hydrophobic interactions (LPS lipid regions interact with hydrophobic domains on the nanoparticle) (62). LPS-contaminated NC stimulated inflammatory responses by co-incubated toll-like receptor 4-expressing immune cells (61). These observations underscore the absolute requirement to test NC for endotoxin contaminations prior to functional testing.

Composition of the Biological Fluids as Determinants of the NC Protein Corona

Besides the physicochemical properties of the NC, the composition of the biological fluid it is immersed into is another relevant factor in the formation of the protein corona (63). In terms of *in vitro* studies, fetal bovine serum, human plasma, or serum are mainly utilized in order to investigate protein-NC interactions and their functional effect on the cellular level (64). The difference between serum and plasma is highly significant in terms of corona composition and ultimately affects the interaction of coated NC with immune cells (65). This difference is caused by the preparation procedure as blood is either naturally coagulated (serum) or supplemented with an anti-coagulant (plasma). Here, it has to be noted that also the choice of the anti-coagulant being either citrate, heparin, or EDTA additionally influences corona formation (63) and cellular outcome (66).

Bringing NC from pre-clinical studies toward the clinical application bears additional challenges. Differences in the corona composition between mice and humans (67) as well as inter-individual variations in plasma protein composition, including dietary factors that affect, e.g., serum lipoprotein composition ("personalized protein corona") have been recognized (68). On top of this, several reports could show that *in vitro* studies cannot fully reflect the situation *in vivo* (69, 70). The interaction

of NC in blood flow is highly dynamic, may be altered by shear stress and hereby strongly alter the composition of the protein corona pattern (71). Based on this, a better understanding of the *in vivo* protein corona formation and composition is still under investigation and is needed to eventually tune the NC properties for targeted cellular interaction.

CELLULAR RECEPTORS FOR NC CORONA PROTEINS

The plasma protein corona around NC can significantly alter their biological behavior *in vivo* (9) and also affect specific targeting moieties that are being used to target NC to specific organs or cell types (11). In many cases, the protein corona may impair the targeting structure on the NC from binding to its receptor on the target cell. This may indeed occur much more frequently than reported, since unsuccessful attempts for targeted delivery of NC are typically not published. In some cases, however, NC corona proteins may also enhance binding of the NC to target cells which bear receptors that recognize specific NC corona proteins (12). As outlined below, among the receptors that bind NC surface molecules in a non-specific manner are CRs, scavenger receptors (SR), immunoglobulin receptors, and lipoprotein receptors. Although not formally shown yet, other phagocytic receptors (72) may also be involved in NC recognition by leukocytes and endothelial cells.

Fc Receptors

Fc receptors bind immunoglobulins *via* their constant (Fc) region (73). There are specific receptors for IgG (FcγRI [CD64]), (FcγRIIA [CD32]), (FcγRIIB [CD32]), (FcγRIIIA [CD16a]), (FcγRIIIB [CD16b]), IgA (FcαRI [CD89]), and IgE (FcεRI and FcεRII [CD23]). These receptors bind immunoglobulins with differential affinity and also modify the functional state of the receptor-bearing cell. Especially for Fcγ receptors, different biological functions of various receptors are known, ranging from antibody-dependent cell-mediated cytotoxicity (73), to phagocytosis, and cell activation (74), or inhibition of cell activity (75). Receptor-specific antibodies are commonly used to enable targeting of NC. They are typically coupled to the NC surface in a non-oriented form (73). Thus, it is often arbitrary whether the antigen-binding Fab or the Fc portion of the molecule is exposed to the outer surface of the NC, resulting in potential binding of the antibody-coated NC to FcR carrying cells *in vivo* (mostly macrophages and liver endothelial cells) (73). Likewise, immunoglobulins derived from plasma may also bind to NC, either in an epitope-specific form (thus "opsonizing" the NC) or *via* non-specific adsorption (76). It is tempting to speculate that this non-epitope-specific binding of antibody-coated NC may interfere with any intended specific targeting of the NC *via*, e.g., the antigen-binding epitope of an NC-coupled antibody. In contrast, immunoglobulin binding to PLGA nanocarriers has also been demonstrated to inhibit non-specific interaction with endothelial cells in human blood flow (77). For clinical applications, it will be imperative to overcome uncontrolled FcR-mediated effects of antibody-targeted NC by using either antibodies coupled *via* the Fc part to the NC to prevent its unintended binding to the FcR (78) or antibody derivatives that lack the Fc portion (79).

Complement Receptors

Complement receptors (CR) are expressed mainly by leukocytes and bind bacteria and other structures opsonized by complement factors as a consequence of classical, alternative, or lectin-mediated complement pathway activation (73). Opsonized material is recognized by CR 1–4. CR1 (CD35), CR3 (CD11b/CD18), and 4 (CD11c/CD18) which mediate phagocytosis by mononuclear cells, whereas CR2 (CD21) is present only on B cells and serves as a co-receptor (80, 81). All types of NC investigated by us that carry glyco-structures on their surface (e.g., dextran and starch) avidly bound and activated the lectin-dependent complement pathway, whereas inorganic NC generally failed to do so (12). Ligation of C3-coated NC by CR2 resulted in efficient binding of iron oxide-dextran NC by murine B cells, resulting in specific targeting of these NC to B cells *in vivo* (12). C3/CR2-mediated B cell engagement of the NC significantly surpassed antibody-mediated targeting, as NC that were additionally coated with an anti-CD205 antibody that is recognized by DC still bound much more abundantly to B cells than to DC *in vivo*. Thus, plasma protein corona components may re-direct NC to certain cell types *in vivo*. This effect can be exploited in an immunotherapeutic fashion, as dextran-iron oxide NC that contained an antigen plus CpG as an adjuvant could be used to efficiently treat B cell-mediated hypersensitivity reactions such as allergic asthma and anaphylaxis.

Scavenger Receptors

Scavenger receptors serve to endocytose diverse polyanionic ligands including modified endogenous (lipo)proteins like oxidized low-density lipoprotein, but also pathogen-derived molecular patterns and endogenous misfolded proteins (82). Low-density lipoproteins are regularly identified in the protein corona of different nanoparticles (53, 83). Due to their interaction with different toll-like receptors, and their association with intracellular signaling complexes like mitogen-activated protein kinases, SR engagement was shown to alter the cellular activation state of DC (84) and macrophages (85). Class A SR (SR-A) that contain a collagen domain were shown to bind negatively charged surfaces on dextran NC (86), polystyrene NC (87), silica NC (88), and superparamagnetic iron oxide NC (89) under standard culture conditions *in vitro*, i.e., at low serum concentration and in the absence of complement and immunoglobulin (53, 83).

Due to the compensatory capacity of single SR-A, binding of NC to this class of receptors is validated most often in blocking studies using fucoidan, Poly(I), and dextran sulfate as competitive high affinity SR-A ligands (90). In this regard, dextran sulfate-based NC were shown to retain their SR-A binding affinity also *in vivo*, and were used to target activated macrophages in a model of murine arthritis (91).

Negatively charged NC surfaces, such as NC conjugated to short linear (anionic) oligonucleotides were efficiently internalized *via* SR-A *in vitro* (92). Consequently, SR-A-mediated internalization of oligonucleotide-conjugated NC was exploited for efficient transfer of drugs and siRNA into different cell types

(93). Pre-incubation of such NC with serum dose-dependently inhibited cell binding, presumably due to shielding of the negatively charged oligonucleotides by yet unknown serum factors (94).

OTHER CORONA PROTEINS THAT AFFECT NC ADSORPTION TO CELLS

Besides corona proteins that mediate binding to classical phagocytosis receptors, other corona proteins also affect the cellular uptake of NC.

Dysopsonins

The main characteristic of "stealth" NC is their reduced interaction with phagocytic cells, which results in a prolonged blood circulation time (95). Overall, stealth NC show less protein adsorption, however, protein corona formation cannot be completely prevented (96). Thus, in general, non-recognition of NC by immune cells is not only due to low amounts of proteins adhering to surfaces but can also dependent on the abundancy of certain corona proteins. Actually, we have identified distinct proteins which inhibit cellular interactions and hereby mediate stealth behavior (63, 97). Those proteins are refereed as "dysopsonins", of which albumin and clusterin (apolipoprotein J) are the most prominent examples. Clusterin has been demonstrated to be required for the stealth effect of poly(ethylene glycol)- and poly(phosphoester)-coated polystyrene NC (53). Albumin, the most abundant protein in serum (98), is a prominent constituent of the protein corona of many types of NC (99). Takeuchi and co-workers (100) demonstrated recently that albumin specifically adsorbs to polymeric nanogels after *in vivo* administration, creating an albumin-rich corona which prolonged blood circulation.

Thus, pre-coating of different types of NC with albumin can improve their circulation half-life and biocompatibility (101). However, when misfolded, albumin coating of NC may also shorten their plasma half-life. Indeed, albumin underwent conformational changes of its alpha-helical domains after adsorption to layered silicate NC (102) and polystyrene NC with a cationic, amino-modified surface (103). In both studies, NC adsorbed with misfolded albumin effectively bound SR-A *in vitro*. Likewise, albumin adsorption to inorganic NC (104–106) also resulted in an unfolding of alpha-helical domains, and similar conformational changes were also reported for other serum proteins like fibrinogen, gamma-globulin, histone, and insulin when adsorbed onto gold NC (107). Further studies need to elucidate whether NC may unintendedly bind SR *in vivo* due to conformationally altered serum factors within their protein corona.

Apolipoproteins

In general, apolipoproteins were identified in high amounts on the surface of various NC formulations (108, 109). For example, ApoE was enriched on the surface of NC coated with the nonionic surfactant polysorbate 80 and hereby enabled the transport of NC across the blood–brain barrier *via* receptor-mediated endocytosis (110). Additionally, recently adopted immuno-mapping techniques (111) offer the possibility to determine functional cell receptor-binding epitopes of corona proteins. Here, it was found that SiO_2 NC are covered by ApoB100 which allows a recognition of NC *via* low-density lipoprotein receptor (112). Moreover, in another study Ritz and coworkers identified a variety of different proteins within the corona of differentially surface-functionalized polystyrene NC, and could correlate their relative abundance with an enhanced or decreased uptake by human mesenchymal stem cells (35). As demonstrated in that study, ApoA4 and C3 were shown to decrease unspecific cell interaction whereas ApoH enhanced cellular uptake.

"Don't Eat Me" Signals

Viable cells, most notably erythrocytes and platelets, express surface receptors like CD31, CD47, and CD200 that interact with counter-receptors on myeloid immune cells to prevent their cytolysis [reviewed in Ref. (113)]. Furthermore, living cells show extensive sialic acid modifications of glycoproteins. Presentation of such "don't eat me" signals has been used to prevent phagocytic clearance of NC. CD47 is ubiquitously expressed and binds SIRPα that is predominantly expressed on phagocytically active leukocytes (114). SIRPα engagement results in the activation of phosphatases that inhibit phagocytic activity. Rodriguez et al. (115) demonstrated that CD47-derived peptides coupled to polystyrene beads reduced their uptake by macrophages, and prolonged their circulation in mice. In line, different types of NC (polystyrene, PLGA) conjugated with an ICAM-1 targeting antibody for endocytic uptake by activated endothelial cells showed clearly reduced unspecific liver accumulation when conjugated in addition with CD47 (116). In a different approach, NC were coated with cell membranes derived from red blood cells to exploit their endogenous high level surface expression of CD47 and other "don't eat me signals" [reviewed in Ref. (117)]. This concept has been broadened by transferring membranes of specific leukocyte populations to make use of the cell type-specific properties of their surface receptors like mediating cell–cell adhesion and homing behavior.

NC DESIGN—AVOID OR EXPLOIT THE PROTEIN CORONA?

Concerning the design of APC targeting nanovaccines, it is necessary to take into account potential intrinsic receptor binding properties of antigen and adjuvant. For example, short oligonucleotides which engage DNA binding danger receptors like TLR9 or STING and thereby activate APC (118) were demonstrated to effectively engage SR-A in a serum-poor environment (94). *In vivo*, however, CpG-rich oligos engage CD205 which is highly expressed by $CD8^+$ DC in mouse (119). We showed that nanovaccines conjugated to this adjuvant retained both their $CD8^+$ DC binding and activating properties *in vivo* (120). Proteins used as a source of antigen may be recognized by receptors if they constitute genuine ligands (e.g., epidermal growth factor) or may bind *via* a protein modification as demonstrated for OVA which is endocytosed *via* the mannose receptor due to mannosylation of the protein (121). In order to prevent interactions of antigen/

adjuvant with cellular receptors (or serum components), nanocapsules may be preferable to shield and to protect the cargo of a nanovaccine (122). If the intrinsic binding properties of cargo components support the intended NC targeting other types of NC that expose their cargo may be employed.

To achieve cell type-specific targeting either antibodies or their fragments, synthetic ligands (e.g., aptamers, DARPins, and cystine-knot miniproteins), or natural ligands of endocytic surface receptors highly expressed by the target APC may be used. However, depending on the orientation of a NC-coupled targeting antibody binding of the exposed Fc part to Fc receptors is possible (73) and may limit cell type specificity. Similarly, a conjugated receptor ligand may bind different receptors as exemplified for mannose-derived oligosaccharides which may engage both the mannose receptor and DC-SIGN (123). Consequently, the efficacy and specificity of NC binding, uptake and subsequent biological effects need to be tested using cell populations comprising also non-target cell types (e.g., human PBMC, mouse spleen, and liver cells).

To predict the *in vivo* behavior of a NC by *in vitro* assays in a more reliable manner, it is necessary to allow formation of a protein corona in a controlled way. One strategy is to minimize adsorption of serum factors to the NC surface which may affect its intended targeting properties (see Physico-Chemical Properties and Surface Functionalization of NC as Determinants of Protein Corona Formation). On the contrary, however, the composition of the protein corona itself may support the biological function of a nanovaccine. For example, we have recently demonstrated that a lectin surface coating of NC resulted in activation of the lectin complement pathway and enabled specific NC targeting to B cells *via* CRs (12). Thus, the protein corona may inhibit or enable cell type-specific targeting.

SUMMARY

In summary, NC are versatile tools to deliver a high amount of antigen plus adjuvant(s) in a targeted manner to APC. Here, we point toward a variety of interesting receptors like CD205 or Clec9A that can focus delivery toward favorable immunological readouts. However, NC are almost inevitably coated with a protein corona after exposure to blood plasma or lymphatic fluid. This plasma protein corona can affect the trafficking of the NC within the body as well as their cellular targeting and uptake to a significant extent, potentially resulting in loss of the desired effects as well as altered functional properties of the NC. Often, antibodies are used as targeting moieties; yet, the interaction of their Fc part with receptors of other cells represents an undesired mistargeting and should be avoided for nanovaccines. On the other hand, the protein corona may also be exploited to extend NC plasma half-life, e.g., by attracting or preadsorbing clusterin, thereby optimizing cell-specific targeting and immunotherapeutic effects, or even to direct NC to specific cell types or organs *in vivo* by exploiting (pre)adsorbed targeting moieties.

AUTHOR CONTRIBUTIONS

All authors contributed writing the manuscript of this Mini Review.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01760/full#supplementary-material.

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MINI REVIEW
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Targeting Cytosolic Nucleic Acid-Sensing Pathways for Cancer Immunotherapies

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The innate immune system provides the first line of defense against pathogen infection though also influences pathways involved in cancer immunosurveillance. The innate immune system relies on a limited set of germ line-encoded sensors termed pattern recognition receptors (PRRs), signaling proteins and immune response factors. Cytosolic receptors mediate recognition of danger damage-associated molecular patterns (DAMPs) signals. Once activated, these sensors trigger multiple signaling cascades, converging on the production of type I interferons and proinflammatory cytokines. Recent studies revealed that PRRs respond to nucleic acids (NA) released by dying, damaged, cancer cells, as danger DAMPs signals, and presence of signaling proteins across cancer types suggests that these signaling mechanisms may be involved in cancer biology. DAMPs play important roles in shaping adaptive immune responses through the activation of innate immune cells and immunological response to danger DAMPs signals is crucial for the host response to cancer and tumor rejection. Furthermore, PRRs mediate the response to NA in several vaccination strategies, including DNA immunization. As route of double-strand DNA intracellular entry, DNA immunization leads to expression of key components of cytosolic NA-sensing pathways. The involvement of NA-sensing mechanisms in the antitumor response makes these pathways attractive drug targets. Natural and synthetic agonists of NA-sensing pathways can trigger cell death in malignant cells, recruit immune cells, such as DCs, $CD8^+$ T cells, and NK cells, into the tumor microenvironment and are being explored as promising adjuvants in cancer immunotherapies. In this minireview, we discuss how cGAS–STING and RIG-I–MAVS pathways have been targeted for cancer treatment in preclinical translational researches. In addition, we present a targeted selection of recent clinical trials employing agonists of cytosolic NA-sensing pathways showing how these pathways are currently being targeted for clinical application in oncology.

Keywords: DAMPs, STING, RIG-1, innate immune system, cytosolic nucleic acid receptors, antitumor response, agonist, clinical trials

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INTRODUCTION

The innate immune system provides the first line of defense against pathogen infection. It relies on a small set of germ line-encoded sensors named pattern recognition receptors (PRRs), which are deputized to detection of pathogen-associated molecular patterns (PAMPs) and danger damage-associated molecular patterns (DAMPs) signals.

Nucleic acid (NA)-sensing is an essential mechanism of the innate immunity that utilizes cytosolic receptors to detect extranuclear DNA or extracellular RNA as DAMPs signals (1).

In mammalian cells, two paradigmatic cytosolic NA-sensing pathways are the cyclic GMP-AMP synthase (cGAS)–stimulator of interferon genes (STING) and the RIG-I-like receptors (RLRs)-MAVS pathways, which are responsible for cytosolic DNA and RNA sensing, respectively (2, 3). The cGAS is a DNA sensor protein, which, upon binding double-strand (ds) DNA independently of DNA sequence, and catalyzes the synthesis of 2′-3′-cyclic GMP-AMP (cGAMP) (4). cGAMP functions as a second messenger that, in turn, engages the endoplasmic reticulum (ER)-membrane adaptor protein STING. After its activation STING traffics from the ER via the Golgi to perinuclear endosomes recruiting tank-binding kinase 1 (TBK1). A phosphorylation cascade allows signal transmission leading to activation of interferon regulatory factor (IRF) 3 and nuclear factor κB (NF-κB) that translocate into the nucleus to drive transcription of type-I interferons (IFNs), interferon-stimulated genes (ISGs), proinflammatory cytokines and chemokines (5, 6). Cytosolic dsRNA sensing involves three sensor proteins, namely retinoic acid-induced gene-I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of physiology and genetics 2 (LGP2) (7), collectively referred to as RLRs. RIG-I sensor preferentially detects 5′-triphosphate (5′-3p)-ending RNA and short dsRNA, while MDA5 recognize long dsRNA (8). The signaling pathway proceeds with interaction of RIG-I or MDA5 with the adaptor mitochondrial antiviral-signaling protein (MAVS) located in the outer mitochondrial membrane and activation of IRF3/IRF7 and NF-κB. The activation usually results in IFNs production, consequent induction of ISGs and activation of NF-kB target genes.

Cancer cells share key hallmarks such as oxidative stress, genome instability and mutations, and altered metabolic rate that can generate nuclear and/or mitochondrial DNA damage (9). Recent studies revealed that damaged NAs released by dying cancer cells can be sensed as DAMP danger signals by PRRs present on CD8α dendritic cells (DCs) in tumor microenvironment (TME), leading to activation of cGAS-STING and/or RIG-I/MDA5 signaling pathways. The consequent type I IFN secretion activates DCs in an autocrine or paracrine manner, resulting in their migration to tumor-draining lymph nodes, where DCs cross-prime naïve $CD8^+$ T lymphocytes (10–13) (**Figure 1**).

Activation of cytosolic DNA sensing pathways impacts on autophagy and tumor antigens (Ags) cross-presentation in DCs. Type-I IFNs production by DCs actually represents the link between NAs sensing and effective Ags cross-presentation to $CD8^+$ T cells, therefore linking innate and adaptive immunity (14). Type-I IFNs stimulate upregulation and consequent surface expression of MHC class I genes. Furthermore, type-I IFNs directly promote Ags intracellular retention in DCs that have engulfed apoptotic tumor cells through slowing the endosomal-lysosomal acidification rate, thus enhancing capacity to cross-present Ag by DCs (15–17). Since MHC class I cross-presentation depends on the time of persistence of Ag within the phagolysosomal compartment (16, 18), autophagy possibly provides an intracellular depot where Ag is stored, rather than degraded and represents an alternative pathway for MHC class I presentation (19–21).

Endosomal tumor-derived NAs escape into the DC cytosol through a yet not completely understood mechanism. Specific internalizing receptors such as CLEC9A and CD205 and high-mobility group box 1 protein can mediate uptake of genetic material from dying tumor cells and affect subsequent endosomal trafficking (22–24). Likewise tumor-derived Ags, released DNA could be retained in the endolysosomal compartment where it is preserved before it gains access to the cytosol where it can be recognized by cGAS and other sensor proteins such as intereferon-γ-inducible protein 16 (IFI16), absent in melanoma 2 (AIM2), and Z-DNA-binding protein 1 (ZBP1) (10, 11, 14). The delayed endosomal acidification may further contribute to reduce DNA degradation by DNAse II protease.

Presence of NA sensor proteins across cancer types suggests that these signaling mechanisms may be involved in cancer biology. Actually the expression of RIG-I was significantly downregulated in human hepatic carcinoma (HCC) tissues (25, 26).

Clinical studies revealed that the expression of STING was significantly reduced in HCC tissues compared to the controls, and lower expression of STING was associated with a more advanced tumor stages and a worse survival (27) as well with poor prognosis for patients with gastric cancer (28). Collectively, these studies suggest that STING and RIG-I sensors may serve as tumor suppressors and have clinical values against certain types of tumors as prognostic/predictive biomarkers.

Furthermore, PRRs mediate the response to NAs in several vaccination approaches, including DNA immunization. As route of dsDNA intracellular entry, DNA immunization leads to expression of key components of the cytosolic NA-sensing pathways.

Recent data showed that natural and synthetic agonists of NA-sensing pathways could trigger cell death in malignant cells and recruit immune cells, such as DCs, $CD8^+$ T cells, and NK cells into the TME.

In this minireview, we will highlight the newest insights from preclinical studies demonstrating the relevance of manipulating the cGAS-STING and RLRs-MAVS signaling pathways for cancer treatment and how these pathways are currently being targeted pharmacologically (**Figure 1**).

Clinical evaluation of these innate immune modulators, with agonists alone and in combination with other immunomodulatory agents demonstrates the high translational potential for cGAS-STING and RLRs-MAVS signaling pathway engagement. In **Table 1** and Table S1 in Supplementary Material, we present a selection of very recent and novel therapies employing agonists of cytosolic NA-sensing pathways in oncology and provide detailed information concerning mechanisms of action, assessments, and outcomes of reported clinical trials.

Results from completed early phase clinical trials with human STING and RIG-I agonists showed biologic and therapeutic effects in patients, leading to combination clinical trials with checkpoint inhibitors (31, 43).

PRECLINICAL EVALUATION OF STING AGONISTS FOR CANCER TREATMENT

Regulation and function of the cGAS-STING pathway has been reviewed elsewhere (3, 46–50), so we will briefly consider newest insights into the topic of STING agonists as potent anticancer agents in preclinical models.

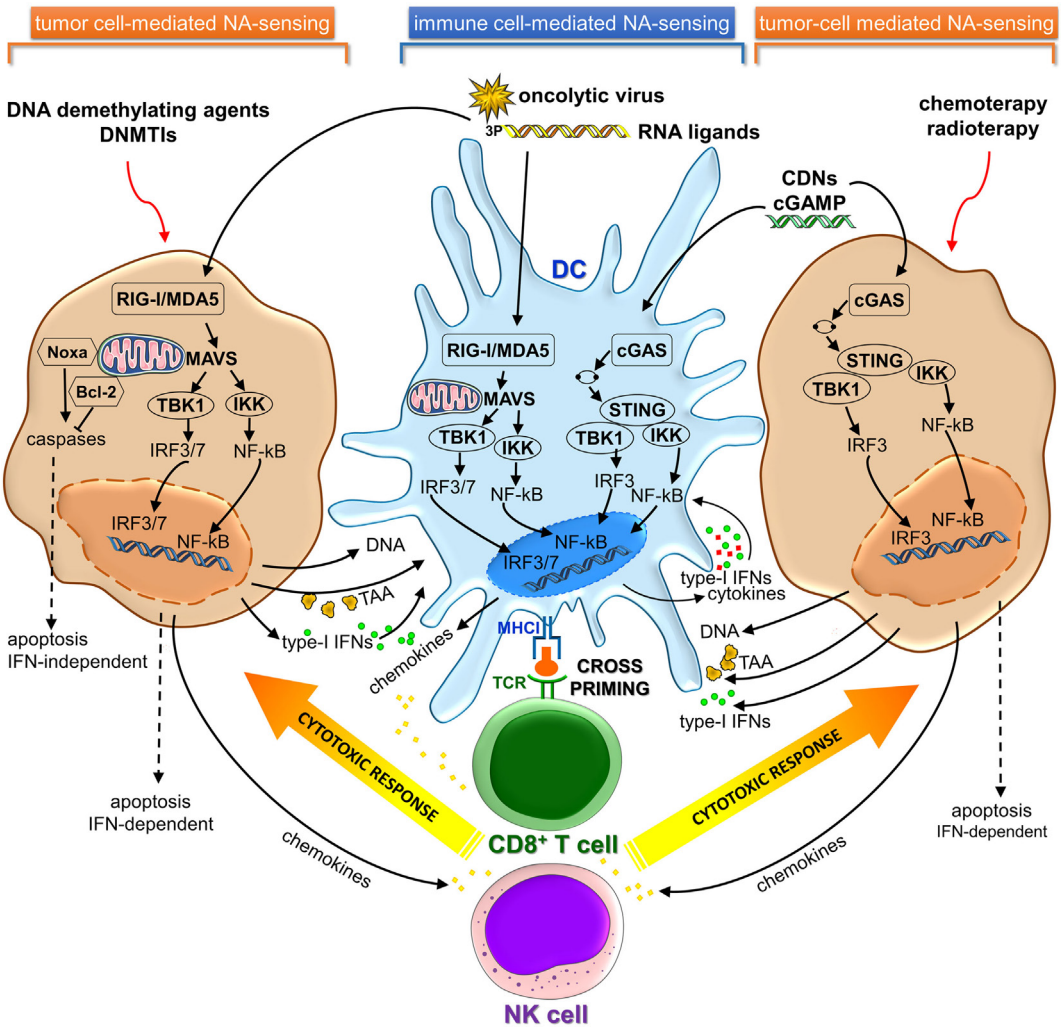

FIGURE 1 | cGAS-STING and RIG-I/MDA5 signaling pathways in immune and cancer cells involved in cancer immunosurveillance and immunotherapy. cGAS, cyclic GMP-AMP synthase; STING, stimulator of interferon genes; RIG-I, retinoic acid-induced gene-I; MDA5, melanoma differentiation-associated gene 5; NA, nucleic acid; TAA, tumor-associated antigen; DNMTIs, DNA methyltransferase inhibitors.

STING pathway has been mostly characterized in APCs, meanwhile in the TME, T cells, endothelial cells, and fibroblasts, stimulated with STING agonists *ex vivo,* have been found to produce type-I IFNs (29). By contrast, most studies indicated that tumor cells developed strategies to inhibit STING pathway activation, likely for immune evasion during carcinogenesis (51, 52).

Recent pieces of evidence have indicated that activation of the STING pathway was correlated to the induction of a spontaneous antitumor T-cell response involving the expression of type-I IFN genes (3, 10, 53). Furthermore, host STING pathway is required for efficient cross-priming of tumor-Ag specific $CD8^+$ T cells mediated by DCs (10, 54) (**Figure 1**).

Based on these findings, direct pharmacologic stimulation of the STING pathway has been explored as a cancer therapy.

Demaria et al. demonstrated $CD8^+$ T and type-I IFNs dependent antitumor effect of cGAMP, a natural STING ligand, in melanoma and colon cancer mice models (55).

In 2016, Li et al. confirmed the potent antitumor effect of intratumoral (i.t.) injection of cGAMP in CT26 colon adenocarcinoma-bearing mice. The antitumor activity of cGAMP relied on DC activation and $CD8^+$T cell cross-priming (56). More recently, Ohkuri et al. demonstrated accumulation and antitumor effect of potent macrophages in mouse TME of breast cancer, squamous cell carcinomas, colon cancer, and melanoma tissues (57) after i.t. injection of cGAMP.

Canonical cyclic-dinucleotides (CDNs), as direct agonists for STING, show a poor ability to activate human STING. Therefore, an increasing number of synthetic CDNs that potently activate all human STING variants have been designed in recent years (29, 58).

New synthetic CDNs agonist has shown potent antitumor efficiency in various tumor models such as B16F10 melanoma, 4T1 mammary adenocarcinoma, and CT26 colon carcinoma, with regression of established tumor, metastasis rejection, and establishment of long-term immune memory (29).

TABLE 1 | Cytosolic DNA sensors targeting clinical trials.

CT Identifier, Phase Study (Reference)	Trial compound	Condition	Target	Status
NCT02675439, I (29, 30)	MIW815 (ADU-S100)	Advanced/metastatic solid tumors or lymphomas	cGAS-STING pathway	Currently recruiting participants Updated on July 2017
NCT03172936, Ib (31)	MIW815 (ADU-S100)/ PDR001	Solid tumors and lymphomas		Currently recruiting participants Updated November 2017
NCT01274455, I (32, 33)	CYL-02/Gemcitabine	Advanced and/or metastatic and/or non resectable pancreatic adenocarcinoma cancer		Completed Updated on March 2016
NCT02806687, II (34)	CYL-02/Gemcitabine	Advanced, non-metastatic and non-resectable pancreatic adenocarcinoma cancer		Currently recruiting participants Updated on February 2017
UMIN000002376, I/II (35–37)	Inactivated Sendai virus particles	Malignant melanoma stage IIIC or stage IV	RIG-I-MAVS signaling pathway	Phase I finished in 2016
UMIN000006142, I/II (38, 39)	Inactivated Sendai virus particles	Castration-resistant prostate cancer		Currently recruiting participants Updated on September 2012
NCT01105377, II (40, 41)	Azacitidine/Entinostat	Metastatic colorectal cancer		Completed Update on August 2014
NCT01349959, II (41, 42)	Azacitidine/Entinostat	Advanced breast cancer; triple-negative and hormone-refractory		Ongoing, but not recruiting participants Update on December 2016
NCT01928576, II (43–45)	Azacitidine/Entinostat/ Nivolumab	Recurrent metastatic non-small cell lung cancer		Currently recruiting participants Updated on October 2017

CT Identifier, Clinical Trial identifier; RIG-I, retinoic acid-induced gene I; STING, stimulator of interferon genes.

Recently, many preclinical studies draw a blueprint for the application of STING agonists in tumor therapy in the context of combination therapies.

Strategies that combine STING immunotherapy with other immunomodulatory agents are being explored in mouse models. The antitumor efficacy of cGAMP administered by i.t. injection into B16.F10 tumors was enhanced when combined with anti-programmed death-1 (PD-1) and anti-cytotoxic T-lymphocyte associated-4 (CTLA-4) antibodies (55). In other studies, CDNs together with anti-PD-1 incited much stronger antitumor effects than monotherapy in a mouse model of squamous cell carcinoma model as well of melanoma (59, 60). Luo et al. showed great synergy by combining a STING-activating nanovaccine and an anti-PD1 antibody, and suggested generation of long-term antitumor memory in TC-1 tumor model (61). STING agonists can enhance antitumor responses when combining with tumor vaccines. CDN ligands formulated with granulocyte-macrophage colony-stimulating factor-producing cellular cancer vaccines, termed STINGVAX, showed strong *in vivo* therapeutic efficacy in several models of established cancer. Antitumor activity was STING dependent and corresponded to activation of DCs and tumor antigen-specific $CD8^+$ T cells. STINGVAX combined with PD-1 blockade induced regression of poorly immunogenic tumors that did not respond to PD-1 blockade alone (62). STING agonists in combination with traditional chemotherapeutic agents or radiotherapy can work synergistically to trigger antitumor response (56, 63).

The focus of STING pathway agonists for clinical use has thus far centered on their role as vaccine adjuvants and as cancer immunotherapeutic agents for treatment of solid tumors. However, induction of type-I IFNs and other inflammatory cytokines through STING pathway activation results in potent leukemia-specific immunity, culminating in impressive improvements in survival of preclinical acute myeloid leukemia models. Thus, Curran et al. provided solid rationale for clinical translation of STING agonists as immune therapy for leukemia and other hematologic cancers (64).

The intricate STING role may be associated with cell type and activated intensity of downstream signaling. Agonist-mediated activation of STING induces apoptosis in malignant B-cells through specific cytotoxicity, suggesting the potential therapeutic use of STING agonists in treating B-cell malignancies (65). Meanwhile, STING activation reverses lymphoma-mediated resistance to antibody immunotherapy through macrophage activation and modulation of intratumoral macrophage phenotype, as showed by Dahal et al. (66). The induction of apoptosis seems to be a general effector response of the STING pathway in lymphocytes. Gulen et al. reported that overt stimulation of the STING pathway in primary and malignant T cells elicits apoptosis through induction of IRF-3-dependent and p53-dependent proapoptotic genes. This phenomenon, which is evident upon strong stimulus delivery, reveals that the signaling strength determines proapoptotic functions of STING (67). In agreement, low and short *in vivo* activation of STING in T cells provokes type-I IFNs production and ISGs expression mimicking the response of innate cells (68).

TARGETING RIG-I/MDA5 PATHWAY FOR CANCER THERAPY

RIG-I-like receptors are expressed in most tissues, including cancer cells (69). Recent studies have demonstrated that promising druggable targets against cancer may be represented by components of antiviral immune response. Tumor cells and virus-infected cells can be regarded as injured host cells sharing common features (70, 71). In fact, cancer cells can be induced to mimic a viral infection using RLRs ligands to activate cytosolic RNA-sensing pathway and IFN response (44, 72). This activation also can result in stimulation of cytotoxic immune cells, such as

NK and CD8$^+$ T cells, which kill cancer cells *via* extrinsic and intrinsic apoptosis (73–75). Consequently, activation of RLRs by using synthetic ligands or oncolytic virus in tumor cells can induce cell death in an IFN-dependent or IFN-independent manner (44, 72–74, 76–78) (**Figure 1**). Several types of bifunctional small interfering RNAs (siRNAs) with 5′-3p ends conferring a non-self RNA PAMP (79) were developed for both silencing oncogenic or immunosuppressive genes and inducing cell death mediated by viral mimicry (12, 13, 73, 77, 80, 81). Systemic administration of a siRNA designed to trigger RIG-I and silence *Bcl2* induced DC-dependent production of IFNs and strongly inhibited tumor growth in B16 melanoma model. These RIG-I-mediated immune responses synergized with siRNA-mediated *Bcl2* silencing to promote massive tumor apoptosis in lung metastases *in vivo* (73). Likewise, in human drug-resistant leukemia cell lines treatment with multifunctional 5′-3P-siRNA downregulated multi-drug resistance 1 (MDR1) expression and triggered RIG-I-dependent intrinsic apoptosis pathway involving upregulation of Noxa protein, cytochrome-*C*, and effector caspases (81). On the other hand, small endogenous non-coding RNAs gave rise to RIG-I:RNA complexes and initiated downstream signaling events, after ionizing radiation treatment (82). Antitumor DNA-demethylating agent, 5-AZA-CdR, and DNA methyltransferase inhibitors (DNMTis) triggered cytosolic sensing of dsRNA in cancer cells activating endogenous retroviruses and, thus, mimicking a viral infection. The increased viral defense gene expression induced a type-I IFN signaling and apoptosis (44, 72). Furthermore, DNMTi treatment potentiated anti-CTLA-4 immune checkpoint therapy in a pre-clinical melanoma model (44).

Besides the dual tumoricidal property, there are several advantages of targeting RIG-I/MDA5 signaling pathway for cancer immunotherapy. It has been reported that malignant cells are highly sensitive to RIG-I/MDA-5 proapoptotic signaling pathway (74, 83), whereas normal cells are less susceptible as they are rescued from apoptosis by upregulation or activation of endogenous *Bcl-xL*, which prevents RIG-I/MDA5-induced cell death (74). Furthermore, RIG-I and MDA5 are able to trigger a p53-independent alternative pathway for the induction of proapoptotic *Noxa*. Hence, RIG-I/MDA5-driven apoptosis is not mediated by the tumor suppressor p53 mutational status in cancer cells (74), which strongly contributes to resistance to chemo- and radiotherapy (84).

In **Table 1** and Table S1 in Supplementary Material are reported some RIG-I/MDA5 ligands that are being utilized in clinical trials. Overall, triggering RIG-I/MDA5 pathway results in eliciting both immunostimulatory and proapoptotic activity conferring to RIG-I/MDA5 a pivotal role in tumor evasion from immune surveillance. Yet it is noteworthy that stimulation of cancer cells by RIG-I ligands not only cause apoptosis but also enhance DCs Ag cross-priming through type-I IFNs release and upregulation of MHC class I gene expression (12, 75, 76).

STIMULATION OF INNATE IMMUNE PRRs BY DNA VACCINES

In the last decade, several DNA vaccines products have been licensed for animal use demonstrating the wide applications of the DNA-based vaccine, such as Apex®-IHN, West Nile-Innovator®, and ONCEPT® (85–88). DNA vaccines can induce both humoral and cellular immune responses. When used in humans, however, DNA vaccines suffer from lower immunogenicity profiles (89).

Several studies confirmed that immunogenicity of DNA vaccines is regulated by critical components of the innate immune system *via* plasmid DNA recognition through the STING–TBK1 pathway.

The DNA vaccine "adjuvant effect" is not TLR9 dependent, indeed, both TLR9- and MyD88-deficient treated mice mount immune responses comparable to wild-type mice (90). Such immunogenicity leads to the production of type-I IFNs that were found to be crucial for both direct- and indirect-antigen presentation *via* distinct cell types (i.e. DCs and muscle cells, respectively), resulting in the adjuvant effect for the encoded antigen (91, 92). However, the requirement for IFN-αβ in generating high-level antibody responses has yielded contradictory results. The necessity for IRF3 in cellular-mediated immune responses was previously demonstrated, but with a more limited impact, as described by Suschak et al. (90). Indeed, the temporary defect in immune priming provided by *Irf3* deletion is overcome by the induction of *Irf7*, allowing for rescue of DNA vaccine immunogenicity.

The acknowledged versatility of plasmid DNA facilitates the co-delivery of genetic adjuvants encoding immune-stimulatory molecules that need to be overexpressed and selected antigen/s, producing a new generation of vaccines that stand out for their safety and feasibility. In a preclinical study, the co-administration of two plasmid vectors one encoding the DNA sensor DAI and the other one the melanoma-associated antigen tyrosinase-related protein-2 (TRP2) resulted in enhanced tumor rejection and protection against B16 melanoma challenge (93).

The concerted stimulation of innate immune PRRs by DNA vaccines can achieve a more potent and broader activation of the immune responses and a long-lasting protective adaptive immunity.

Co-expressing TBK1 and the serine repeat antigen, a candidate vaccine antigen expressed in the blood-stages of *Plasmodium falciparum*, in the same plasmid backbone enhanced the antigen-specific humoral immune responses, but failed to improve cellular immune responses (91).

Favorable safety profile and potential clinical benefit were achieved after the phase I clinical trial on the activity of CYL-02, a non-viral gene therapy product that sensitizes pancreatic cancer cells to gemcitabine, a chemotherapic acting as a STING pathway agonist, and a non-invasive biomarkers for patient selection was identified (32).

RIG-I and MDA5-activating DNA vaccines can elicit apoptotic and immunostimulatory effects and, thus, could induce growth inhibition or apoptosis of multiple types of cancer cells. Plasmid vector backbones expressing composite immunostimulatory RNAs that act as synergic RIG-I agonists lead to type-I IFNs production (92, 94).

CONCLUSION AND PERSPECTIVES

The spatiotemporal orchestration of innate stimulation with antigen cross-presentation in APCs represents a crucial challenge

in reaching a strong tumor specific T-cell response, which in turn is crucial for cancer immunotherapy.

Several studies suggest that cytosolic NA-sensing plays a central role in inducing and bridging innate immunity and adaptive immune responses against tumors and that triggering innate immune system contribute to counteract tumor-induced immunosuppression. Employment of RIG-I/MDA5 and cGAS-STING agonists could represent a novel strategy for cancer immunotherapy.

The role of cGAS-STING and RLRs-MAVS pathways in tumor immunity remains complex and numerous questions still remain unanswered.

Noteworthy, some studies suggest that STING activation may induce a suppressive TME, contributing to tumor growth and metastasis and that STING agonists may not be effective against all tumors, particularly those with tolerogenic responses to DNA and low tumor antigenicity (95, 96). Sensing of DNA by specific innate immune or other cell types and different routes of acute or chronic DNA exposure influence immune responses to DNA.

In head and neck squamous cell carcinoma, dose-dependent activation of RIG-I resulted in divergent effects on cancer cell proliferation. Actually, low dose of dsRNA promoted NF-kB- and Akt-dependent cell proliferation and metastasis (97).

The functional consequences of the cGAS-STING and RIG-I-MAVS pathways regulation/activation in different cells within the TME require deeper characterization. Targeting multiple pathways may be required for efficacious therapeutic responses in some patients and the crosstalk between RIG-I and STING pathways through direct interactions between downstream signaling components may amplify the innate response.

The complex role of STING and RIG-I signaling in cancer underlines how innate immune pathways in the TME alter tumorigenesis in distinct tumors, with effects in designing efficacious immunotherapy trials.

AUTHOR CONTRIBUTIONS

SI: searched for literature articles, conceived and wrote the manuscript, conceived the figure, and revised and approved the final version of the manuscript. DF: performed search for clinical trials, collected and analyzed clinical trials data, prepared the tables, conceived and prepared the figure, contributed to the manuscript preparation and editing, and revised and approved the final version of the manuscript. MR: searched for literature articles, conceived and wrote the manuscript, edited the manuscript, and revised and approved the final version 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.2018.00711/full#supplementary-material.

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Humoral Responses Elicited by Adenovirus Displaying Epitopes Are Induced Independently of the Infection Process and Shaped by the Toll-Like Receptor/MyD88 Pathway

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The use of serotype 5 adenovirus (Ad)-derived vectors in vaccination is confronted to preexisting anti-Ad immunity. Epitope display on Ad capsid is currently being investigated as an alternative approach of vaccination. The present study seeks to better understand virus- and host-related factors controlling the efficacy of this new vaccination approach. In contrast to an Ad vector expressing ovalbumin as a transgene, Ad displaying an ovalbumin-derived B-cell epitope inserted into the fiber protein was able to elicit antibody responses in both Ad-naive and Ad-immune mice. Moreover, introduction of a set of mutations abrogating Ad interaction with its receptors did not modify the virus capacity to elicit a humoral response against the inserted epitope while reducing its capacity to mount antibody responses against the transgene product. Taken as a whole these data indicate that the efficacy of Ad displaying epitopes requires neither Ad binding to its receptors nor the infection process. In addition, the use of genetically deficient mice demonstrated that both toll-like receptor (TLR)/MyD88 and RIG-I/mitochondrial antiviral-signaling (MAVS) innate immunity pathways were dispensable to mount anti-epitope antibody responses. However, they also revealed that TLR/MyD88 pathway but not RIG-I/MAVS pathway controls the nature of antibodies directed against the displayed epitope.

Keywords: adenovirus, fiber, innate immunity, antibody isotype, MyD88, mitochondrial antiviral-signaling

INTRODUCTION

Adenoviruses (Ad) belong to a family of non-enveloped DNA viruses containing a linear double-strand DNA genome. Knowledge accumulated over more than 20 years on their biology has led to the development of Ad-derived vectors (1). Ease of Ad manipulation, their production at high titers, as well as the strong level of gene expression achieved by these vectors makes them an attractive tool not only for gene therapy but also for vaccination. Indeed, Ad-mediated gene transfer of DNA fragments encoding heterologous proteins was shown to elicit strong humoral and cellular

responses toward transgene-encoded proteins (2). The efficacy of this approach of vaccination (hereafter referred to as the classical approach) stems from Ad's ability to transduce *in vivo* a large set of cells and in the intrinsic immunogenic properties of this vector (3).

Several studies investigated Ad capsid proteins and cell receptors controlling Ad infection. Thus, in the case of the well-characterized serotype 5 Ad (Ad5), interaction of fiber protein, and more precisely its knob, with Coxsackie and Ad receptor (CAR) was shown to be responsible for initial virus attachment. Subsequent binding of penton base-located RGD motif to cellular integrins allows virus endocytosis through a clathrin-dependent pathway (3). The role of integrins and CAR in controlling Ad distribution *in vivo* was, for a long time, a matter of debate. CAR was shown to play a minor role in the transduction of different tissues, including liver and spleen (4, 5). Integrin-ablated Ad led to a reduced transgene expression in spleen and lungs (6). Of note, ablation of both CAR and integrin binding was unable to reduce liver gene transfer (5, 7) [for review, see Ref. (3)]. Besides CAR and integrins, different studies demonstrated a role for of Ad shaft in controlling liver and spleen transduction (4, 8, 9). More recently, different Ad serotypes including serotype 5 were shown to bind to plasma proteins such as vitamin K-dependent coagulation factors, leading to liver transduction (10). Among numerous coagulation factors, factor X (FX) plays a key role in liver transduction by bridging Ad capsid to liver heparan sulfate proteoglycans. Moreover, mutations of Ad capsid helped to identify Ad hexon protein as the capsomer directly involved in FX binding (11–13).

Apart from their role in cell transduction, Ad receptors contribute to the intrinsic immunogenic properties of this vector. For example, interaction with CAR and integrins were at the origin of pro-inflammatory cytokine and chemokine production in epithelial cells and macrophages [for review, see Ref. (3)]. Innate immune responses to Ad are also triggered through the stimulation of pathogen recognition receptors. Several studies reported a role of membrane-anchored sensors, such as toll-like receptor (TLR) 9 and more surprisingly TLR2 in controlling cytokine production (14, 15). In addition, mice deficient in Myeloid differentiation primary response gene 88 (MyD88)—an adaptor protein common to different TLR signaling pathways—displayed reduced levels of plasma pro-inflammatory cytokines and chemokines upon intravenous Ad administration (14). After endosome escape, one could anticipate Ad to stimulate cytosolic sensors. Indeed, following Ad infection, synthesis of viral-associated RNA elicits type I interferon (IFN) through retinoic acid-inducible gene (RIG)-I mediated pathway (16). Finally, comparison of the transcriptome in the spleen after administration of wild-type and FX-ablated Ad revealed an unanticipated key role of FX in activating NFκB pathway leading to pro-inflammatory cytokine production (17).

Despite their efficacy in transducing cells *in vivo* and their strong adjuvant properties, the use of Ad in the classical vaccination approach is hampered by the highly prevalent anti-Ad5 immunity. Moreover, Ad vector immunogenicity impairs the efficiency of homologous prime-boost administrations. Several strategies were developed to overcome these limitations [for review, see Ref. (2)]; among them, epitope display relying on genetic insertion of relevant epitopes on Ad capsid. This approach was successful at inducing antibody responses against *P. aeruginosa* (18), *B. anthracis* (19), or *Plasmodium* (20). Using a B cell epitope derived from a model antigen, ovalbumin, we previously uncovered that anti-Ad preexisting antibodies (Abs) strongly increased the antibody response elicited by Ad displaying the epitope into the fiber protein (21). The present results seek to go further in our understanding of this strategy of vaccination by defining the role of Ad interaction with their receptors, as well as the influence of innate immune pathways.

MATERIALS AND METHODS

Mice

Seven-week-old C57BL/6 female mice were purchased from Harlan (Gannat, France). MyD88- ($MyD88^{-/-}$) (22) and mitochondrial antiviral-signaling (MAVS)- ($MAVS^{-/-}$) (23) deficient mice were bred in animal facilities of TAAM-UPS 44 (Orléans) and UMR 0892 (VIM, Jouy-en-Josas), respectively. All mice were conditioned for at least 1 week in our animal facilities before beginning of the experiments. All animal experiments were approved by Ethics Committee No. 26 (officially recognized by the French Ministry for Research) in accordance with the European Directive 2010/63 UE and its transposition into French Law.

Virus Construction and Production

AdWT [described as AE18 in Ref. (24)] is based on Ad5 and is deleted in E1 and E3 regions. The expression cassette cloned instead of the E1 region contains the promoter/enhancer from the immediate early gene of human cytomegalovirus, the *Escherichia coli lacZ* gene with a nuclear localization signal, and the SV40 late polyA signal (SV40pA). AdH-3OVA2 and AdF-3OVA2 derived from AdWT and displaying $OVA_{320–341}$ (3OVA2) epitope, respectively, in hexon or fiber protein were described previously (21). AdP*F-3OVA2, AdH*F-3OVA2, AdH*P*F-3OVA2, and AdS*F-3OVA2 disabled to a different extend in Ad interactions with their natural receptors, were derived from AdF-3OVA2 (**Table 1**). More precisely, AdP*F-3OVA2 was derived from AE74 (9) bearing a deletion of penton base RGD motif, impairing interaction with integrins. AdH*F-3OVA2 was derived from AdH[GA]24 (12) bearing a deletion of hypervariable region 5 (HVR5) of hexon protein, impairing FX binding. AdH*P*F-3OVA2 contains both deletions. Finally, AdS*F-3OVA2 was obtained from AdWT by replacing the fiber shaft with the shaft from Ad serotype 3 (25). All capsid-modified viruses (**Table 1**) were constructed by recombinational cloning in *E. coli* (26).

AdOVA (provided by Dr. D. Descamps, INRA, Jouy-en-Josas) has a wild-type capsid and encodes the complete amino-acid sequence of ovalbumin protein. AdControl encoding no transgene was described previously (27).

All viruses were obtained using previously described procedures (5), stored at −80°C in PBS-7% glycerol, and titrated by spectrophotometry [1 $OD_{260} = 1.1 \times 10^{12}$ viral particle (vp)/ml].

TABLE 1 | Characteristics of Ad displaying ovalbumin-derived epitopes.

Virus	Transgene	Capsid modifications			Titer[b] ($\times 10^{12}$ vp/cell)
		Penton	Hexon	Fiber	
AdWT	βgal	–	–	–	4.4 ± 0.8
AdOVA	Ovalbumin	–	–	–	7.3 ± 2.1
AdH-3OVA2	βgal	–	3OVA2[a]	–	4.5 ± 2.9
AdF-3OVA2	βgal	–	–	3OVA2	4.6 ± 1.9
AdP*F-3OVA2	βgal	RGD deletion	–	3OVA2	2.3 ± 1.0
AdH*F-3OVA2	βgal	–	Hypervariable region 5 (HVR5) deletion	3OVA2	2.1 ± 0.6
AdH*P*F-3OVA2	βgal	RGD deletion	HVR5 deletion	3OVA2	2.5 ± 1.2
AdS*F-3OVA2	βgal	–	–	3OVA2 + Ad3 shaft	1.7 ± 0.6

[a]*3OVA2 refers to ovalbumin 320–341 epitope.*
[b]*Mean ± SD.*

Cell Lines

The 293A human embryonic kidney cell line (R705-07, Invitrogen) was maintained in modified Eagle medium supplemented with 10% FBS and 1% of non-essential amino acids. CHO-k1-hCAR and CHO-k1-pCDNA were kindly provided by J. M. Bergelson (School of Medecine, University of Pennsylvania). L929 murine fibrosarcoma cells were kindly provided by Dr. U. Greber (Institute of Molecular Biology, Zurich, Switzerland) and maintained in DMEM supplemented with 10% FCS.

SDS-PAGE and Western Blot

Purified viruses (10^{10} vp) were resuspended in Laemmli lysis buffer, boiled for 10 min and loaded onto a 10% NuPage gel (Novex, Invitrogen, CA, USA). After electrophoresis, the gel was stained with a silver staining kit (Invitrogen, Carlsbad, CA, USA). Alternatively, the gel was transferred on nitrocellulose membrane and the membrane was incubated with a rabbit polyclonal antibody directed against the fiber protein.

In Vitro Cell Transduction

In order to evaluate virus infectivity, cells were plated out in 12-well plates at 1×10^5 cells/well 48 h prior to infection. On the day of infection, cells were counted and infected with the indicated multiplicity of infection (MOI) of different Ad in 400 µl of serum-free medium. After 24 h, βgalactosidase (βgal) activity was measured using a chemiluminescent assay (BD Biosciences, Clontech, CA, USA) and protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, Marnes-la-Coquette, France). Results were presented as relative light units (RLU) per microgram of proteins.

In order to evaluate FX-dependent cell transduction, viruses were mixed with or without human FX (1 U/ml, Cryopep). Then, virus-FX solution was added to CHO-k1-pCDNA cells and cells were incubated for 24 h at 37°C. βgal activity was measured as described above.

Epitope Detection on Virions

To confirm the presence and accessibility of the epitopes on the capsid surface, viral particles were coated on 96-well plates (Nunc, Roskilde, Denmark). Viruses were inactivated by incubation at 56°C for 30 min, followed by addition of 0.1% SDS. Spectrophotometric measurements at 215 and 225 nm allowed to determine viral protein concentrations, and subsequently 100 ng was coated on 96-well plates. To analyze epitope presence and accessibility on virions, non-denaturated viruses (100 ng) were coated on the plates. After overnight incubation at 4°C, non-specific sites were blocked with 5% milk PBS-Tween for 2 h, then plates were washed and incubated for 1 h with an anti-ovalbumin rabbit polyclonal antibody (AB1225, Millipore, MA, USA). Upon washing, an anti-rabbit IgG peroxidase-linked Ab (NA934, Amersham Biosciences, Saclay, France) was added for 1 h and peroxidase activity was revealed by incubation with the substrate *O*-phenylenediamine dihydrochloride (Sigma-Aldrich, Lyon, France) for 30 min. The reaction was stopped by addition of 3 N HCl and spectrophotometric readings were performed at 490 nm. Each virus was assayed in sexdecaplicates and the experiments were repeated at least twice.

In Vivo Experiments

Capsid-modified viruses (10^{10} vp) in PBS (200 µl) were injected intraperitoneally. Repeated injections were performed at 2 weeks intervals with a total number of injections ranging between two and three. Blood samples were collected from the submandibular vein, before virus injection and at different time points thereafter. Mice sera were prepared and analyzed for the presence of anti-ovalbumin, anti-βgal, and anti-Ad Abs by ELISA as described below.

In some experiments, mice were depleted of coagulation factors by subcutaneous injection of 133 µg of warfarin in 100 µl of PBS at days 3 and 1 prior to virus administration as described previously (10). Ad-naive or Ad–immune mice were obtained after injection of PBS or AdControl (10^{10} vp), respectively.

Measurement of Specific Abs

Sera were analyzed for the presence of specific Abs by ELISA. After coating of 96-well plates (Nunc) with 1 µg of ovalbumin (Sigma), 100 ng of βgal (Sigma), or 100 ng of denaturated AdWT viral particles, serial dilutions of the sera in 5% milk PBS-Tween were added. Bound Abs were detected with peroxidase-conjugated anti-mouse IgG, IgG1, IgG2b, or IgG2c isotype goat Abs (Southern Biotechnology Associates, Birmingham, AL, USA). The peroxidase activity was revealed by incubation with the

substrate *O*-phenylenediamine dihydrochloride (Sigma-Aldrich) for 30 min. The reaction was stopped by addition of 3 N HCl and spectrophotometric readings were performed at 490 nm. Titers were defined as the reciprocal of the highest dilution giving an OD_{490} twofold above background values.

Statistical Analysis

Data from *in vivo* experiments (titers) were log_2-transformed before analysis. Comparison between two groups was done using unpaired Mann–Whitney test. Comparison between multiple groups were done using Kruskal–Wallis test followed with Dunn's *post hoc* test. Two-way repeated measures ANOVA was used for comparison of responses measured for different groups at different time points, then Bonferroni *post hoc* test was used to compare between groups at each time point. Differences were considered significant when $P < 0.05$. All graphs and statistical tests were obtained with the use of GraphPad Prism software.

RESULTS

Antibody Responses Elicited by Ad Displaying Epitopes Do Not Require Gene Transfer

Our previous results have shown that pre-existence of anti-Ad Abs strongly enhances anti-ovalbumin antibody responses elicited by AdF-3OVA2 bearing $OVA_{320–341}$ B cell epitope (21), suggesting that cell transduction is dispensable for the efficacy of this vector. To address specifically this point, we compared the ability of capsid-modified AdF-3OVA2 and AdH-3OVA2 (**Table 1**) to elicit anti-ovalbumin antibody responses relative to AdOVA, encoding the whole ovalbumin protein as a transgene, in both naive and immune mice. C57BL/6 mice were injected with PBS or with an Ad bearing a wild-type capsid (AdWT, 10^{10} vp) and 2 weeks later received one intraperitoneal injection of AdH-3OVA2, AdF-3OVA2, or AdOVA (10^{10} vp). In Ad-naive mice, AdOVA induced high levels of anti-ovalbumin Abs from day 14 p.i. up to 209 days p.i (**Figure 1**, upper panel). In accordance with our previous study (21), AdH-3OVA2 led to a significantly higher anti-ovalbumin Ab responses compared to AdF-3OVA2. However, these responses remain lower than the ones observed in AdOVA-injected mice (**Figure 1**, upper panel). In Ad-immune mice, AdF-3OVA2 triggered strong anti-ovalbumin antibody responses compared to AdH-3OVA2 as described previously (21) while AdOVA was unable to trigger any antibody response (**Figure 1**, middle panel). Interestingly, the immune responses elicited by AdF-3OVA2 were long-lasting with strong Ab titers detectable up to 7 months after the immunization. While AdF-3OVA2 and AdH-3OVA2 elicit strong anti-βgalactosidase (βgal) Ab responses in Ad-naive mice, none of them triggered significant Ab responses in Ad-immune mice (Figure S1 in Supplementary Material).

Altogether, our data indicate that, in contrast to AdOVA, AdF-3OVA2 was able to trigger Ab responses in both Ad-naive

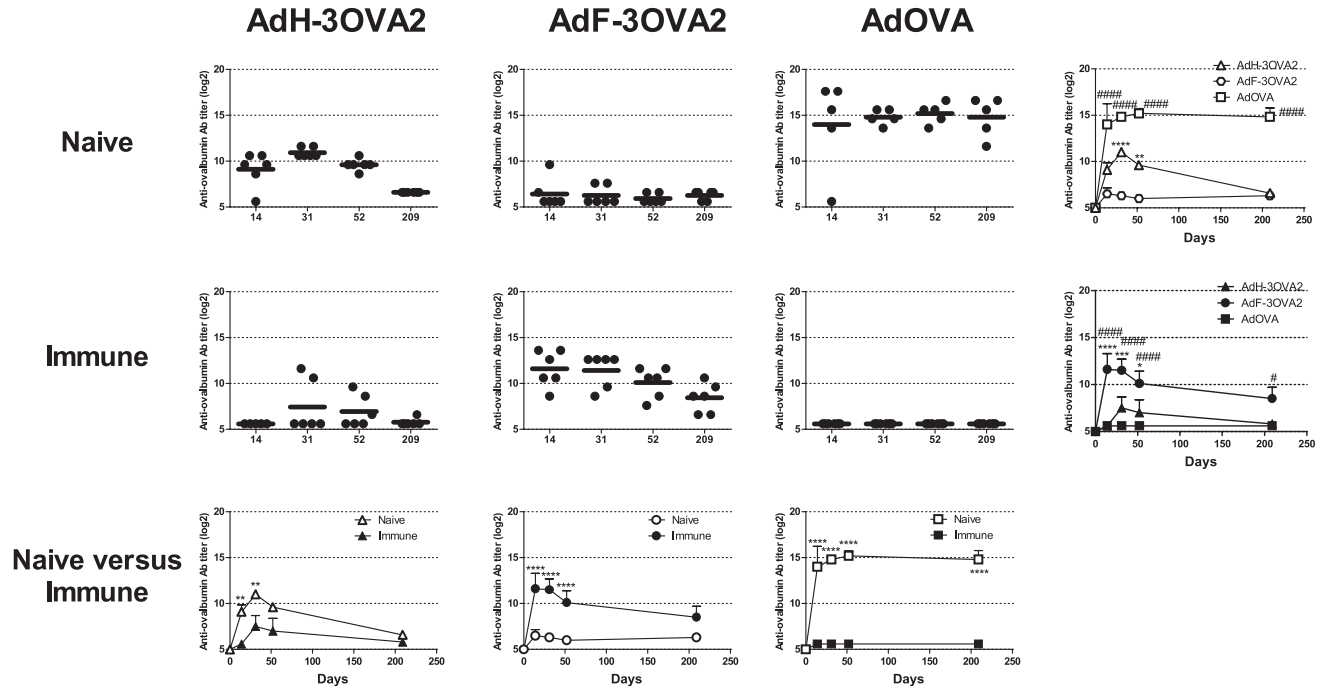

FIGURE 1 | Kinetics of anti-ovalbumin humoral response. Naive- (upper panel) or Ad-immune (medium panel) C57Bl/6 mice were immunized intraperitoneally with 10^{10} vp of capsid-modified Ad (AdH-3OVA2 or AdF-3OVA2) or with an Ad vector encoding ovalbumin as a transgene (AdOVA). Anti-ovalbumin IgG titers were determined by ELISA at different time points after injection. Titers below 100 were plotted as 50. Left part, circles and bars represent results of individual mice (n = 5–6) and means, respectively. Right part, means + SEM for each group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ difference between AdF-3OAV2 and AdH-3OVA2. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$ difference between AdF-3OVA2 and AdOVA. (Lower panel) comparison between naive and immune mice. $^{**}p < 0.01$ and $^{****}p < 0.0001$.

and Ad-immune mice, suggesting that virus transduction is not mandatory for the efficacy of this vector.

Production and Characterization of Ad Displaying Ovalbumin Epitope in the Fiber Protein and Ablated in their Native Receptor Interactions

To examine more precisely whether virus transduction plays any role in controlling the efficacy of AdF-3OVA2, different vectors were produced displaying both 3OVA2 epitope inserted into the fiber protein and capsid modifications impairing binding to specific receptors. AdP*F-3OVA2 presents a deletion of the RGD motif in penton base protein in order to ablate interaction with integrins (9). AdH*F3-OVA2 contains a mutation in hexon HVR5 impairing binding to FX (12). AdH*P*F-3OVA2 possesses both hexon and penton base mutations. Finally, AdS*F-3OVA2 contains an Ad3 fiber shaft instead of Ad5 shaft (25) impairing binding to CAR receptor. All vectors were produced at titers comparable to AdF-3OVA2 (**Table 1**). SDS-PAGE analyses showed no difference in virus composition between the different Ad. As expected, all viruses but AdS*F-3OVA2 displayed a similar migration pattern for the modified fiber (MW = 63.3 kDa, **Figure 2A**). For AdS*F-3OVA2, the migration pattern of the fiber protein is consistent with its reduced size (MW = 33.8 kDa, **Figure 2A**). Using a polyclonal serum specific for the fiber protein, we confirmed the modification of fiber size (long fiber or short fiber) for all vectors displaying 3OVA2 epitope (**Figure 2B**). Additionally, 3OVA2 epitope was detected on native virions by ELISA using a polyclonal anti-ovalbumin antibody (**Figure 2C**). Interestingly, the detection of 3OVA2 epitope on AdS*F-3OVA2 was reduced compared to AdF-3OVA2 ($p < 0.001$).

With the exception of AdS*F-3OVA2, all vectors possess the same capacity to transduce CHO-k1-CAR cells, a cell line over-expressing Ad primary receptor. AdS*F-3OVA2 led to a reduced cell transduction (**Figure 3A**) consistent with previous results of our laboratory (Raddi et al., in revision). Next, we examined transduction efficiency using specific cell lines allowing to monitor the detargeting from native Ad receptors. AdP*F-3OVA2 and AdH*P*F-3OVA2 were tested on CAR-negative integrin-positive L929 cells. Both vectors led to a reduced βgal activity compared to AdF-3OVA2, confirming their reduced ability to bind integrins (**Figure 3B**). AdP*F-3OVA2 and AdH*P*F-3OVA2 were used to transduce CAR-negative CHO-pcDNA cells in the presence or absence of FX (**Figure 3C**). The results showed that both vectors displayed reduced ability to use FX in accordance with the role of hexon protein in FX binding (**Figures 3C,D**).

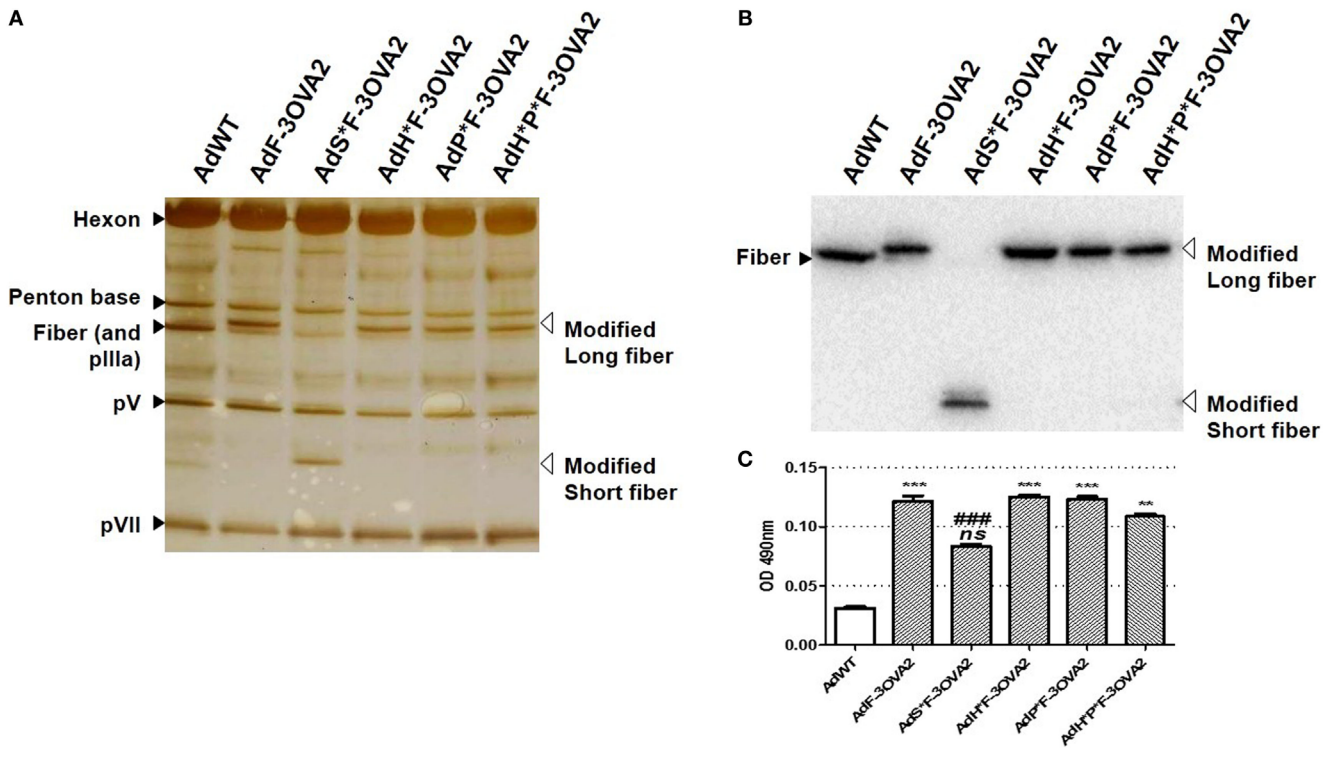

FIGURE 2 | Epitope detection on capsid-modified vectors. **(A)** Silver staining of capsid-modified Ad. Similar amount (10^{10} vp) of either a control Ad (AdWT) or a capsid-modified Ad (AdF-3OVA2, AdH*F-3OVA2, AdP*F-3OVA2, AdH*P*F-3OVA2, and AdS*F-3OVA2) were separated on a 10% polyacrylamide gel. Modified fibers are indicated with white arrows while hexon, penton base, polypeptide IIIa (pIIIa), and native fiber proteins are labeled with black arrows. **(B)** Detection of fiber protein by western blot. **(C)** Detection of 3OVA2 epitopes on virions. ELISA plates were coated with 100 ng of native viruses and incubated with a rabbit polyclonal antibody against ovalbumin protein. The binding was detected with a HRP-conjugated secondary antibody. One of two experiments is shown, means + SEM of 16 replicates. ns, non significant; $^{**}p < 0.01$ and $^{***}p < 0.001$ versus AdWT; $^{###}p < 0.001$ versus AdF-3OVA2.

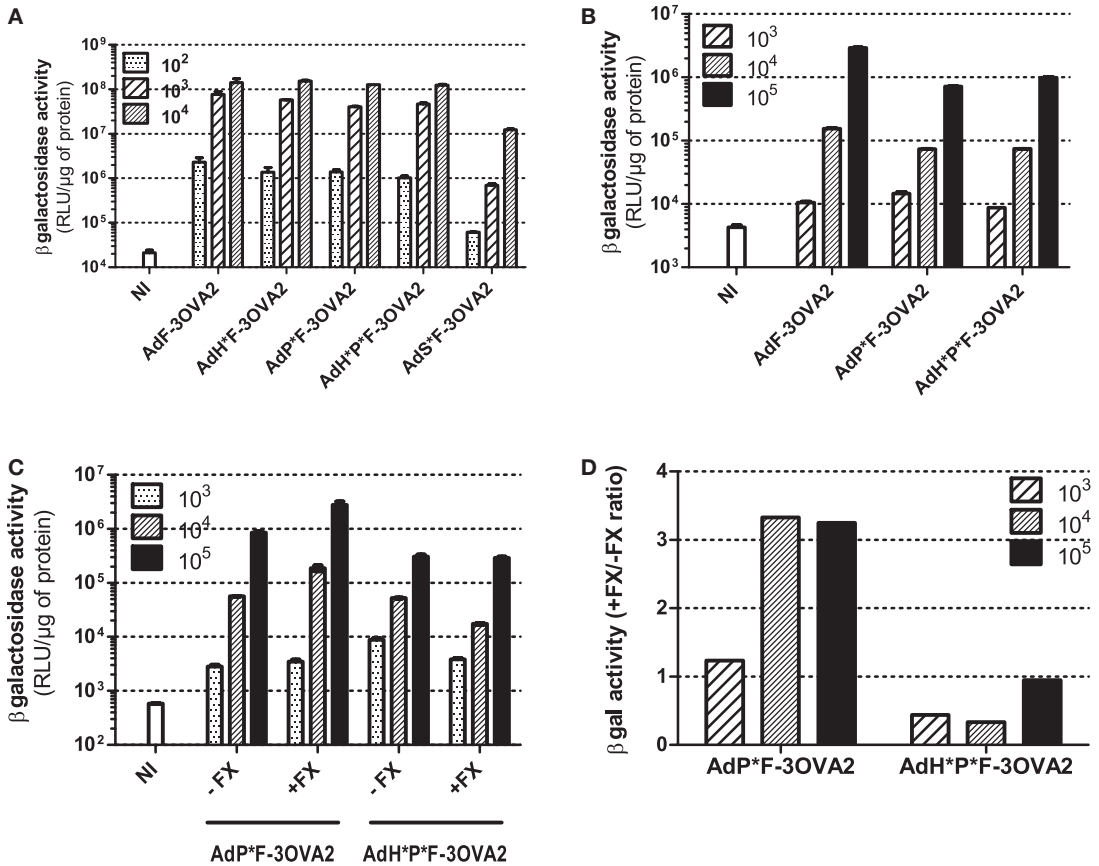

FIGURE 3 | *In vitro* infectivity of capsid-modified vectors. **(A)** Transduction of CHO-k1-hCAR. Cells were mock-infected (NI) or infected with indicated multiplicity of infection (MOI) of capsid-modified Ad (AdF-3OVA2, AdH*F-3OVA2, AdP*F-3OVA2, AdH*P*F-3OVA2 and AdS*F-3OVA2). Vectors were added at different MOI to CHO-k1-hCAR cells and incubated for 24 h. **(B)** Transduction of L929 cells. Cells were mock-infected or infected with different MOI of capsid-modified Ad (AdF-3OVA2, AdP*F-3OVA2 and AdH*P*F–3OVA2). **(C,D)** Transduction of CHO-k1-pCDNA. Cells were mock-infected or infected with different MOI of capsid-modified Ad (AdH*F-3OVA2 and AdH*P*F-3OVA2) with or without physiological levels (1 U/ml) of factor X (FX). In all panels, βgal activity (means + SD of duplicates) was measured in cells harvested 24 h post-infection and expressed as relative light unit (RLU) per microgram of protein **(A–C)** or as a ratio of βgal activity with FX relative to the one without FX **(D)**. The representative results of at least two experiments are shown.

Humoral Responses Elicited by Ad Displaying Ovalbumin Epitope on the Fiber Protein and Ablated in their Native Receptor Interactions

After the characterization of produced vectors, we examined their capacity to mount anti-ovalbumin humoral responses. C57BL/6 mice were injected intraperitoneally twice 2 weeks apart with 10^{10} vp. Sera were collected 2 weeks after each administration and anti-ovalbumin, anti-βgal, and anti-Ad Abs were quantified by ELISA. Remarkably, no difference in ability to trigger anti-ovalbumin Abs was observed between detargeted vectors (AdH*F-3OVA2, AdP*F-3OVA2, AdH*P*F-3OVA2, and AdS*F-3OVA2) and AdF-3OVA2, neither after priming nor after boosting (**Figure 4A**). In contrast, anti-βgal antibody titers were significantly reduced after priming in AdH*P*F-3OVA2-injected mice (**Figure 4B**, $p < 0.05$) compared to AdF-3OVA2-injected mice. After boosting AdH*P*F-3OVA2- and AdS*F-3OVA2 were significantly impaired in their ability to trigger anti-βgal Ab (**Figure 4B**, $p < 0.05$ and $p < 0.01$ respectively). Of note, all vectors induced comparable anti-Ad Ab responses at all time points (data not shown). Altogether, these results underline that while detargeting Ad from its natural receptors reduces its ability to trigger humoral responses against the transgene, it has no impact on its ability to trigger humoral responses against an epitope displayed on the capsid.

In parallel to FX-detargeting through hexon modification, we also used a pharmacological approach based on administration of warfarin, a drug able to deplete all vitamin K-dependent blood factors. To do so, mice were pre-treated with warfarin or PBS before each AdF-3OVA2 administration. Blood factor depletion was confirmed by the measurement of FX activity in mice sera harvested prior to virus delivery (data not shown). Measurement of anti-ovalbumin titers showed no significant difference between warfarin- and PBS-pre-treated mice neither after priming nor after boosting (Figure S2A in Supplementary Material). Additionally, levels of anti-βgal

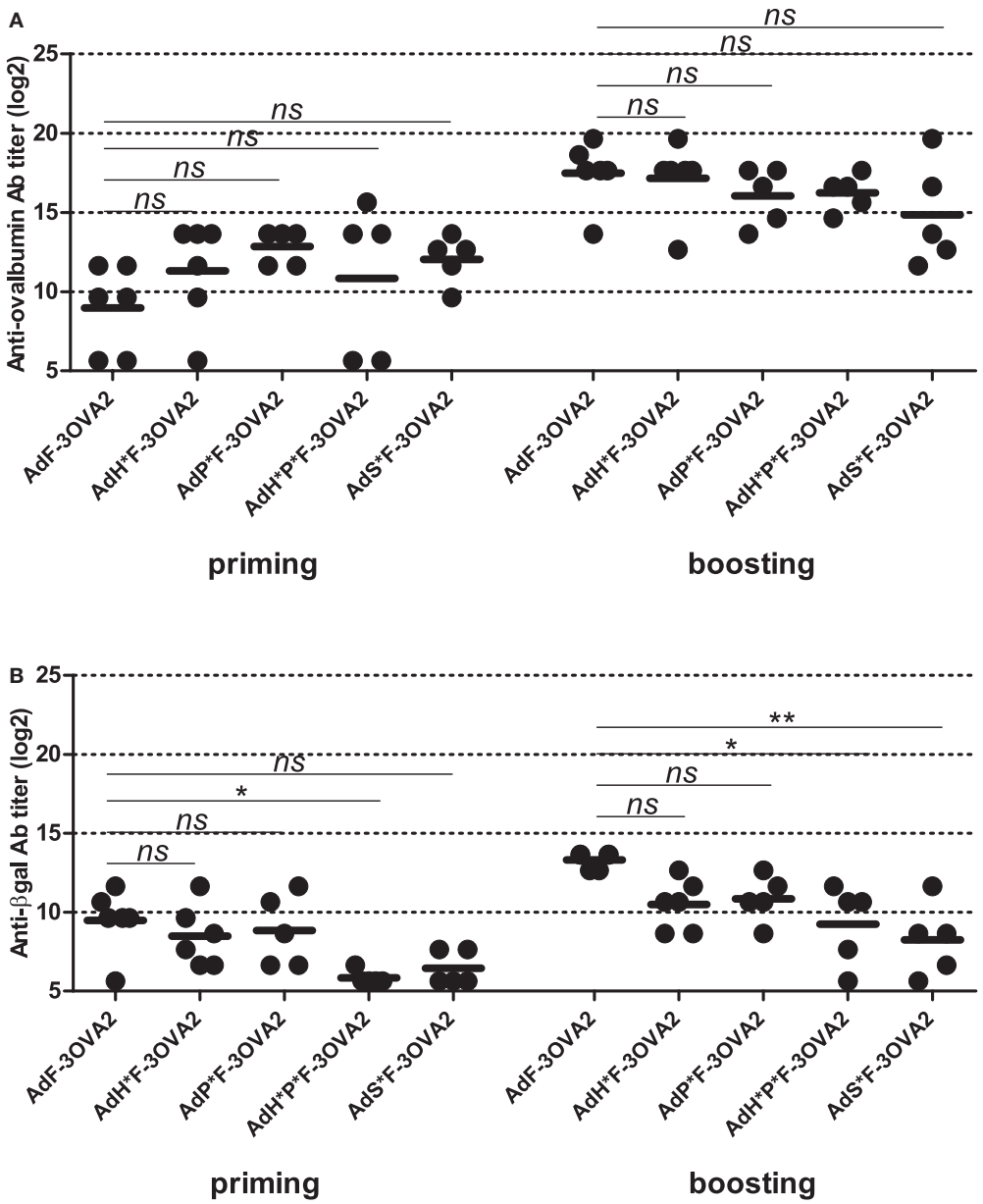

FIGURE 4 | Humoral responses elicited by Ad displaying 3OVA2 epitopes and ablated in their native receptor interaction. C57BL/6 mice were immunized intraperitoneally with 10^{10} vp of capsid-modified AdF-3OVA2, AdH*F-3OVA2, AdP*F-3OVA2, AdH*P*F-3OVA2, and AdS*F-3OVA2. Anti-ovalbumin **(A)** and anti-βgal **(B)** IgG titers were determined by ELISA at day 14 after first (priming) and second (boosting) administration. Titers below 100 were plotted as 50. One of two experiments is shown, dots and bars represent results from individual mice (n = 5–6 mice) and means, respectively (*$p < 0.05$; **$p < 0.01$ versus AdF-3OVA2).

and anti-Ad Abs also remained unmodified in warfarin-pre-treated mice (Figures S2B,C in Supplementary Material). Thus, both genetic (**Figure 4A**) and pharmacological (Figure 2A in Supplementary Material) approaches used to detarget Ad from FX were unable to decrease humoral responses toward 3OVA2 epitope.

Collectively, these results underline that in contrast to humoral responses against βgal transgene, humoral responses against 3OVA2 epitope displayed on the fiber protein do not rely on Ad interaction with their cellular receptors.

Role of Innate Immune Pathways in Humoral Responses Elicited by Ad Displaying Ovalbumin Epitope Inserted into the Fiber Protein

To get further insight into molecular bases controlling the efficacy of vaccination with Ad displaying epitopes, we investigated the role of innate immune pathways. First, since TLR and MyD88 were shown to participate in Ad innate immunity, we examined their role in shaping humoral responses by using MyD88-deficient

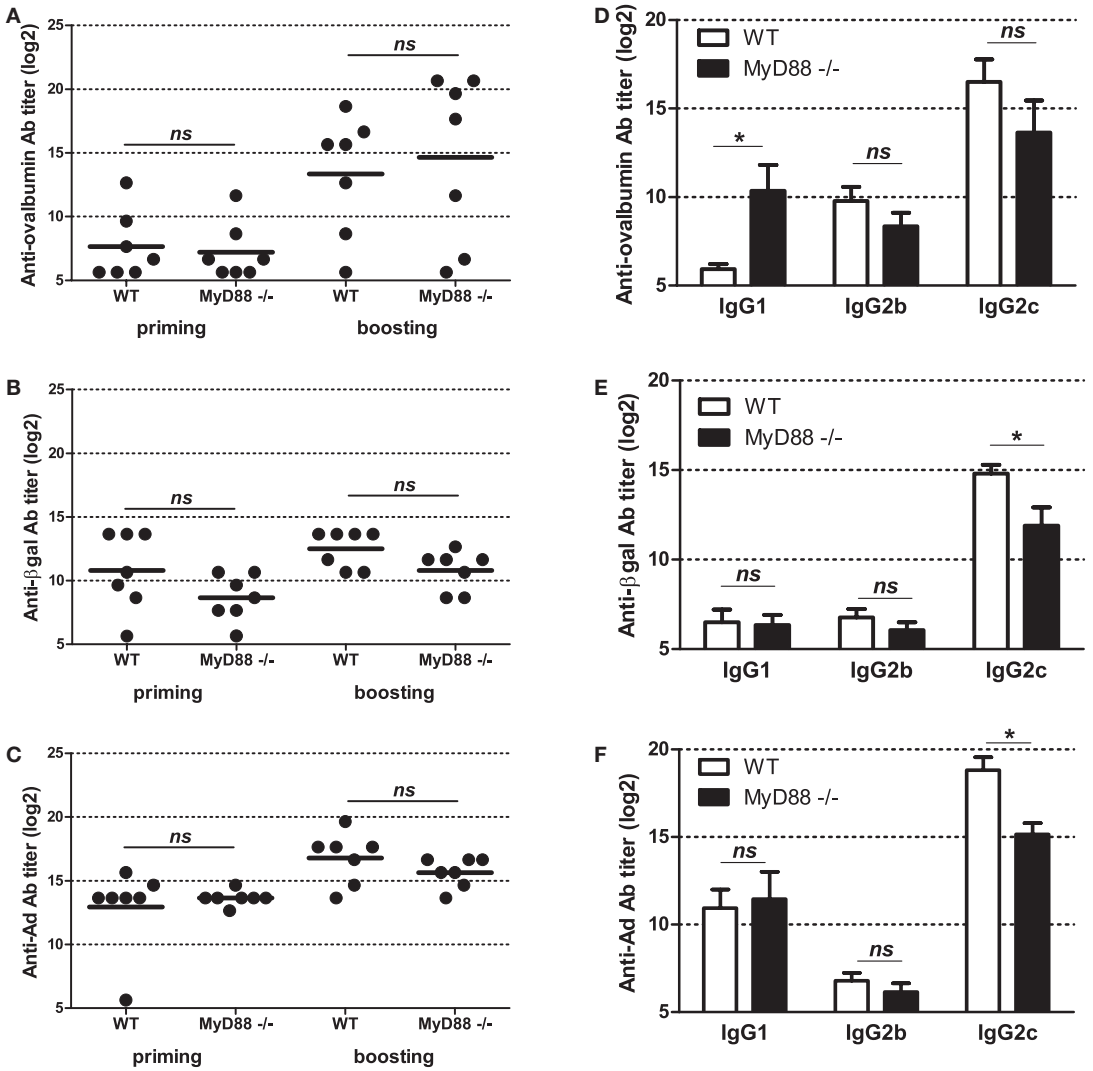

FIGURE 5 | Influence of MyD88 on humoral responses elicited by Ad displaying 3OVA2 epitope. Mice were immunized intraperitoneally with 10^{10} vp of AdF-3OVA2. Anti-ovalbumin **(A)**, anti-βgal **(B)**, and anti-Ad **(C)** IgG were measured by ELISA at day 14 after first (priming) and second (boosting) administration. Dots and bars represent results from individual mice (n = 6–7 mice) and means, respectively. Titers below 100 were plotted as 50. ns, non significant. Anti-ovalbumin **(D)**, anti-βgal **(E)**, and anti-Ad **(F)** antibodies of IgG1, IgG2b, and IgG2c isotypes were measured by ELISA at day 42 after the first administration. One of two experiments is shown, means + SEM (n = 6–7). *$P < 0.05$.

mice. No significant difference was found in anti-ovalbumin IgG Ab responses in wild-type and MyD88-deficient mice, neither after priming nor after boosting (**Figure 5A**). In addition, both strains elicited comparable levels of anti-βgal (**Figure 5B**) and anti-Ad Abs (**Figure 5C**). Interestingly, compared to AdF3-OVA2-injected wild-type mice, AdF3-OVA2-injected MyD88$^{-/-}$ mice displayed a strong increase in IgG1 anti-ovalbumin Abs (**Figure 5D**, $p < 0.05$) and a trend toward reduced levels of IgG2b and IgG2c anti-ovalbumin Abs. In contrast, MyD88$^{-/-}$ mice displayed very low titers of anti-βgal IgG1 and IgG2b and a strong reduction in IgG2c (**Figure 5E**, $p < 0.05$). A reduction in IgG1 anti-Ad Abs was also found in MyD88$^{-/-}$ mice (**Figure 5F**, $p < 0.05$). Then, we examined the role of RIG-I-induced innate immune pathway using mice deficient in MAVS protein. The levels of total IgG (**Figure 6A**) as well as IgG1, IgG2b, and IgG2c anti-ovalbumin Abs (**Figure 6B**) were comparable in wild-type and in MAVS$^{-/-}$ mice, ruling out a major role of RIG-I/MAVS pathway in controlling anti-epitope humoral responses. In addition, no difference was found in anti-βgal (**Figure 6C**) or anti-Ad (**Figure 6D**) Ab responses between both strains.

Altogether, these results underline that TLR/MyD88 and RIG-I pathways are both dispensable in mounting antibody responses against 3OVA2 epitope. However, they also reveal that TLR/MyD88 pathway influences the isotype nature of antibody responses against the displayed epitope.

DISCUSSION

Vectors derived from Ad were used in different preclinical studies as well as in clinical trials for the vaccination purpose. The

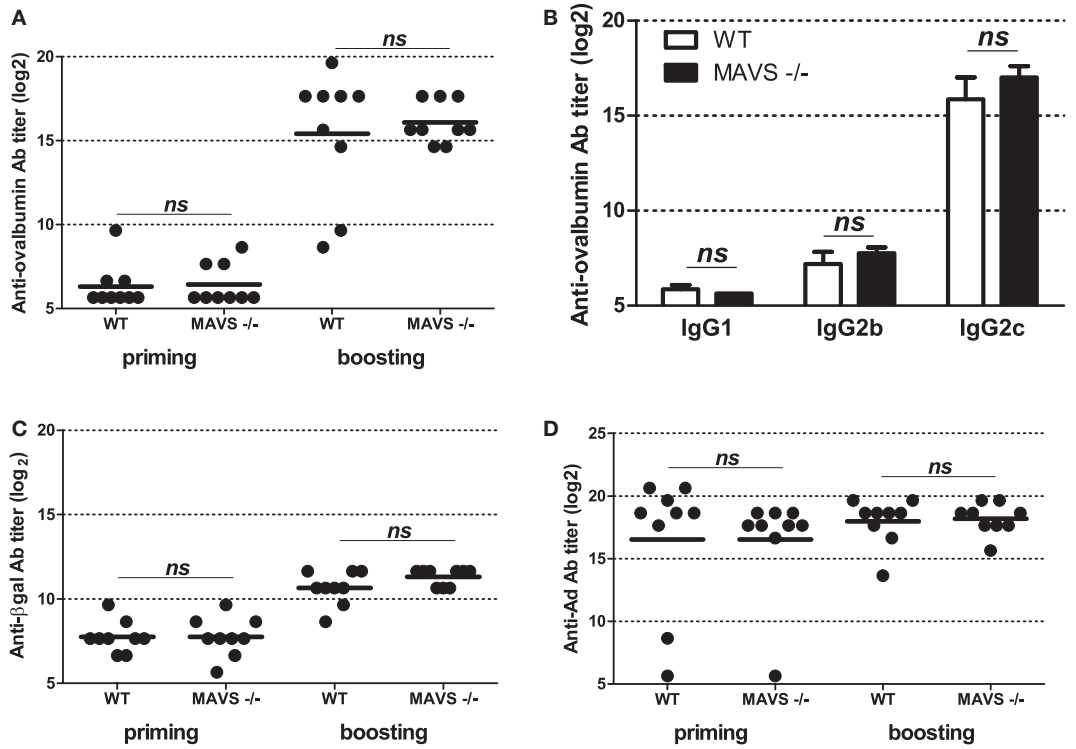

FIGURE 6 | Influence of RIG-I/mitochondrial antiviral-signaling (MAVS) pathway on humoral responses elicited by Ad displaying 3OVA2 epitope. Mice were immunized intraperitoneally with 10^{10} vp of AdF-3OVA2. Total IgG specific for ovalbumin **(A)**, βgal **(C)**, and Ad **(D)** were determined by ELISA at day 14 after first (priming) and second (boosting) administration. One of two experiments is shown. Titers below 100 were plotted as 50. Circles represent individual mice (n = 8–9) and bars reflect means. ns, non significant. **(B)** IgG isotypes specific for ovalbumin were determined by ELISA at day 28 after the second administration. Means + SEM (n = 8–9). ns, non significant.

high seroprevalence of neutralizing Abs as well as the induction of strong anti-vector immunity after first administration led to the development of different strategies allowing to overcome these limits (2). Among them is the use of other Ad serotypes or even xenotypes, but also the epitope display on Ad capsid. This latter approach relies on the insertion of peptides within a capsid protein (most frequently the hexon protein). Commonly, those peptides were B cell epitopes and they were found to successfully elicit antibody responses against model antigens (21) but also against several pathogens such as influenza virus (18), *B. anthracis* (19), or *Plasmodium yoelii* (20). While several studies investigated the role of the epitope insertion site (and thus the number of introduced motifs per capsid) (21, 27) or the size of the peptides (19), to the best of our knowledge no study investigated molecular mechanisms controlling the efficacy of Ad displaying epitopes.

In a previous study, we discovered a key role of anti-Ad Abs in increasing antibody responses induced by Ad displaying ovalbumin-derived epitopes in the fiber protein. At the same time, anti-Ad Abs were impeding the efficacy of Ad displaying epitopes into the hexon protein (21). These previous results suggested that when anti-Ad Abs were able to neutralize the particle, Ad displaying epitopes were still able to trigger antibody responses. Two observations of the present paper confirmed that Ad infectious process is not mandatory to allow triggering of antibody responses by AdF-3OVA2. First, *lacZ* recombinant AdF-3OVA2 failed to mount significant anti-βgal Ab responses in Ad-immune mice while being more efficient in inducing anti-ovalbumin Ab responses than in Ad-naive mice (**Figure 1**). Second, detargeting AdF-3OVA2 from its native receptors reduced its ability to elicit antibody responses against βgal transgene without significant impairment of Ab production against 3OVA2 epitope (**Figure 4A**). Altogether, these results indicate that epitope display strategy does not rely on gene delivery in contrast to the classical Ad vaccine approach that requires transgene expression.

Detargeting Ad from integrin receptor or from FX did not impact Ad's capacity to induce humoral responses toward βgal transgene. However, ablation of both integrin and FX binding (AdH*P*F-3OVA2) or modification of Ad5 shaft (AdS*F-3OVA2) translated into a significant reduction of anti-βgal antibody titers (**Figure 4B**). These results may stem from the reduced ability of AdH*P*F-3OVA2 to transduce the spleen and to induce cytokine production, due to its impaired hexon:FX and penton:integrin binding (17, 28). The decrease in anti-βgal responses for AdS*F-3OVA2 could be related to the reduced ability of shaft-mutated Ads to transduce different cells and tissues *in vivo* (Raddi et al. in revision) but may also be linked to their reduced potential to trigger pro-inflammatory cytokine and chemokine production (25).

Innate immune responses are key factors in the establishment of adaptive immune responses. Among the different innate immune pathways, Ad was shown to activate TLR/Myd88 signaling. At all analyzed time points, no significant modification of total anti-IgG Abs against Ad, βgal or 3OVA2 epitope was found in MyD88$^{-/-}$ mice compared to their wild-type counterparts (**Figure 5A**). These data suggest that TLR/MyD88 pathway is dispensable in mounting efficient humoral responses or, alternatively, that other innate immune pathways could compensate the lack of MyD88. However, it should be noticed that Hartman et al. reported previously a reduction in anti-Ad IgG Ab in MyD88$^{-/-}$ mice compared to heterozygous MyD88$^{+/-}$ mice (29). The discrepancies between their and our study could be linked to differences in mouse strains, virus dose or mode of administration.

Increase of anti-epitope IgG1 Abs (**Figure 5D**) unraveled a role of MyD88 in shaping Ig isotype balance. Interestingly, MyD88 was not mandatory for the production of different anti-βgal (**Figure 5E**) and anti-Ad (**Figure 5F**) IgG isotypes, but it influences the level of production of anti-βgal and anti-Ad IgG2c production. The difference in MyD88 requirement for the production of Abs against the inserted epitope, the vector or the transgene product could be linked to intrinsic nature of the antigen (soluble protein or particle, monomeric or multimeric protein). The precise TLR involved in MyD88 activation was not investigated in this study. However, previous studies have unraveled Ad's capacity to trigger different TLRs, such as TLR2 (30), TLR4 (17), and TLR9 (14, 30).

Modification of IgG isotype balance in MyD88$^{-/-}$ mice compared to their wild-type counterparts was previously reported for other non-enveloped DNA viruses (31–33) but also for enveloped RNA viruses (34, 35). The role of MyD88 may be linked to its ability to trigger type I IFN production by dendritic cells. This cytokine was shown to promote IgG2b and IgG2c production while reducing IgG1 level (36, 37). MyD88 expressed in B cell could also directly promote isotype switching and affinity maturation as described previously (32, 38).

Several studies have formerly shown that Ad triggers RIG-I pathway (16). Our study revealed that mice deficient in MAVS, a protein acting downstream of RIG-I, did not show modification in total IgG nor specific IgG isotype production against the epitope displayed into the capsid (**Figure 6**). This suggests that MAVS/RIG-I pathway did not play any significant role in modifying humoral responses either against the epitope or against the transgene product. Alternatively, other innate immune sensor pathways such as TLR/MyD88 may compensate the absence of functional RIG-I/MAVS pathway.

To summarize, our results show for the first time that the efficacy of epitope display strategy depends neither on Ad infection process nor on Ad interaction with its natural receptors. Interestingly, whereas mice deficient in TLR/MyD88 or RIG-I/MAVS pathways mount IgG antibody responses comparable to control mice, we unmasked a key role of TLR/MyD88 pathway in shaping antibody isotype production against the epitope inserted into Ad capsid. Taken as a whole, the present study improved our understanding of molecular bases controlling the efficacy of Ad displaying epitopes on their capsid. The results pave the way for the development of vaccines based on epitope display on Ad capsid.

ETHICS STATEMENT

All animal experiments were approved by Ethics Committee No. 26 in accordance with the European Directive 2016/63 UE and its transposition into French Law.

AUTHOR CONTRIBUTIONS

AA performed the experiments, analyzed and interpreted data, and wrote the first draft of the manuscript. NR constructed adenovirus mutants, performed experiments, analyzed data, and corrected the manuscript. PP performed the experiments, analyzed, and interpreted data. LZ performed the experiments and analyzed data. RG and BR provided MAVS and MyD88-deficient mice, respectively. KB supervised the study, designed the experiments, performed the experiments, analyzed data, and wrote the manuscript. All authors read and approved the final version 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.2018.00124/full#supplementary-material.

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Conflict of Interest Statement: 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.

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MINI REVIEW
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Approaches to Improve Chemically Defined Synthetic Peptide Vaccines

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Progress made in peptide-based vaccinations to induce T-cell-dependent immune responses against cancer has invigorated the search for optimal vaccine modalities. Design of new vaccine strategies intrinsically depends on the knowledge of antigen handling and optimal epitope presentation in both major histocompatibility complex class I and -II molecules by professional antigen-presenting cells to induce robust CD8 and CD4 T-cell responses. Although there is a steady increase in the understanding of the underlying mechanisms that bridges innate and adaptive immunology, many questions remain to be answered. Moreover, we are in the early stage of exploiting this knowledge to clinical advantage. Several adaptations of peptide-based vaccines like peptide-adjuvant conjugates have been explored and showed beneficial outcomes in preclinical models; but in the clinical trials conducted so far, mixed results were obtained. A major limiting factor to unravel antigen handling mechanistically is the lack of tools to efficiently track peptide vaccines at the molecular and (sub)cellular level. In this mini-review, we will discuss options to develop molecular tools for improving, as well as studying, peptide-based vaccines.

Keywords: peptide vaccination, click chemistry, antigen presentation, intracellular processing, targeted vaccination, tumor immunology, toll-like receptors, bioorthogonal

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INTRODUCTION

Recent breakthroughs in immunotherapy of cancer have unveiled that clinical responses correlate with activation and expansion of tumor-specific T lymphocytes that mostly target mutation-based neo-antigens (1–6). Alongside, induction of tumor-specific T-cell responses has been achieved with well-defined peptide-based vaccines in preclinical and clinical settings (7–10). This indicates that therapeutic vaccination with well-defined synthetically produced neo-antigenic peptides is a viable strategy.

Immunogenicity of synthetic peptide-based vaccines can be significantly influenced by the mode of delivery (11–17). For example, efficiency of cytotoxic T-cell activation and anti-tumor immune responses is improved when peptides are encapsulated in liposomes or covalently conjugated to adjuvants (18, 19). Such modifications will allow optimal uptake of antigenic peptides from the vaccination site by specialized antigen-presenting cells (APCs) with efficient proteolytic processing for major histocompatibility complex (MHC) class I and class II presentation to CD8+ cytotoxic- or CD4+ helper-T cells, respectively (20). However, the development of optimal peptide delivery modalities is non-trivial and largely remains a process of trial-and-error based on time-intensive and indirect read-out systems. Most of what is known about *in vivo* processing routes of peptides is based on murine models and little data are available in humans. Additionally, the sequence and amino acid composition may alter the physical properties and immunological behavior of individual peptides. Therefore, mechanisms of intracellular routing and processing of administered peptides in APC require in depth examination.

The processing machineries for peptide loading in MHC class I or -II for presentation is characterized by distinct protease systems. While MHC class I processing pathways involves cytosolic proteasomes, peptidases and ER-resident trimming aminopeptidases, MHC class II peptide production takes place in endolysosomal compartments involving cathepsin-like proteases (21, 22). The cell biology of antigen presentation and cross-presentation, a specialized mechanism to process exogenous engulfed protein antigen for MHC class I, by dendritic cells (DCs) is not fully elucidated in detail but is crucial knowledge for optimal vaccine design (20, 22). Improving our knowledge on vaccine behavior is therefore essential for rationally designing peptide-based vaccines. However, tracking of vaccine components through the developing stages of an immune response remains difficult with present day techniques. Bulky labeling groups that are used to visualize peptides affect their physiochemical properties, which likely alters the way a peptide is internalized, processed, and presented. It is therefore vital to apply detection strategies that minimally impact the processing of peptides.

This mini-review encompasses our current knowledge of peptide-based vaccine modalities, the possibilities to properly target them through defined alterations, novel options for rational design, and the development of (bio)chemical visualization tools to improve our understanding of peptide-based vaccine behavior *in vivo*.

PEPTIDE VACCINATION HISTORY

Peptide vaccination is based on the biological concept that induction of a T-cell response relies on the specificity of the T-cell receptor to recognize a presented oligopeptide-epitope. This epitope corresponds to only a fraction of the entire protein (polypeptide) antigen. Therefore, to initiate a T-cell response against a specific protein, a vaccine essentially needs to include only the minimal immunogenic peptide sequence which can be produced synthetically.

Vaccination with minimal epitopes in form of synthetic peptides was shown to raise antigen-specific T-cell responses (23) and represented an exciting step forward in modern vaccination biology. Immunogenicity studies in preclinical models showed effective induction of T-cell responses and the potential for its application in cancer immunotherapy was recognized. However, clinical translation of this concept did not lead to the results anticipated by the first studies (24–29). As an example, vaccination with the immunogenic peptide of the differentiation antigen gp100 for the treatment of melanoma, failed to elicit sufficiently effective T-cell responses in several clinical trials, even when relatively high numbers of antigen-specific cells were detected (30–32).

Comprehensive *in vivo* studies have revealed that, rather than the exact epitope, peptides consisting of a termini-extended sequence (long peptides) promotes higher quality T-cell responses (33). In fact, exact epitopes can directly bind on MHC class I molecules present on the surface of any somatic cell, most of which are non-professional APCs, which causes suboptimal T-cell priming. On the other hand, long peptides are processing-dependent and can be presented only by professional APC, which are specialized and equipped for engulfing, processing, and presenting the antigenic peptides coinciding with optimal T-cell co-stimulation (34, 35).

The first peptide vaccination studies in humans were carried out with long peptides derived from self-antigens mucin and HER-2/neu, and mutated K-RAS. These studies reported safety of synthetic peptide administration and an observation of tumor- or antigen-specific T-cell responses (36–39). These clinical studies provided the basis for the use of long peptides as a strategy to design more efficacious vaccines for cancer treatment. In a study conducted in an HPV-induced preclinical model, vaccination with a 35 amino acid long synthetic peptide covering a CTL and a T helper epitope of the HPV16 E7 protein, improved T-cell responses compared with vaccination with minimal epitopes and controlled tumor growth (40). The use of long peptides bolstered priming by professional APCs that resulted in higher T-cell expansion, memory formation, and markedly improved efficacy. This paved the way for clinical testing of a mixture of overlapping peptides of 32–35 amino acids covering the sequence of the E6 and E7 HPV16 proteins for the treatment of HPV-associated gynecological tumors (10, 41).

Synthetic peptide vaccination also holds high potential for the novel field of cancer vaccination against mutation-derived neo-antigens. The ambition of raising an immune response against tumor-specific mutated proteins by vaccination represents an exciting challenge that has animated cancer therapeutic research over the last few years. Efforts needed in determining the MHC-restricted epitopes may be bypassed by designing a peptide that spans the amino acid sequence on either side of the mutation. Interestingly, this concept has been successfully applied in a recent phase I study on melanoma patients (9). In this study, six patients were vaccinated with 13–20 different peptides of 15–30 amino acids designed to target an equal amount of patient-specific somatic mutations of the sequenced tumor. All patients exhibited enhanced neo-antigen-specific T-cell populations after peptide vaccination and displayed objective clinical responses, even though two patients required a supplemental treatment with anti-PD1 immuno-modulatory antibody to reach complete tumor regression. In perspective, the use of multiple long peptides for vaccination may be complicated, as the behavior of different amino acids sequences, in terms of physico-chemical properties, solubility, and bio-distribution may differ.

Concurrently, a similar approach has been developed by encoding selected patient-specific epitopes in RNA molecules (8), as the window of physico-chemical properties is smaller for these oligomers than for peptides. Also this RNA-based vaccination was able to induce a personalized tumor-specific T-cell response with clinical benefits. Both studies represent an important proof of concept for the field of neo-antigen vaccination and stimulate research to progress toward the most effective vaccination approach.

SYNTHETIC PEPTIDES: VERSATILE VACCINE ANTIGENS

One advantage in the use of synthetic peptides as vaccines from both an immunological and a chemical point of view is their

versatility. Immunologically, peptide vaccines induce better T-cell responses compared with full protein vaccines (42, 43). In fact, peptides are more efficiently endocytosed, processed, and presented on MHC molecules compared with full proteins. Other, less understood, aspects of antigen handling by APC, indicate that antigen cross-presentation—on a mole-for-mole ratio—is more optimal for peptides than protein. This is perhaps due to efficient translocation of peptides into the cytoplasm from endosomes (44).

On the other hand, peptides are chemically easier to produce than protein antigens as they do not necessitate folding into a tertiary structure. The high throughput and parallel production set-ups for synthetic peptides allows that several variations in the linear sequence can be made to refine vaccine formulation. Collateral problems such as induction of tolerance or suboptimal priming (45, 46) can potentially be circumvented by conjugation to "adjuvant" molecules that allow targeting of APCs and contribute to adequate immune-stimulation.

A feasible strategy to improve APC targeting of synthetic peptides and at the same time deliver the right signals is to integrate ligands of pattern-recognition receptors (PRRs), such as C-type lectin- (CLR), toll-like- (TLR), and NOD-like-receptors. These receptors are highly expressed by professional APCs and are essential for pathogen sensing and immune-stimulation. Different ligands have been identified for these receptors which can be employed for targeting and immune-stimulation. This approach can also modulate the internalization routing of endocytosed antigen (47). For example, the CLR-specific mannosylation of long peptides canalized intracellular trafficking toward early endosomal low-degradative compartments rather than lysosomes for degradation, compared with non-mannosylated peptide. This, favored antigen presentation and enhanced T-cell activation both *in vitro* and *in vivo* (15).

A second approach that has resulted in improved T-cell activation has been the direct conjugation of long peptides to TLR ligands. TLR-mediated trafficking was described to impact antigen presentation. A study shows that the presence of antigen and TLR ligand in the same endosomes determines entrance to the presentation pathways, suggesting that TLRs or other PRRs might have an important role in determining efficient presentation after antigen uptake (48). Conjugation of antigenic peptides to TLR ligands like the TLR9-ligand CpG or the TLR1/2 heterodimer agonist Pam_3CSK_4 have been shown to strongly improve T-cell priming *in vivo* thanks to the combined effect of increased uptake of long peptides and co-delivery with the immune-stimulatory signal (49, 50). Furthermore, the Pam_3CSK_4-conjugates were able to establish potent anti-tumor immune responses in multiple preclinical models and are now being tested in a phase I/II clinical trial evaluating synthetic peptide vaccination for treatment of HPV-induced cancers (19, 50) (ClinicalTrials.gov Identifier: NCT02821494). This represents a promising platform for potentiating neo-epitope-based personalized peptide vaccines.

A third targeting strategy includes the conjugation of peptide to a DC-targeting antibody, as reported in a study evaluating the DC-specific receptor DEC205 antibody (51). Targeting viral-specific long peptides to DEC205 promotes peptide uptake by DEC205+ cells and leads to enhanced presentation on MHC class I, which resulted in improved protection to viral challenge. Interestingly, no effect was observed in the efficiency of MHC class II presentation. This highlights the fact that peptide targeting does not only influence which cells will engulf the antigen, but also impacts intracellular trafficking and fate of the antigen for presentation on either MHC class I or II. This becomes more evident in a comparative study on antibody-mediated targeting to either mannose receptor, DEC205, or CD40 in human DCs (52). Targeting of different receptors leads to differential uptake efficiency and endosomal antigen localization. While targeting of the co-stimulatory molecule CD40 was associated to the lowest uptake, it was also associated to the most efficient MHC class II and cross-presentation. In this setting, DEC205-targeting was associated to routing to degradative compartments and low-MHC class I presentation, which could be rescued by inhibiting degradation. These observations expose the complex relations between APC subsets, endosomal routing, and antigen presentation efficiency.

Lastly, an efficient approach is the encapsulation of long peptides in structures such as nanoparticles, liposomes, or nano/hydrogel-systems to enhance T-cell priming by DCs (53–56). Particulate vaccines have been shown to be well internalized by various professional APCs. Properties of these particles, e.g., charge, size, composition, can be modulated to influence uptake by different cells, and vaccine dispersion after injection (57). In the case of liposomes, smaller particles are better internalized by DCs than larger, and positively charged cationic liposomes increase ROS production and cross-presentation (58, 59). The added benefit of nano/hydrogels is the possible incorporation of environmental ques which are slowly released during the induction of DC maturation while peptide can be processed and presented (14). A shared advantage of these delivery systems is the ability to prevent the rapid release of high quantities of free peptide.

MODULATING THE VACCINE RESPONSE

Recent reports has highlighted that initiation of an adaptive immune response is more than an APC meeting a T cell. Complex interactions of several APC subsets and their crosstalk with other cell types within the vaccination-draining lymph node will determine the outcome of the immune reaction (60–63). Additionally, different APCs can induce different types of immune reactions due to their intrinsic characteristics (61–63). By the application of alternative formulations or conjugations with PRR ligands of peptides-based vaccines, modulation of the immune reaction may be possible by delivering the antigen toward the proper APC to initiate the proper immune response. To optimally design peptide-based vaccines in the future; it is thus necessary to understand the consequences of modifications in APC targeting.

Recently, the importance of cross-presenting DCs in the initiation of an effective anti-tumor immune response was exemplified in several studies (64–66). Tissue originating cross-presenting DCs were shown to be required to migrate from the tumor microenvironment (TME), loaded with antigens from the tumor, toward the draining lymph node, to induce CD8 T cell-dependent delay of tumor outgrowth. This special DC type was characterized by the expression of CD103 and is a DC subtype closely related

to the cross-presenting CD8α-expressing DCs that reside in the secondary lymphoid organs (67). These DC subtypes are indicated as part of the type 1 conventional DC (cDC1) group, have a common expression of the previously mentioned C-type lectin receptor DEC205 that was exploited successfully for improved cross-presentation, as well as the "dead cell-receptor" CLEC9A, and has an homolog in the human DC family (68–70). Closely related is the macrophage lineage originating Langerhans cell, which shows similar cross-priming capabilities as cDC1s and is a shared population between mice and humans. Their characteristic expression of c-type lectin receptor Langerin-1 has been used in antibody-mediated targeting to improve cross-presentation and CTL activation (17).

Additionally, the induction of effective CD4 helper responses are crucial for improved CD8 T-cell priming and memory formation, increased tumor infiltration, and local effectiveness (58–60). The DC family has, likewise the conventional DC type 1, a type 2 conventional DC with the characteristic expression of CD11b, which is considered specialized in their capacity to induce T-cell help while lacking CD8 priming capacity (67). However, exploration of specific targeting of this DC subtype for improved helper T-cell priming had negligible attention, due to the intrinsic capacity of most DCs to present in MHC class II. Therefore, it appears more effective to incorporate vaccine modalities which harbor both CD4 and CD8 epitopes and target a wide range of DC subsets, including cross-presenting DCs.

Furthermore, potent peptide-based vaccines can modulate the TME as shown by shifts in the myeloid subpopulations in the tumor (19, 71). Most likely, polarization of CD4 T-cell subsets will regulate the TME to a more proinflammatory state. This is supported by TLR2 ligand-conjugated HPV long peptides which can strongly activate CD4 and CD8 T cells from tumor-draining lymph nodes of cervical cancer patients (50). Other options to modulate the suppressive TME can be achieved by combining cancer vaccines with classical chemotherapy (72) or widely used checkpoint blocking antibodies like anti-PD-1 or anti-PD-L1 (65, 73–78).

The application of TLR-ligand Pam_3CSK_4 as targeting moiety was highly promising due to a broad expression of its receptor in dermal DCs (79, 80). The added benefit of a TLR ligand is the combination of a maturation signal with an antigen. Maturation of the DC is known to strongly influence the intracellular machinery and processing of exogenous antigens (81–83). By conjugation of a maturation signal with the antigen, the survival of internalized antigen is increased by the formation of antigen storage depots for prolonged presentation and priming (84). The application of ligands for other PRRs is of interest as well (85). However, care should be taken in using combinations of different PRR ligands in the same modality. Different PRR pathways may affect each other upon simultaneous activation and reduce DC proinflammatory responses, which is exploited by some pathogens (86, 87).

In conclusion, these findings shows that the field is steadily progressing to unravel the relevant cell types involved in optimal (cross-)presentation of antigens. Peptide-based vaccination studies using antigen-bound fluorophores show co-localization with endosomal markers in DC, which correlated with a robust antigen-specific T-cell immune response. However, the strong influence of the relatively large fluorophore on the physicochemical properties of the antigenic peptide to gain trustworthy physiological information and the limitations to detect peptide intermediates makes interpretations of this complex process difficult. To unravel how activation of APCs orchestrate molecular and cellular mechanisms of antigen processing and presentation operate *in vivo*, and how we can incorporate this knowledge in peptide-based vaccination modalities requires better tracking and visualization tools of vaccine moieties.

NOVEL CHEMICAL VISUALIZATION TOOLS

Several technologies to visualize antigens in APCs, other than using T-cell readouts, have been developed in the last decades. Most of these have relied on tracking the activity of an enzyme through a cell. Examples of enzymes used for this are ß-lactamase, luciferase, and horseradish peroxidase. Using these approaches, the endocytic compartments involved in (cross-)presentation could be observed, as well as the cytosolic location of proteins during this event (88–90). Fluorophore-labeled antigens have also been used to study the intracellular movement of antigen in an APC. Using this approach, the presence of intracellular antigen depots was, for example, identified (84).

However, these approaches also have their constraints. For reporter proteins, the main constraint is that degradation is the hallmark of antigen (cross-)presentation: during antigen presentation any protein must be degraded into peptides to allow for its MHC-loading. As enzyme activities are reliant on largely intact proteins, this means that later stages of the pathway will be invisible using this approach. The use of fluorophore-modified antigens partially solves this by making the detectable signal independent of the intactness of the protein. However, the physicochemical properties of fluorophores must also be considered. Due to their bulky and hydrophobic structure compared with relatively small peptides, fluorophores could strongly influence the behavior of the antigenic peptide and mask epitope residues as well as proteolytic cleavage sites. Moreover, the size of conjugated fluorophores may hamper these peptides to pass through the proteasomal α-annulus of several ångström wide (91). Similarly, peptide translocation by TAP (transporter associated with antigen presentation) to the ER lumen for presentation in MHC class I molecules may be strongly influenced (92). Furthermore, it is difficult, if not impossible, to rule out that constructs lose their fluorophores during processing. As a consequence, not much data exists of later processing stages that could directly visualize antigen. And even in rare cases in which it has been possible [e.g., the H-2K^b-SIINFEKL pMHC complex antibody 25-D1.16 (93)], translation to other antigens is not obvious. Therefore, a method to thoroughly and accurately apply tracking across a manifold of peptide-based vaccine modalities and the complex cellular interactions involved is highly wanted (see **Figure 1**).

One field of chemistry of which we are currently exploring the potential is click chemistry (94). This type of chemistry involves a defined ligation reaction between a small *bioorthogonal* chemical group—a chemical group which can be selectively

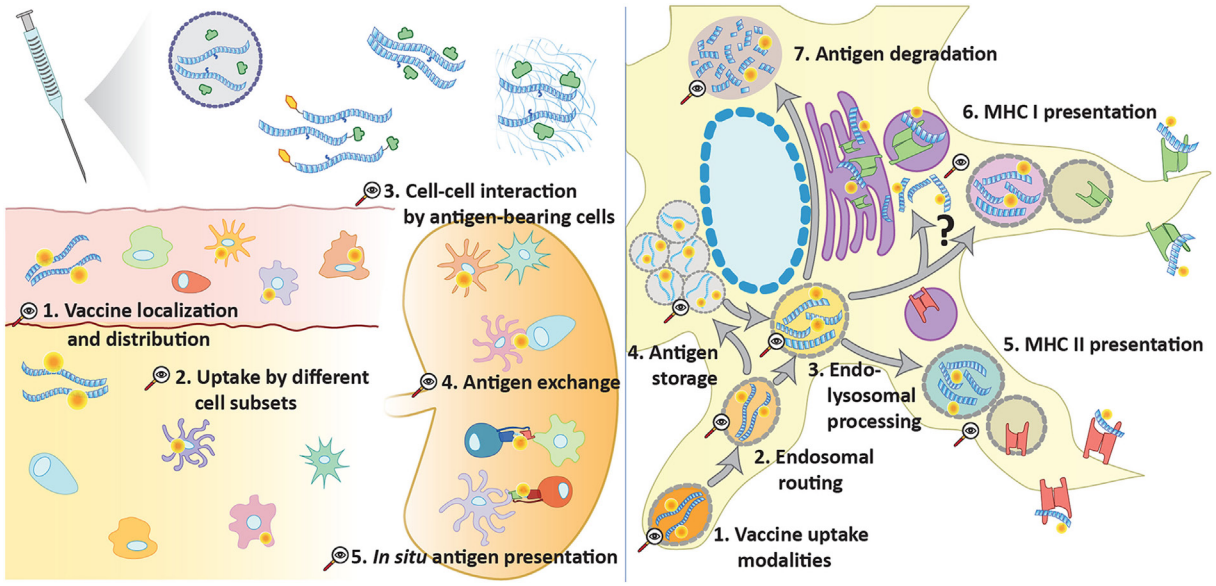

FIGURE 1 | Opportunities for advanced tracking techniques in vaccination. Several forms of peptide antigen can be traced (e.g., *via* click chemistry) at multiple levels to provide further understanding of vaccine processing and induction of adaptive immunity. At a supracellular level, it can unveil vaccine diffusion, drainage, and the main cellular recipients. Tracking of antigen in different cell types can also help to understand the steps involved in the initiation of an immune response, such as cell–cell interaction, antigen exchange, and *in situ* antigen presentation. At a subcellular level, peptide tracking is an important tool to explore the intracellular events that lead to antigen presentation following antigen uptake: endosomal trafficking and sorting to storage compartments, class I or class II presentation or degradation.

ligated within the context of the living cell or organism—to form a covalent linkage to a detectable group *after* the biological time course has been completed. It is relatively easy (in *Escherichia coli*) to produce bioorthogonally labeled recombinant proteins (95–97) using methionine auxotrophic producer strains in combination with bioorthogonal methionine analogs (98, 99). This chemistry has been applied widely, but its application to immunology is still in its infancy. We ourselves have applied this chemistry to label surface loaded minimal epitopes on the surface of APCs (100) to allow their quantification without using T-cell reagents. However, the reaction is still limited by poor signal-to-noise ratios that cannot compare with the sensitivity of T cells. Detection of the handles in antigens after routing and processing is therefore not yet possible using this approach, despite the groups surviving the antigen presentation pathway (101–103). Once the sensitivity issues can be solved this technique could prove valuable in the imaging of the entire antigen routing pathway with minimal bias. Additionally, this approach may be suited to analyze the *in vivo* fate of chemically defined peptide vaccines. By *ex vivo* secondary staining of relevant cell types or tissues using fluorescent microscopy or histological analysis, the presence and location of the peptide vaccine can be determined. This could be valuable information to improve peptide vaccine design.

CONCLUDING REMARKS

Our current knowledge on innate and adaptive immune system allows us to design molecularly well-defined vaccine moieties. Adjuvant molecules that bind PRR can be synthetically coupled to antigenic peptide sequences. Even though these defined peptide vaccines have strong vaccination capacity, the mechanisms underlying these improvements are only understood to a basal level. To improve the design of peptide-based vaccines, we need to better our understanding of chemically altered vaccines on the events unfolding during vaccination *in vivo*. A major limitation to this understanding is the lack of techniques that allow the study of late stages of antigen processing, and presentation on a cellular and molecular level. Fundamental questions about transfer of peptides within and between cells are currently troublesome since tags or fluorophores are lost and prone to altering essential physicochemical properties due to their bulkiness. The introduction of novel types of chemistry may in future circumvent these problems, which in turn may lead to novel insight in the complex cellular and molecular interactions in immune response induction.

AUTHOR CONTRIBUTIONS

BH and ET have contributed equally to the manuscript in writing and figure design. SK and FO have been responsible for scientific input and revisions of the final manuscript.

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The Isoform Selective Roles of PI3Ks in Dendritic Cell Biology and Function

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Phosphoinositide-3 kinases (PI3Ks) generate 3-phosphorylated phosphoinositide lipids that are implicated in many biological processes in homeostatic states and pathologies such as cancer, inflammation and autoimmunity. Eight isoforms of PI3K exist in mammals and among them the class I PI3K, p110γ, and PI3Kδ, and class III Vps34 being the most expressed and well characterized in immune cells. Following engagement of pathogen recognition receptors (PRRs), PI3Ks coordinate vital cellular processes of signaling and vesicular trafficking in innate phagocytes such as macrophages and professional antigen presenting dendritic cells (DCs). Although previous studies demonstrated the involvement of PI3K isoforms in innate and adaptive immune cell types, the role of PI3Ks with respect to DC biology has been enigmatic. Thus, this review, based on studies involving PI3K isoforms, highlight how the different PI3Ks isoforms could regulate DC functions such as antigen processing and presentation including PRR responses.

Keywords: dendritic cell, antigen presentation, PI3K, toll like receptors, phospholipids

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INTRODUCTION

PI3Ks are activated by diverse signaling pathways including small G proteins of Ras and Rac family, tyrosine kinases- or G-protein- coupled receptors (1). PI3Ks comprise three class I, II, and III family of enzymes. Except for class II PI3K (isoforms α, β, δ) which has only catalytic subunits, class I (isoforms α, β, δ, γ) and class III (Vps34) PI3Ks form heterodimeric molecules, which consist of the assembly of a catalytic and a regulatory subunit(s) (**Figure 1**). PI3Ks phosphorylate the 3-hydroxyl group of the inositol ring of three species of phosphatidylinositol (PI) lipid substrates; namely, PI, PI-4-phosphate PI(4)P, and PI-4,5-bisphosphate PI(4,5)P_2. They catalyze the formation of 3-phosphorylated phosphoinositide lipids, such as phosphoinositol-4,5 biphosphate PI(4,5)P_2 into phosphoinositol-3,4,5 triphosphate PI(3,4,5)P_3 for class I, and generation of phosphoinositol triphosphate PI(3)P from phosphoinositol (PI) or phosphoinositol-3,4 bisphosphate PI(3,4)P_2 from phosphoinositol phosphate PI(4)P for class II, and finally only the production of phosphoinositol triphosphate PI(3)P from PI for class III.

PI3Ks are evolutionarily conserved from soil dwelling amoeba, *Dictyostelium discoideum* to mammals (2). The evolutionary conservation of PI3K families and its functions from the *Dictyostelium,* an archetypical phagocyte, to mammals in generating membrane phospholipids highlights the importance of these kinases in endocytic and phagocytic processes and their non-redundant functions in innate phagocytes including dendritic cells (DCs, **Figure 2**).

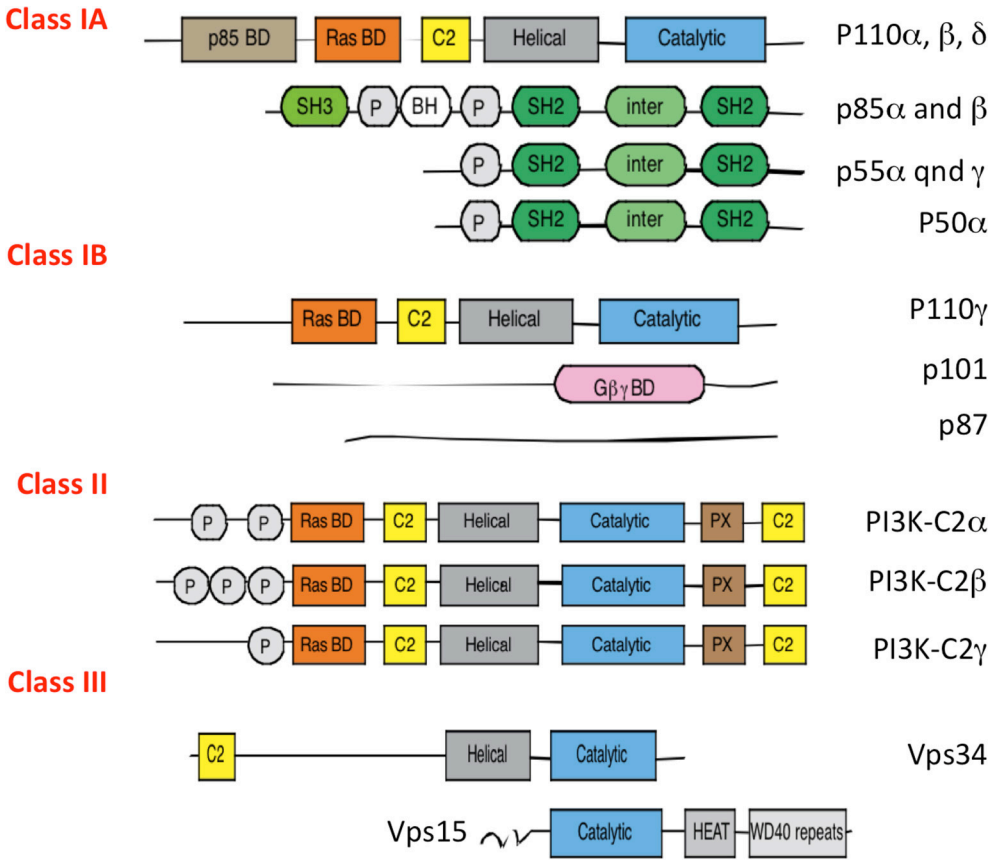

FIGURE 1 | Different classes of PI3Ks. All PI3Ks isoforms (p110 α, β, δ, γ, C2α, C2β, C2γ, Vps34) have 3 to 5 domains: an N terminal domain which can bind the regulatory p85 proteins, a Ras binding domain, a C2 domain which binds membranes, an helical domain of unknown function and a catalytic subunit with kinase activity. They associate with a regulatory subunit, p85 isoforms (for p110 α, β, δ), p101, and p87 for p110γ and Vps15 for Vps34. P85 regulatory subunits are encoded by different genes: PI3KR1 (depending on the promoter will give p85α, p55α, p50β), PI3KR2 (p85β), and PI3KR3 (p55γ). PI3Ks use phospatidylinositol lipids as their substrates.

THE ROLE OF PI3KS IN DC MEDIATED HOMEOSTATIC REGULATION OF INFLAMMATION AND IMMUNITY

PI3K isoforms, particularly class I family of enzymes, have distinct tissue and cell distribution in mammals. Whereas PI3Kδ and PI3Kγ are preferentially expressed in immune cells of hematopoietic origin at high levels, PI3Kα, and PI3Kβ are ubiquitous and broadly expressed in all somatic cells (4). However, data from published microarrays suggest differential expression of PI3Kγ and δ in subset of DCs. PI3Kδ mRNAs are well expressed in plasmacytoid DCs (pDCs), subset of DCs producing high amount of type I interferon (IFN) following viral infection, and PI3Kγ mRNA expression was found exclusively restricted to type 2 DCs or cDC2 (5). These data remain to be confirmed at the protein level but might imply a preferential role for PI3Kγ in major histocompatibility class II (MHCH II) antigen presentation as cDC2 are the main DCs subset to present antigens to $CD4^+$ T cells (6).

Over the last decade, a number of PI3K isoform-selective gene-targeted mouse models for class I PI3K catalytic and regulatory subunits and class III PI3K have been generated and together with the development of isoform specific inhibitors have greatly advanced the understanding of PI3K signaling in mammalian biology. Due to the cell type specific expression of PI3Kγ and PI3Kδ, targeting these isoforms affect innate and adaptive immune responses (7). Studies using genetic and pharmacological targeting of PI3Kδ isoform has shown PI3Kδ is a homeostatic regulator of activation, downstream of Mal-MyD88-coupled TLR2 and TLR4 signaling pathways in DCs. PI3Kδ achieves this by dampening pro-inflammatory cytokine secretion, while supporting production of IL-10 (8). PI3Kγ was shown to be needed for the development of lung $CD11b^+$DC and

Abbreviations: APC, antigen presenting cells; PI3Ks, Phosphoinositide−3 kinases; PRRs, pathogen recognition receptors; DC, dendritic cells; PI, phosphatidylinositol; PI(4)P, phosphoinositol phosphate; $PI(4,5)P_2$, phosphoinositol-4,5-bisphosphate; $PI(3,4,5)P_3$, phosphoinositol-3,4,5 triphosphate; $PI(3,4)P_2$, phosphoinositol-3,4 bisphosphate; PI(3)P, phosphoinositol triphosphate; IFN, interferon; MHCI, major histocompatibilty class I; pDCs, plasmacytoid DCs; OVA, ovalbumin; MHCII, major histocompatibility class II.

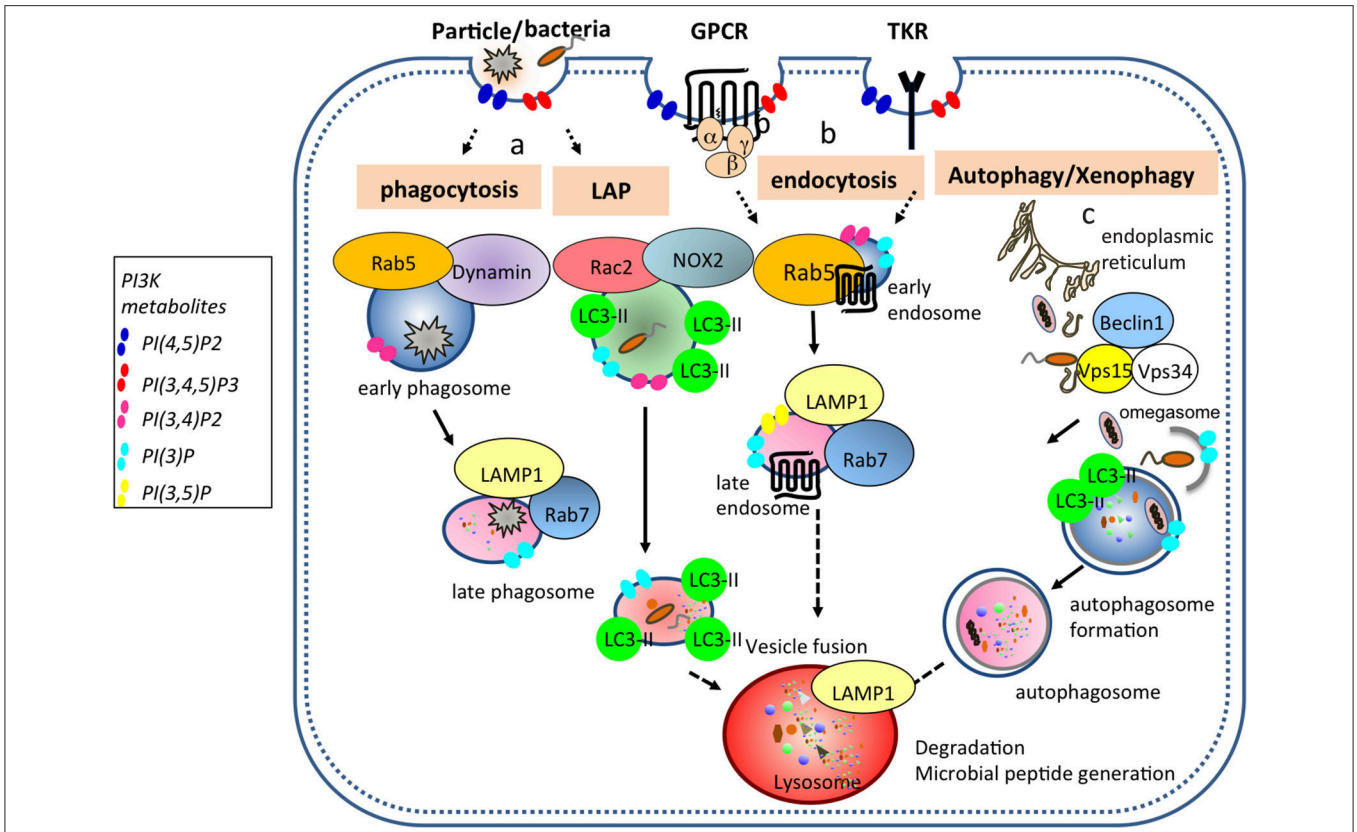

FIGURE 2 | PI3Ks generate membrane phospholipids important for vesicular trafficking events in DCs. (a) Bacteria, cell debris, and large particles are taken up in Rab5$^+$ dynamin$^+$ early phagosomes that undergo maturation to generate Rab7$^+$ LAMP1$^+$ late phagosome. Pathogens such as bacteria and yeast can be internalized into LC3 associated phagosome (LAP) where Rac2 and NOX2 are recruited and are required to generate reactive oxygen species (ROS). (b) During endocytosis, ligands bound to G protein coupled- and tyrosine kinase-receptors are taken up from the plasma membrane into Rab5$^+$ early endosomes and then either traffic to Rab7$^+$ LAMP1$^+$ late endosomes or are recycled back to the plasma membrane. (c) During autophagy, a double membrane organelle is generated from an omegasome (specific ER structure) where Vps34, Vps15, and Beclin1 are recruited. This double membrane organelle will form later the autophagosome by ingesting cytoplasmic material. Ultimately, all transport vesicles (late phagosome, late endosome, autophagosome) will fuse with the lysosomes for cargo degradation. PI(4,5)P_2 is enriched proximal to the plasma membrane and in lysosomal location. Class I PI3Ks produce PI(3,4,5)P_3 from PI(4,5)P_2, while class II and III PI3Ks produce PI(3)P following phagosome enclosure, and PI(3,4,5)P_3 is converted into PI(3,4,)P_2 by SHIP1 and SHIP2 (3) phosphatases. PI(3)P and PI(3.5)P_2 are present in late endosomes.

CD103$^+$DCs especially by regulation of signaling downstream of Flt3, whereas it was dispensable for DC development in many other tissues (9). PI3Kγ deficiency was, in mice, demonstrated to increase susceptibility to influenza virus infection due to impaired T cell priming by lung resident DC and delayed viral clearance owing to the pre-existing DC developmental deficiency in the lung compartment. In another study, PI3Kγ deficient mice showed selective reduction in the number of skin Langerhans cells and in lymph node CD8α$^-$DC (10). Pan PI3K inhibitors such as wortmannin and LY294002 and deficiency in the PI3Kδ or p85α regulatory isoform of class I PI3Ks (that couples to PI3Kα, β or δ isoforms) were found to enhance TLR4 mediated pro-inflammatory responses by LPS in myeloid cells including DC and macrophages (8, 11–15). TLR4 activation triggers Mal-MyD88 signaling originating from the plasma membrane via phosphoinositol 4,5-bisphosphate [PI(4,5)P_2]-localized TIRAP signaling, which is consecutively followed by phosphoinositol–binding TRAM mediated endocytic pathway leading to type I IFNβ production (16, 17). This study has shown that PI3Kδ mediates the switch between TIRAP-dependent pro-inflammatory pathway coupled to TLR4 endocytosis and TIRAP degradation subsequently leading to TRAM-dependent type I IFNβ and IL-10 secretion (8). This type of homeostatic control indicates that PI3Kδ signaling pathway is a physiological regulator of inflammation by dampening endotoxemia and sepsis.

While PI3Kγ and PI3Kδ roles in TLR mediated pro-inflammatory reactions has been extensively addressed, their role in DC antigen presentation and DC-dependent T cell-mediated immunity in infection models have yet to be examined. Indeed, a recent study, reporting the diminished ability of WT OT1 T cells to provide help for the p110δ kinase-deficient T cells in *Listeria* expressing ovalbumin (OVA) infection model, indicates a possible role of DCs supporting antigen-specific CD8$^+$ T cell expansion in a PI3Kδ dependent manner (18). In this study, WT OT1 cells, injected into p110δ^{D910A} hosts, showed reduced

primary immune responses and proliferation. Likewise in other studies, the PI3Kδ and p85α regulatory subunit deficient mouse strains were found to exhibit enhanced resistance to *Leishmania major* infection, despite mounting impaired T cell responses and yet intact or enhanced DC pro-inflammatory cytokine response (19). Also, DCs lacking SHIP1, the phosphatase converting $PI(3,4,5)P_3$ into $PI(3,4)P_2$, were able to mature and induce autoimmunity by promoting $CD8^+$ T cells expansion and INFγ production in an *in vivo* model of diabetes (20). In agreement with this, SHIP1 overexpression led to an inability of DCs to trigger T cell auto immunity (20) suggesting that PI regulated by SHIP phosphatases and PI3Ks play a major role in DCs antigen presentation.

Recently, mice deficient for Class III PI3K or Vps34 in $CD11c^+$DCs were generated (21). These mice showed a specific reduction in the number of $CD8^+$DCs, subset of DCs specialized in MHC class I (MHCI) antigen cross presentation, in the spleen and were defective at presenting dying cell-associated antigens to the MHCI antigen cross presentation pathway. The defect was linked to a reduced expression of Tim4, a phospatidylserine receptor require for uptake of apoptotic cells, in $CD8^+$DCs lacking Vps34. In contrast, presentation of antigens by the classical MHC class I and II pathways was increased and might be linked to an overall enhancement of DCs activation at the steady state in the Vps34-$CD11c^+$DCs deficient mice (21). In addition, a highly selective and potent class III PI3K inhibitor, SAR405 was reported to influence vesicle trafficking and autophagy (22) and it will be of importance to unravel the exact role of Vps34 kinase and scaffolding functions regulating DC biology.

The PI3Kδ and PI3Kγ isoforms are key targets, being harnessed in chronic inflammatory and autoimmune conditions such as asthma, psoriasis rheumatoid arthritis (RA) and systemic lupus erythematous (SLE), with single or dual inhibitors for both isoforms being tested in clinical trials (23). In OVA-induced allergic inflammation models, genetic or pharmacological targeting of PI3Kδ was reported to reduce inflammatory cell infiltrates and IL-17 secretion (24), while PI3Kδ deficiency in mice resulted in suppression of Th2 cell mediated responses to OVA following immunization with OVA antigen *in vivo* (25). Although mice lacking PI3Kγ exhibited reduced levels of eosinophilic airway inflammation, they did not show significant differences in serum OVA-specific IgE and IgG1 levels and CD4/CD8 T cell balance (26). However, PI3Kδ deficient animals display reductions in the levels of eosinophil recruitment and Th2 cytokine response, indicating that DC-mediated antigen priming of T cells might be altered under PI3Kδ deficiency, a topic unaddressed so far (26).

THE ROLE OF PI3Ks IN DC MIGRATION

The activation of class I PI3K downstream of several receptors for chemo attractants, such as chemokines, complement component C5a, Nformylmethionyl- leucyl-phenylalanine and sphingosine-1-phosphate explains the pivotal role of these enzymes in cell migration (27). Interestingly, the role of class I PI3K-dependent signaling in migratory responses to chemokines was mainly explored in leukocytes, neutrophils and to a lesser extent, in macrophages (28–31). Nevertheless, it is very likely that class I PI3K signaling, especially PI3Kγ and PI3Kδ, have also crucial roles in DCs migration, a process necessary for DCs to reach secondary lymphoid organs to present antigens to T cells, in a similar way as they act in neutrophils and macrophages. The major class I PI3K activated downstream chemokine receptors in neutrophils is considered to be the class IB PI3Kγ, although an important degree of cooperation, still incompletely understood, exists between PI3Kγ and PI3Kδ in the control of neutrophil migration. Thus, early steps of neutrophil migration depend on PI3Kγ, while late steps of neutrophil long-term migration requires the cooperative action of PI3Kγ and PI3δ (32).

The central molecular event connecting PI3K activation with cell migration is the production of $PI(3,4,5)P_3$, which interacts with proteins containing pleckstrin homology domains (PH). Proteins containing PH-domains include several master regulators of cytoskeleton remodeling that are essential for cell migration. These are the guanine nucleotide exchange factors for Rac (P-REX1 and 2 and VAVs 1, 2 and3) (33, 34) that regulates F-actin polymerization and myosin assembly (35). The biosensors, formed by GFP fusion with typical PH domains, specific for $PI(3,4,5)P_3$, which allows the visualization of $PI(3,4,5)P_3$ in cells clearly established that $PI(3,4,5)P_3$ is concentrated to the leading edge of the cell and this is mandatory for Rac activation, cytoskeleton rearrangements and finally, orientated neutrophil migration (36, 37). Indeed, neutrophil migration and wound repair were enhanced in a zebrafish model where SHIP phosphatases were depleted (38) but were restored to control level when cells deficient for SHIP were incubated with PI3Kγ inhibitor. Whether a similar role of PI3Kγ-mediated distribution of $PI(3,4,5)P_3$ occurs during DC migration remains unknown. However, studies involving experimental dextran sodium sulfate (DSS)-induced colitis reported that genetic or pharmacological inhibition of PI3Kγ is protective, which may be in part due to inhibitory effects on inflammatory leukocyte migration.

Investigation of the role of PI3Kγ and PI3Kδ in DC and DC-like cell lines migration is an important issue, which should be studied in the future. Significant layers of complexity exist in the study of class I PI3K, notably PI3Kγ and PI3Kβ, in DC migration. One is the diversity of DC populations and their different roles in the immune response (39), which arise the possibility that the inhibition of one member of class I PI3K will affect the migratory and chemotactic abilities of a particular DC subset. Thus, it would be interesting to investigate if pDC migration is depending on PI3Kδ, while cDC2 migration depends on PI3Kγ (5). PI3Kγ was shown to play a key role in DC trafficking and in the activation of specific immunity since PI3Kγ deficient DCs show inhibited migration to the lymph nodes (LN) in response to CCR7, which was correlated with reduced DC numbers in LNs (10). Another level of complexity is given by the existence of two regulatory sub-units for the master PI3K kinase involved in immune cell migration, the PI3Kγ (class IB). Unlike all other class I PI3K, this enzyme has two regulatory subunits, both of them expressed, but never studied in DCs. It remains to be investigated if these two regulatory subunits of PI3Kγ have similar functions in DC migration, by means of differential coupling to distinct signaling

receptors. And, as a final level of complexity of the class I PI3K involvement in DCs migration, it should be underlined that essential regulators of Rac activity, such as Vav1, 2, 3 and P-Rex1, 2 are differentially expressed in DC subsets according with the mRNA expression data (5). Thus, Vav1 and 2 proteins are expressed mainly in pDCs, Vav3 in type 1 DCs, while type 2 DCs express P-Rex 1 and 2 (5). This pattern of Rac GEFs expression suggest that downstream targets of class I PI3K activation might also contribute to different molecular pathways regulating DC migration in a subset specific way.

PI3K ROLE IN DC TOLEROGENIC FUNCTIONS AND THEIR ANTIGEN PRESENTATION POTENTIAL

Although the exact roles of PI3K isoforms in antigen processing and presentation in DCs remains unknown, there are indications that tolerogenic DC functions may rely on PI3K-Akt signaling. PI3K/Akt/mTOR pathway is central to the regulation of glycolytic metabolism (40, 41), and equally important in DC immunometabolic activities and maturation associated increase of co-stimulatory molecules and MHCII surface expression (42). Consistent with this, PI3K/Akt axis was shown to be essential for sustained commitment to glycolysis in TLR activated DCs (43). Furthermore, the existence of close links between glycolysis-hypoxia and PI3K-Akt signaling in immune cells indicated that hypoxic conditions may influence PI3K signaling and thus impact DC functions involving their migration capacity and their ability to induce Tregs in DSS model of colitis as reported (44, 45). This has come to attention since a recent work using human 1,25D3-DCs demonstrated that tolerogenic DCs, generated by 1,25(OH)2D3, rely on glucose accessibility and aerobic glycolysis to maintain their tolerance-inducing properties (46). Indeed, inhibition of the PI3K/Akt/mTOR pathway was shown to reverse the tolerogenic function of 1,25(OH)2D3-modulated DCs by a transcriptional reprogramming of glycolysis associated genes. Furthermore, pan PI3K inhibitors and rapamycin were found to hinder tolerogenic DCs function, in part, by partial restoration of $CD4^+$ T cell proliferation (46).

PI3Kδ and PI3Kγ single and dual isoform selective inhibitors and mice deficient in p110δ or p110γ do not manifest overt pathogenic phenotypes, despite exhibiting a wide spectrum of immunological defects. Nevertheless, patients treated with the PI3Kδ-selective inhibitor idelalisib (Zydelig) manifest serious side effects, which results in colitis, diarrhea, neutropenia, pneumonitis and some level of liver damage (47, 48). This overt clinical phenotype in humans remarkably coincides with the occurrence of spontaneous colitis in PI3Kδ deficient mice (49). These mice progressively develop colitis (49) which depends on the presence of enteric microbiota for colitis development in germ free p110δ deficient animals (50, 51). The cellular and molecular mechanisms for increased colitis susceptibility, under PI3Kδ deficiency requires careful examination of the host immune status both in mouse studies and in human trials (52). PI3Kδ deficiency may increase susceptibility to common infections due to defects in mounting T cell immunity, not only owing to the diminished function of PI3Kδ deficient T cells, but also because of faulty function of DC in peripheral tissues, enriched in microbiome. A clear dissection between the T cell- and DC-intrinsic role of PI3Ks in mounting the adaptive immune response require in the future the generation of tissue-specific PI3K-deficient mouse strains.

PI3K ROLE IN DC-MEDIATED CANCER IMMUNITY

PI3K pathway-targeted therapies have been tested in oncology trials and several pharmaceutical companies have developed selective PI3K inhibitors to target PI3K pathway in diverse types of cancer cells. Due to the restricted tissue and cell expression, PI3Kδ and PI3Kγ are attractive drug targets in hematological cancers, and a distinguished success of efficacy was reported with PI3Kδ-selective idelalisib in treating Chronic Lymphocytic Leukemia (CLL) and non-Hodgkin's lymphoma, and idelalisib is now approved for clinic (47, 48, 53).

Evidence from genetic or pharmacological targeting studies indicate that inhibiting class I PI3K isoforms may be beneficial in improving cancer immunotherapy (54, 55). In parallel to PI3K research in oncology, several studies have uncovered exciting and unexpected roles for PI3K catalytic and regulatory subunits in cancer immunity, potentially by boosting the efficacy of PI3K-targeted therapies by adapting the immune compartment (1). Previously, genetic or pharmacological inhibition of PI3Kδ reduced tumor burden and metastasis in a range of mouse cancer models including melanoma, thymoma, lung, and breast cancer (54). In these models, PI3Kδ inhibition was found to attenuate Treg function and tumor infiltration and surprisingly did not alter cytotoxic T cell responses, resulting in enhanced anti-tumor immunity. Interestingly, one of the common side effect of checkpoint inhibitors in cancer therapy using PDL1, PD-1, and CTLA blocking antibodies concerns colitis development due to inhibition of Treg functions (56, 57). It is plausible that combined with Treg functions, DC-mediated tolerance induction may be altered in PI3Kδ deficiency in both humans and mice.

Genetic or pharmacological inhibition of p110γ was reported to reduce tumor growth and metastasis in melanoma, lung, pancreatic breast, and colon cancer models. The efficacy of inhibiting PI3Kγ signaling was suggested to involve myeloid cell recruitment to the tumor microenvironment through integrin α4β1 mediated adhesion in response to chemotactic signals. Therefore, intervention of p110γ signaling appears as an effective target in reducing tumor-associated inflammation and subsequent angiogenesis response (58, 59).

Overall based on a number of studies, isoform selective PI3K inhibition in cancer therapy appears to be efficacious, but it will be critical in the near future to uncover DC-intrinsic roles of PI3K isoforms, particularly in the context of antigen presenting cells (APC) functions, since DC orchestrate immune responses by activating antitumor immunity. Because PI3K inhibitors, particularly PI3Kδ inhibitors, have been reported to boost proinflammatory TLR responses and IL12p70 production,

it will be interesting to find out whether APC functions and Th1 inducing capacity of DCs will be affected under PI3K inhibition. Understanding the role of PI3K inhibition in both innate and adaptive immune functions of DCs will indicate if PI3K isoform selective inhibitors may be utilized as novel "innate" check point inhibitors to boost current cancer therapies.

CONCLUSION

Overall, the exact role and contribution of PI3K isoforms in APC function of DC in T cell priming is still enigmatic and new tools such as tissue-specific PI3K-deficient mouse strains should be developed in the future. These will allow underpinning DC-intrinsic roles of PI3K isoforms in antigen presentation during cancer, auto inflammatory and autoimmune diseases.

AUTHOR CONTRIBUTIONS

EA, LS, and BM wrote the manuscript. EA and BM contributed critically in the conceptualization and arrangement of the review.

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