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Cancer stem cell antigen nanodisc cocktail elicits anti-tumor immune responses in melanoma

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ABSTRACT

One of the major reasons for poor cancer outcomes is the existence of cancer stem cells (CSCs). CSCs are a small subpopulation of tumor cells that can self-renew, differentiate into the majority of tumor cells, and maintain tumorigenicity. As CSCs are resistant to traditional chemotherapy and radiation, they contribute to metastasis and relapse. Thus, new approaches are needed to target and eliminate CSCs. Here, we sought to target and reduce the frequency of CSCs in melanoma by therapeutic vaccination against CSC-associated transcription factors, such as Sox2 and Nanog, and aldehyde dehydrogenase (ALDH). Toward this goal, we have identified novel immunogenic peptide epitopes derived from CSC-associated Sox2 and Nanog and synthesized synthetic high-density lipoprotein (sHDL) nanodisc vaccine formulated with Sox2, Nanog, and ALDH antigen peptides together with CpG, a Toll-like receptor 9 agonist. Vaccination with nanodiscs containing six CSC antigen peptides elicited robust T cell responses against CSC-associated antigens and promoted intratumoral infiltration of CD8+ T cells, while reducing the frequency of CSCs and CD4+ regulatory T cells within melanoma tumors. Nanodisc vaccination effectively reduced tumor growth and significantly extended animal survival without toxicity toward normal stem cells. Overall, our therapeutic strategy against CSCs represents a cost-effective, safe, and versatile approach that may be applied to melanoma and other cancer types, as well as serve as a critical component in combined therapies to target and eliminate CSCs.

1. Introduction

According to the World Health Organization, cancer is responsible for one out of every six deaths, making it the second most common cause of death globally. Cancer is difficult to combat which is partially attributed to tumor heterogeneity and an immunosuppressive microenvironment. With regard to heterogeneity, tumors consist of differentiated, mature tumor cells as well as cancer stem cells (CSCs). CSCs are a small subpopulation of cells that can self-renew in a de-differentiated state, differentiate into a larger population of tumor cells, and maintain tumorigenicity. CSCs are attributed to tumor recurrence after chemotherapy and/or radiation therapy. More specifically, CSC-mediated resistance to chemotherapy and radiation therapy results in limited treatment options, tumor dormancy, more aggressive tumors, and in some case metastasis [1].

CSCs are hypothesized to originate from normal stem cells or progenitor cells that reside in a quiescent state maintained by a specialized niche [2]. However, these cells can undergo mutations to genes such as oncogenes, tumor suppressor genes, or genes involved in DNA repairs that lead to tumorigenesis [3]. The primary mechanism by which CSCs

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initiate and maintain cancer *via* self-renewal is through stemness transcription factors that regulate pluripotency. These transcription factors, such as Sox2 and Nanog, are critical for self-renewal and pluripotency in CSCs and they mediate tumor proliferation and differentiation [4–7]. They are also considered biomarkers of poor prognosis in multiple types of cancers [8]. Their normal functions are critical for embryonic development and tissue repair in adults [9]. Thus far, CSCs have been identified in many types of hematologic and solid cancers, including melanoma, leukemia, breast cancer, brain cancer, colorectal cancer, and pancreatic cancer [10–15]. With the identification of CSCs, there is an urgent need to target and eliminate this sub-population of cancer cells.

CSCs are resistant to traditional cancer therapies, including chemotherapy and radiation therapy [16]. Current strategies under development to target CSCs have shown limitations: (1) Adoptive T cell transfer: CSC antigen-specific T cells are difficult to isolate from patients. (2) Dendritic cell (DC)-based vaccines: similar to adoptive T cell transfer, it is difficult to isolate a sufficient number of CSCs from patients to pulse DCs. Moreover, DC-based vaccines have shown suboptimal therapeutic efficacy in clinic. (3) Oncolytic virus therapy: while oncolytic viruses may infect and replicate selectively in CSCs, physical barriers within the tumor microenvironment (TME) limit viral spread to CSCs. (4) Immune checkpoint blockade (ICB): although ICBs can prevent exhaustion of immune cells, they have only demonstrated 10–30% response rates in the clinic [17–20]. One potential approach to target CSCs is peptide-based vaccination, where immunodominant peptides classified as tumor-associated antigens or tumor-specific antigens are administered, normally with an adjuvant, thus providing signals for innate and adaptive immune activation. Peptide-based cancer vaccination has shown promise in clinical trials, which is safe, inexpensive, and amendable for targeting multiple antigens [21–24]. However, direct injection of antigen peptides admixed with an adjuvant can result in precipitation, accumulation, and sustained inflammation at the injection site [25–27]. Instead, it is ideal to co-deliver peptide antigens and adjuvants to immune activation sites, such as lymph nodes (LNs), where antigen-presenting cells (APCs) reside and can trigger potent anti-tumor immunity. One such method is through nanoparticle delivery platforms.

We have previously reported the development of synthetic high-density lipoprotein (sHDL) nanodiscs for delivering peptide antigens and a Toll-like receptor 9 (TLR-9) agonist CpG to APCs in LNs and inducing a robust CD8 T cell response with sustained anti-tumor efficacy [28,29]. Here, we have sought to employ the sHDL nanodisc platform to deliver CSC antigen peptides and induce CSC-specific T cell responses in a syngeneic model D5, which is a poorly immunogenic murine model of melanoma [30]. Given the role of Sox2 and Nanog transcription factors in CSCs, which are responsible for cancer initiation and maintenance, we sought to elicit T cell responses against these stemness factors.

Toward this goal, we have identified new immunogenic sequences from Sox2 and Nanog, which are transcription factors associated with the stem-like properties exhibited by CSCs. Sox2 and Nanog are mainly expressed by embryonic stem cells during embryonic development [31], and normal adult stem cells have minimal or only transient expression of these factors [32,33]; thus, vaccination against Sox2 and Nanog may allow us to immunologically target CSCs. In this work, we identified immunogenic sequences from Sox2 and Nanog utilizing *in silico* and *in vivo* methods. In addition to the Sox2 and Nanog sequences, we added two immunogenic sequences derived from Aldehyde dehydrogenase (ALDH), a CSC biomarker, based on our previous work [34]. We have prepared sHDL nanodiscs carrying 6 CSC-derived peptides and CpG and shown that vaccination with these sHDL nanodiscs elicited robust CSC antigen-specific CD8 T cell response and achieved potent anti-CSC immunity in syngeneic mouse D5 melanoma model. Furthermore, we find that vaccination with these nanodiscs did not alter normal adult stem cell levels.

2. Materials & methods

2.1. Study design

The aim of this study was to develop a nanoparticle vaccine system to deliver antigenic peptides derived from CSCs to direct and elicit a comprehensive immune response against CSCs. We first identified immunogenic sequences from Sox2 and Nanog transcription factors that are overexpressed in CSCs from many cancer types. Using our previously developed nanodisc platform [28], we co-loaded the identified Sox2 and Nanog antigen peptides and CpG, a Toll-like Receptor 9 agonist. We characterized nanodiscs using dynamic light scattering (DLS), liquid chromatography-mass spectrometry (LC-MS), and gel permeation chromatography (GPC). We then evaluated its anti-tumor efficacy in a subcutaneous D5 melanoma model, a clone from the B16-BL6 tumor line that is a poorly immunogenic melanoma of spontaneous origin [30]. The nanodisc therapy containing all ALDH, Sox2, and Nanog peptides was compared to control groups ($n = 8$ mice per group). Mice were randomly assigned to treatment groups. Anti-tumor efficacy was assessed by monitoring tumor growth. We performed immunological assays to measure antigen-specific T cell responses, CSC depletion, and TME modulation.

2.2. Reagents and materials

$Sox2_{105–121}$, $Sox2_{162–180}$, $Nanog_{91–108}$, $Nanog_{235–248}$, $ALDH_{88–96}$ (ALDH-A1), and $ALDH_{99–107}$ (ALDH-A3) epitope peptides modified with a cysteine-serine-serine linker at the N-terminus were synthesized by Genemed Synthesis Inc. (San Antonio, Texas). Dipalmitoylphosphatidylcholine (DMPC) lipids were purchased from NOF America (White Plains, NY). 22A apolipoprotein-A1 mimetic peptide was synthesized by GenScript (Piscataway, NJ). 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[3-(2-pyridyldithio)propionate] (DOPE-PDP) was purchased from Avanti Polar Lipids (Alabastar, AL). Cholesterol-modified CpG1826 (chol-CpG1826) and unmodified CpG1826 were synthesized by Integrated DNA Technologies (Coralville, IA). Cell medium was purchased from Invitrogen (Carlsbad, CA). Female C57BL/6 mice aged 5–7 weeks were purchased from Jackson Laboratories (Bar Harbor, ME). The following antibodies used for flow cytometry were obtained from BioLegend: BV421-CD19 (clone HIB19); Pacific Blue-CD8 (clone 53–6.7); BV510-MHC-II (clone M5/114.15.2); BV570-CD45 (clone 30-F11); BV605-CD206 (clone C068C2); BV650-CD4 (clone RM4–5); BV570-CD11c (clone N418); BV785-CD62L (clone MEL-14); AF532-F4/80 (BM8); PerCp-Cy5.5-CD11b (clone M1/70); PE-NK1.1 (clone PK136); PE-Cy5.5-Foxp3 (clone 150D); APC-CD3 (clone 17A2); A647-Ki67 (clone 16A8); AF700-CD44 (clone IM7). IFN-γ ELISPOT kits were obtained from Fisher Scientific (Hampton, NH). The ALDEFLUOR™ Kit for ALDH Assays was from STEMCELL TECHNOLOGIES.

2.3. Synthesis of CSC antigen-loaded nanodiscs

The nanodiscs were prepared as we previously reported [1]. Briefly, DMPC and 22A were dissolved in acetic acid, lyophilized, and rehydrated in 10 mmol/L sodium phosphate buffer, followed by thermocycling. Lipid-peptide conjugates were prepared by reacting thiol-modified lipid DOPE-PDP with the cysteine-serine-serine (CSS) modified peptides in DMF for 2–3 h on an orbital shaker at room temperature. For incorporation of lipid-peptide conjugates into nanodiscs, the lipid-peptide conjugate mixture was added dropwise to un-loaded nanodiscs and incubated on an orbital shaker for 1 h at 200 rpm. After incorporation, unincorporated lipid-peptide conjugates were removed through ultracentrifuge filtration (MilliporeSigma Amicon Ultra Centrifugal Filter, 10 kDa). Chol-CpG was loaded by simply mixing the peptide-loaded nanodiscs with 2.3 nmol chol-CpG followed by incubation at room temperature for 1 h. Zetasizer Nanoseries Nano-ZS90 (Serial No: Mal1074171) was used to measure the size and zeta potential of the

nanodiscs. 100 μl nanodiscs was diluted in 900 μl PBS and loaded into a DTS1070 folded capillary cell prior to loading into the instrument. Reverse-phase ultra-performance liquid chromatography/mass spectrometry (UPLC/MS) was used to quantify the conjugation and loading efficiencies of the lipid-peptide conjugate. GPC equipped with a TSKgel G3000SWxl was used as a secondary confirmation of nanodisc size homogeneity and measure the amount of CpG loading.

2.4. CSC antigen nanodisc formulations

The following nanodisc formulations were used in the *in vivo* studies: free peptides + CpG; free peptides + CpG + Montanide; nanodiscs co-loaded with chol-CpG, $ALDH_{88–96}$, and $ALDH_{99–107}$ (A-Nanodisc); nanodiscs co-loaded with chol-CpG, $Sox2_{105–121}$, $Sox2_{162–180}$, $Nanog_{91–108}$, and $Nanog_{235–248}$ peptides (S-N-Nanodisc); nanodiscs co-loaded with chol-CpG and $ALDH_{88–96}$, $ALDH_{99–107}$, $Sox2_{105–121}$, $Sox2_{162–180}$, $Nanog_{91–108}$, and $Nanog_{235–248}$, peptides (A-S-N-Nanodisc); The free peptides + CpG nanodisc formulation contains all six CSC antigen peptides at 20 μg per peptide mixed with 2.3 nmol (~ 15 μg) CpG in PBS. The free peptides + CpG + Montanide formulation contains all six CSC antigen peptides at 20 μg per peptide mixed with CpG in PBS emulsified with Montanide at a 1:1 volume ratio.

2.5. Animals

C57BL/6 female mice 6–8 weeks old were purchased from Jackson laboratory (Bar Harbor, ME). All animal experiments were conducted in accordance with the approval by the Institutional Animal Care and Use Committee at the University of Michigan (Ann Arbor, MI).

2.6. In vivo immunotherapy studies

To establish the D5 melanoma-bearing tumor mouse model, C57BL/6 mice were inoculated subcutaneously in the right flank with 5×10^4 D5 cells in HBSS (Hank's balanced salt solution). Mice were randomly divided into six groups ($n = 8$) and received either PBS, soluble peptides + CpG, soluble peptides + CpG + Montanide, A-Nanodisc, S-N-Nanodisc, or A-S-N-Nanodisc. Bilateral vaccines were administered subcutaneously at the tail-base containing 20 μg per peptide and 2.3 nmol CpG or chol-CpG. Tumors were measured every 3 days using calipers. Tumor size was measured, and the volume was calculated as ½ (L x W^2). Tumor-bearing mice were euthanized when the tumor size reached 1.5 cm in any diameter or when the animals became moribund with severe weight loss (> 20%) or tumor ulceration.

2.7. Anti-tumor responses induced by nanodiscs

The IFN-γ Enzyme-linked Immunospot (ELISPOT) assay purchased from BD Biosciences (Catalog # BDB551083) was used to measure antigen-specific immune responses according to the protocol. An ELISPOT plate was coated with IFN-γ capture antibody for 24 h and blocked with DMEM medium containing 10% fetal bovine serum for 2 h. Splenocytes in a single cell suspension *via* 70 μm strainer and obtained from the treated mice were added to the 96-well ELISPOT plate at a concentration of 2×10^5 live cells per well. ALDH-A1 and ALDH-A3, $Sox2_{105–121}$, $Sox2_{162–180}$, $Nanog_{91–108}$, and $Nanog_{235–248}$ (all at 20 μg/ml) was added to stimulate the splenocytes. Ionomycin and phorbol myristate acetate were employed as the positive control. After 18 h, the IFN-γ spots were detected with biotinylated detection antibody, followed by streptavidin-horseradish peroxidase and AEC substrate kit. The number of the IFN-γ spot forming units were measured. The ELISA assay was performed on sera collected from mice treated with either PBS, free peptides + CpG (ALDH-A1, ALDH-A3, $Nanog_{235–248}$, and $Sox2_{162–180}$), or A-S-N-nanodisc (ALDH-A1, ALDH-A3, $Nanog_{235–248}$, and $Sox2_{162–180}$). IFN-γ, TNF-α, CXCL-9, and CXCL-10 were measured.

2.8. Analysis of CSCs

Mice were inoculated subcutaneously in the right flank with 5×10^4 D5 melanoma cells, followed by bilateral subcutaneous injections of PBS, free peptides + CpG, or A-S-N-Nanodiscs at the tail-base using the four CSC antigens with significant immune responses observed in the ELISPOT assay. Tumors were excised and passed through a 70 μm strainer to prepare a single cell suspension. Tumor cells were stained using the ALDEFLUOR™ Kit ALDEFLUOR to detect $ALDH^{hi}$ cells. We characterized the cancer cell stemness of the $ALDH^{hi}$ D5 cells previously [18]. In addition, tumor cells were stained with anti-CD45-BV570 and anti-CD3-APC-Cy7. $ALDH^{hi}$ CSCs were analyzed with the ZE5 Cell Analyzer from Bio-Rad Laboratories.

2.9. Immune profiling

For immune population analyses in the spleen, splenocytes were washed with fluorescence-activated cell sorting (FACS) buffer and blocked with CD16/32 antibody. The cells were then stained with the designated antibodies: BV421-CD19 (clone HIB19); Pacific Blue-CD8 (clone 53–6.7); BV510-MHC-II (clone M5/114.15.2); BV570-CD45 (clone 30-F11); BV605-CD206 (clone C068C2); BV650-CD4 (clone RM4–5); BV570-CD11c (clone N418); BV785-CD62L (clone MEL-14); AF532-F4/80 (BM8); PerCp-Cy5.5-CD11b (clone M1/70); PE-NK1.1 (clone PK136); PE-Cy5.5-Foxp3 (clone 150D); APC-CD3 (clone 17A2); A647-Ki67 (clone 16A8); AF700-CD44 (clone IM7). The cells were washed and stained with fixable viability dye Near-IR (Thermo Fisher Scientific) for flow cytometric analysis.

2.10. Safety of nanodisc vaccination

D5 melanoma tumor-bearing mice subjected to nanodisc vaccine treatments as shown in Fig. 5 were euthanized on day 15 for the toxicity evaluation. Blood was collected for a complete blood count (including white blood cells, platelets, red blood cells, and hemoglobin). Additionally, a fraction of the blood collected was used for biochemical analysis for alanine aminotransferase, aspartate aminotransferase, albumin, total bilirubin, blood urea nitrogen, alkaline phosphatase, and creatine phosphokinase. The lung, liver, spleen, heart, and kidneys were collected for hematoxylin and eosin staining. All these tests were performed at the *In-Vivo* Animal Core of the University of Michigan.

2.11. Statistical analysis

Sample sizes were chosen based on preliminary data from pilot experiments and previously published results in the literature. All animal studies were performed after randomization. Data were analyzed by one- or two-way analysis of variance (ANOVA), followed by Tukey's *post doc* test for comparison of multiple groups with Prism 9.0 (GraphPad Software). Animal survival was analyzed by log-rank (Mantel–Cox) test with Prism 9.0 (GraphPad Software). Data were normally distributed and *P values* < 0.05 were considered statistically significant. Statistical significance was denoted with $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. No samples were excluded from analysis.

3. Results and discussion

3.1. Overall study design

The main objective of our work was to develop a new vaccine against CSCs and examine its anti-tumor effects (Fig. 1). We have previously shown that nanodiscs are phagocytosed by resident DCs in LNs, where cleavage of the peptide and subsequent antigen presentation provides Signal 1 while CpG promotes upregulation co-stimulatory molecules, including CD40, CD80, and CD86, on DCs, thus providing Signal 2 [28]. Activated DCs then initiate cross-priming of T cells, leading to anti-

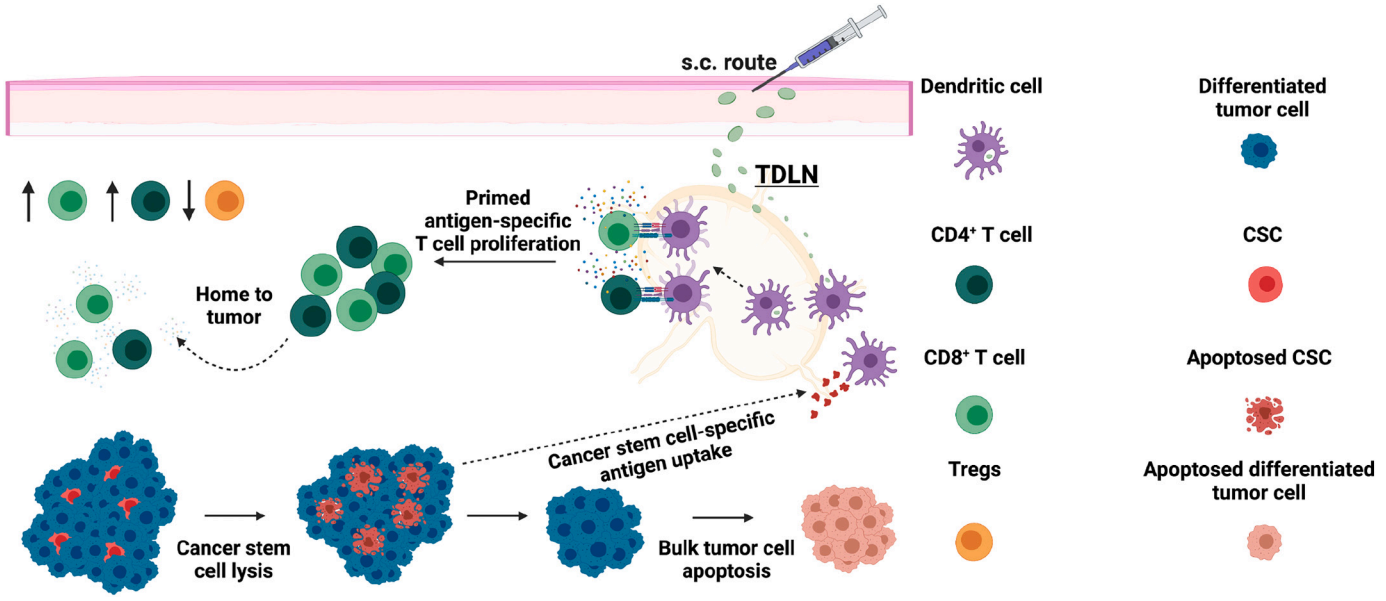

Fig. 1. Schematic illustration of CSC antigen-loaded nanodisc immunotherapy. CSC nanodiscs injected subcutaneously traffic to lymph nodes where resident DCs phagocytose and present CSC antigen to T cells for activation. Primed CSC-specific T cells proliferate and home to tumor where they reduce the frequency of CSCs. Elimination of CSCs, the precursor to differentiated tumor cells, leaves only differentiated tumor cells which eventually undergo apoptosis, thus reducing the tumor size. Nanodisc immunotherapy also remodels the immunosuppressive TME to be pro-inflammatory and reduces the frequency of regulatory T cells (Tregs). Created with BioRender.com.

tumor immunity. Given the ability of CSCs to initiate, maintain, and support tumor growth [1,3], here we sought to test our hypothesis that nanodisc-mediated delivery of CSC-derived epitopes elicits CD8 T cells against CSCs, leading to reduction of CSCs and induction of anti-tumor effects.

Toward this goal, we used the Immune Epitope Database Analysis Resource to identify new epitopes within Sox2 and Nanog that are highly upregulated among CSCs [35–38]. $Nanog_{91–108}$, $Nanog_{235–248}$, $Sox2_{105–121}$, and $Sox2_{162–180}$ were predicted to have high binding affinity to major histocompatibility complex I (MHC-I) molecules. Thus, we vaccinated mice with these peptides, anti-CD40 IgG antibody, and polyIC on days 0 and 7, followed by IFN-γ ELISPOT assay on splenocytes on day 10 [39,40]. The results indicated immunogenicity of $Nanog_{91–108}$, $Nanog_{235–248}$, $Sox2_{105–121}$, and $Sox2_{162–180}$. We originally identified 20 sequences predicted to have high affinity to MHC-I derived from stemness factors Oct4, Sox2, and Nanog, but the remaining 14 sequences showed minimal immunogenicity *in vivo* (data not shown); therefore, in the subsequent studies, we have focused on $Nanog_{91–108}$, $Nanog_{235–248}$, $Sox2_{105–121}$, and $Sox2_{162–180}$. In addition to these 4 antigens, we have previously used 2 CSC antigens, $ALDH_{88–96}$ (ALDH-A1) and $ALDH_{99–107}$ (ALDH-A3) peptides derived from ALDH, a polymorphic enzyme known to detoxify intracellular aldehydes through an oxidation process and extensively used as a functional biomarker to isolate CSCs from > 20 types of cancer [5,34,41–45]. Therefore, we evaluated T cell responses and anti-tumor efficacy of nanodisc vaccination using these 2 ALDH-derived epitopes as well as 4 new CSC-derived epitope peptides in this study.

3.2. Synthesis and characterization of nanodiscs

We synthesized sHDL nanodiscs co-loaded with each antigen and CpG (Fig. 2). Fig. 3a shows the schematic for the sHDL synthesis process. Blank sHDL nanodiscs were prepared using DMPC lipid and 22A apolipoprotein-A1 mimetic peptide. The peptide antigens were synthesized with a cysteine-serine-serine linker and conjugated with the functionalized lipid DOPE-PDP to produce antigen-lipid conjugates, which were then added to blank nanodiscs (Supplementary Figs. 1). Each nanodisc formulation was first analyzed for its hydrodynamic size and charge (Fig. 2 and Supplementary Figs. 2–6). Blank and antigen-loaded nanodiscs were similar in size ranging from 9 to 15 nm and had zeta potential ranging from +6 to −6 mV (Fig. 2b). Nanodiscs loaded with ALDH-A1, ALDH-A3, $Nanog_{235–248}$, and $Sox2_{162–180}$ antigens were negatively charged [46]. Nanodiscs loaded with $Nanog_{91–108}$ and $Sox2_{105–121}$ antigens were slightly positively charged.

The amount of CSC antigen loaded into the nanodiscs was quantified using high performance liquid chromatography (HPLC) as shown in Fig. 2c. We observed successful conjugation of all 6 CSC peptides to DOPE lipid and incorporation into nanodiscs with > 90% efficiency, as quantified by the amount of antigen-lipid conjugates remaining before and after filtration of nanodiscs. HPLC chromatograms also showed disappearance of the un-conjugated peptide peaks after filtration. In addition to quantifying the CSC antigens, mass spectrometry (MS) was used to confirm that antigen peptides did not undergo any modifications during the synthesis process [47,48]. Fig. 2d shows the MS peak for the antigen-lipid conjugate alone at 1324.40 m/1 and loaded into the nanodisc at the same mass, 1324.35 m/1 and 678.55 m/2, indicating no chemical modification. Finally, nanodiscs were incubated with 2.3 nmol (~15 μg) cholesterol-modified CpG (chol-CpG) by simply mixing at room temperature. GPC was used to confirm > 95% incorporation of chol-CpG and size homogeneity of nanodiscs co-loaded with CSC peptides and CpG (Fig. 2e).

3.3. Therapeutic efficacy of nanodisc vaccination

To evaluate the therapeutic efficacy of the nanodisc-based vaccine, we used the D5 melanoma cell line, a clone from the B16-BL6 tumor line that is poorly immunogenic and syngeneic to B6 mice [18,30]. D5 cells have CSCs characterized as $ALDH^{hi}$. We have shown previously that as few as 500 $ALDH^{hi}$ D5 cells implanted subcutaneously in mice formed tumors, whereas as many as 2×10^5 $ALDH^{low}$ cells did not form tumors [18]. For our current study, C57Bl/6 mice were inoculated subcutaneously in the right flank with 5×10^4 D5 tumor cells on day 0 and treated subcutaneously at tail base on days 1, 8, and 15 with CSC peptides in nanodisc or soluble forms containing 20 μg antigen peptides and 15 μg CpG (Fig. 3a). As a benchmark, we also treated mice with CSC peptides and CpG emulsified in water-in-oil Montanide, which is a potent, clinical

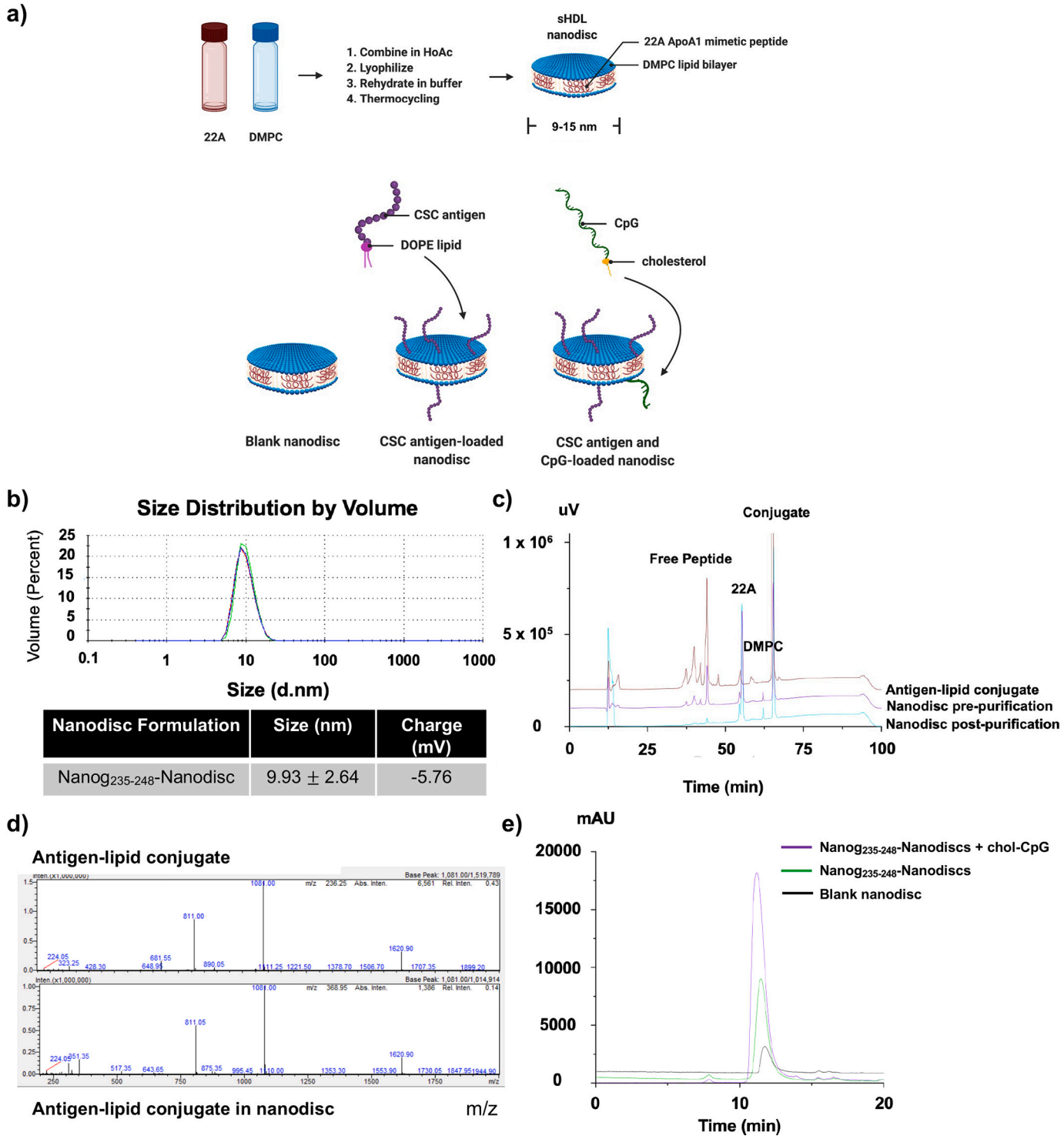

Fig. 2. Synthesis and characterization of CSC antigen-loaded nanodiscs. a) Schematic illustration of CSC antigen-loaded nanodisc synthesis using $Nanog_{235–248}$ as example. Created with BioRender.com. b) DLS of $Nanog_{235–248}$-Nanodiscs, showing the average size and zeta potential. c) Reverse-phase chromatography with HPLC of antigen-lipid conjugate (top), nanodisc before purification (middle), and nanodisc after purification (bottom). d) MS of antigen-lipid conjugate alone (top) and loaded into the nanodisc (bottom). e) GPC of blank nanodisc (black), $Nanog_{235–248}$-Nanodiscs (green), and cholesterol-modified CpG loaded into $Nanog_{235–248}$-Nanodiscs (purple). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stage adjuvant system for peptide-based vaccines [49,50].

We tested the following vaccine formulations *in vivo*: 6 antigen peptides + CpG (termed "free peptides + CpG); 6 antigen peptides + CpG + Montanide (termed "free peptides + CpG + Montanide); nanodiscs co-loaded with ALDH peptides and chol-CpG (termed "A-Nanodisc"); nanodiscs co-loaded with Sox2 and Nanog peptides and chol-CpG (termed "S-N-Nanodisc"); and nanodiscs co-loaded with ALDH, Sox2, and Nanog peptides and chol-CpG (termed "A-S-N-Nanodisc").

Vaccination with free peptides + CpG and free peptides + CpG + Montanide significantly slowed tumor growth, compared with PBS-treated mice (Fig. 3b–c), showing the potential efficacy of CSC peptide vaccination. Importantly, vaccination with A-S-N-Nanodisc exerted

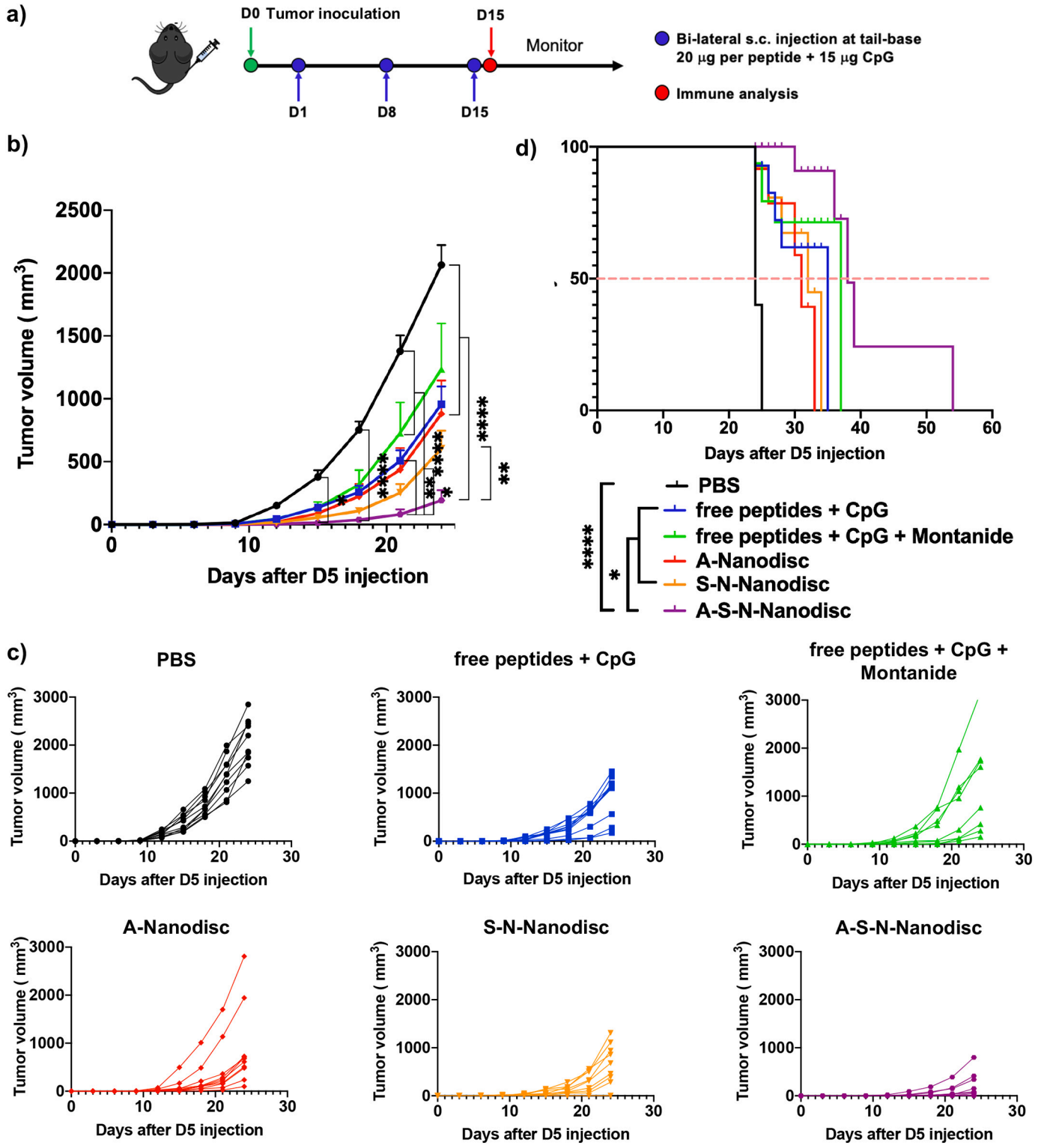

Fig. 3. Therapeutic efficacy of nanodisc vaccination in a D5 melanoma model. a) Treatment regimen and study timeline. C57bl/6 mice were inoculated with 5×10^5 D5 cells at SC flank on day 0 and treated with vaccines administered SC at tail base on days 1 and 8. b) Average tumor growth for all treatment groups. c) Individual tumor growth curves for all treatment groups. d) Kaplan-Meier overall survival curves. The data show mean ± SEM with $n = 8$ mice/group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

stronger anti-tumor efficacy, compared with all other treatment groups, as shown by statistically significant decrease in the average tumor size of mice treated with A-S-N-Nanodisc (Fig. 3b–c). Moreover, vaccination with A-S-N-Nanodisc resulted in statistically significantly extension in the animal survival, compared with all other groups (Fig. 3d). While vaccination with A-Nanodisc as well as S-N-Nanodisc exhibited anti-tumor efficacy, they showed reduced anti-tumor efficacy, compared with the A-S-N-Nanodisc group (Fig. 3b-c), thus showing the importance of having multiple antigen targets.

Taken together, these results demonstrate that vaccination against

CSC antigens derived from stemness factors can inhibit melanoma tumor growth. Nanodisc-mediated vaccination against a cocktail of CSC antigens significantly inhibited tumor growth and extended animal survival, compared with soluble peptide vaccination.

3.4. Systemic T cell response and immune activation

Having shown anti-tumor effects of nanodisc vaccination against CSCs, we analyzed T cell responses against CSC antigens. We performed vaccinations after inoculation of D5 cells as shown in Fig. 3A, followed by analysis of CSC antigen-specific T cell responses using IFN-γ ELISPOT assay on day 15. We focused on IFN-γ since it is a Type I interferon primarily produced by immune cells and plays a critical role in anti-tumor responses by activating effector immune cells, enhancing antigen presentation, and orchestrating activation of the innate immune system [51].

Free peptides + CpG and free peptides + CpG + Montanide groups showed minimal IFN-γ responses, compared with PBS group (Fig. 4). In contrast, when restimulated with ALDH-A1, ALDH-A3, $Nanog_{235–248}$, and $Sox2_{162–180}$, the A-S-N-Nanodisc group showed significantly increased IFN-γ responses, compared with other vaccine groups (Fig. 4). Specifically, A-S-N-Nanodisc group had 30.5-fold, 3.7-fold, and 12.8-fold increase in IFN-γ Elispots when restimulated with ALDH-A1 + ALDH-A3, $Nanog_{235–248}$, and $Sox2_{162–180}$, compared with the free peptides + CpG group, respectively (Fig. 4). A-S-N-Nanodisc also induced modest IFN-γ + T cell responses against $Nanog_{91–108}$, and $Sox2_{105–121}$, although the results were not statistically significant. This indicates that $Nanog_{235–248}$ and $Sox2_{162–180}$ are more immunodominant epitopes. To corroborate these results, we isolated $CD8^+$ T cells from spleen of vaccinated mice and restimulated with epitope peptides for 3 days. Analysis of the supernatant by ELISA indicated robust secretion of IFN-γ and TNF-α (Supplementary Fig. 7), thus indicating robust antigen-specific $CD8^+$ T cell response induced by nanodisc vaccination.

Interestingly, vaccination with A-Nanodisc led to IFN-γ responses against ALDH-A1 + ALDH-A3 as well as $Nanog_{235–248}$ and $Sox2_{162–180}$ (Fig. 4) although the differences were not statistically significant due to the large number of comparison groups. This suggests A-Nanodisc induced epitope spreading, which is the development of an immune response to epitopes distinct from the administered epitopes, to other CSC-associated antigens. Taken together, these results demonstrate that nanodiscs carrying CSC antigens elicit antigen-specific T cell responses and induces epitope spreading across CSC-associated antigens.

3.5. Immune modulation after nanodisc vaccination

As the A-S-N-Nanodisc group exhibited most potent anti-tumor effects (Fig. 3), we subsequently performed immunological profiling studies after treating tumor-bearing mice with A-S-N-Nanodiscs and compared with free peptides + CpG or PBS control. We performed vaccinations after inoculation of D5 cells as shown in Fig. 3A, followed by measuring pro-inflammatory IFN-γ, TNF-α, CXCL-9, and CXCL-10 secreted in serum (Fig. 5a). TNF-α is a mediator of innate immunity capable of inducing necrosis in tumors, and CXCL-9/10 are chemokines that regulate immune cell migration, differentiation, and activation [52,53]. Serum collected 24 h after the first immunotherapy dose was analyzed for cytokines and chemokines by ELISA. Mice treated free peptides + CpG produced a minimal level of IFN-γ but a significant amount of CXCL-10 (Fig. 5a). Vaccination with A-S-N-Nanodisc led to 2.3-fold, 14-fold, 14.3-fold, and 1.6-fold higher levels of IFN-γ (n.s.), TNF-α ($P < 0.05$), CXCL-9 ($P < 0.0001$), and CXCL-10 ($P < 0.0001$) in serum, compared with free peptides + CpG, respectively (Fig. 5a). We have observed a similar trend of elevated IFN-γ, TNF-α, CXCL-9, and CXCL-10 after the 2nd and 3rd vaccinations with A-S-N-Nanodisc (Supplementary Fig. 8). Importantly, however, the elevated cytokines and chemokines returned to the baseline within 48 h after vaccination, likely indicating minimal immunotoxicity for local and systemic immune systems (data not shown).

Next, we analyzed changes in the TME after nanodisc vaccination. In particular, $CD8^+$ T cells are considered the primary immune cell population responsible for cytotoxicity against tumor cells [54,55], whereas regulatory $CD4^+$ T cells (Tregs) suppress pro-inflammatory immune responses and are recruited to the TME by tumor cells as part of their immune escape mechanisms critical to tumorigenesis [56]. Hence, we performed vaccinations after inoculation of D5 cells as shown in Fig. 3A, followed by immunological analysis of $CD8^+$ T cell, $CD4^+$ T cells, and Tregs on day 15. Vaccination with the A-S-N-Nanodisc elicited significantly higher frequency of intratumoral $CD8^+$ T cells, with 2-fold and 6-fold increases, compared with free peptides + CpG ($P < 0.05$) and PBS ($P < 0.0001$), respectively (Fig. 5b). A-S-N-Nanodisc vaccination also promoted strong proliferation of intratumoral $CD8^+$ T cells as shown by significantly elevated Ki67 staining, compared with other groups (Fig. 5b). A-S-N-Nanodisc vaccination triggered robust induction and

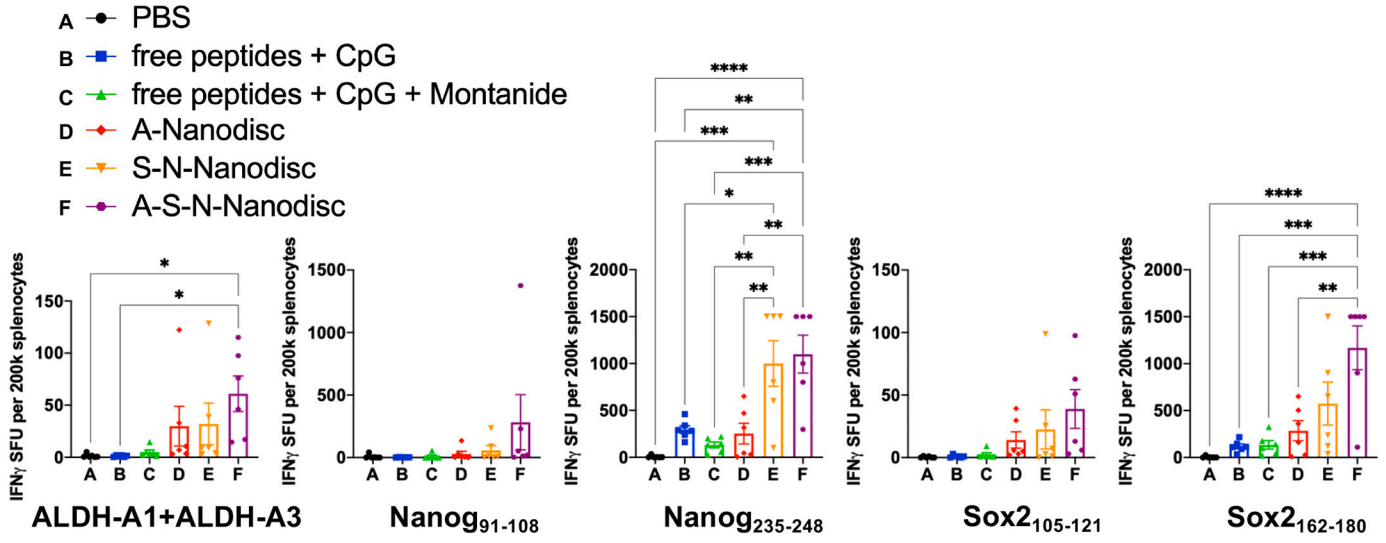

Fig. 4. Antigen-specific T cell responses induced by CSC antigen-loaded nanodisc immunotherapy. Tumor-bearing mice were treated as shown in Fig. 3A, and IFN-γ ELISPOT assays were performed on splenocytes on day 15. The data show mean ± SEM with $n = 6$ mice/group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

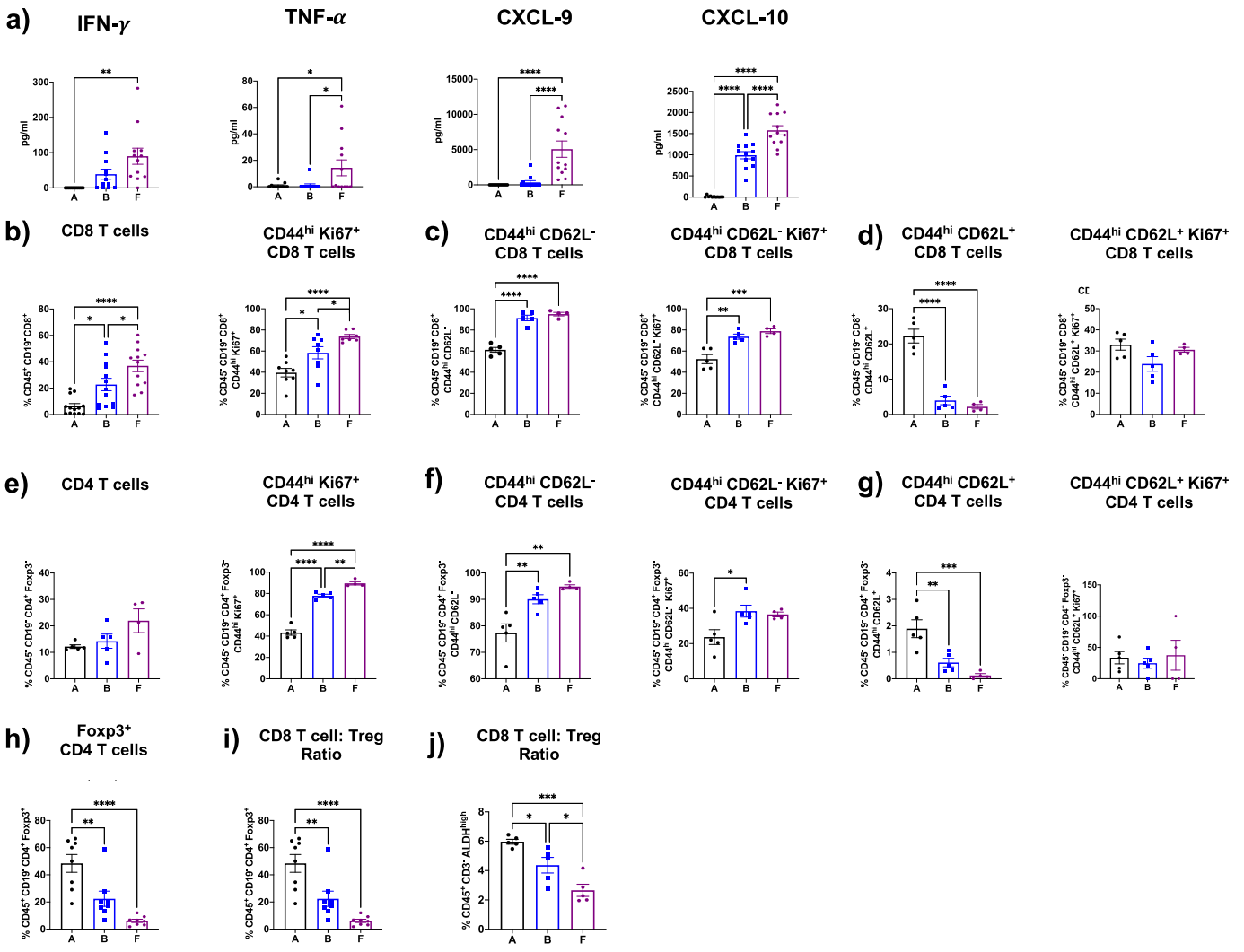

Fig. 5. Impact of nanodisc vaccination on serum cytokines, intratumoral lymphocytes, and CSCs. C57BL/6 mice were inoculated subcutaneously with 5×10^5 D5 melanoma tumor cells on day 0 and were vaccinated on days 1 and 8. a) Serum concentrations of IFN-γ, TNF-α, CXCL-9, and CXCL-10 were measured after 24 h of the first vaccination on day 1. b-j) On day 15, cells from tumor tissues were stained with antibodies and analyzed by flow cytometry for b-d) $CD8^+$ T cells, e-g) $CD4^+$ T cells, and h) Tregs. i) Shown is the ratio of $CD8^+$ T cells to Tregs and j) $ALDH^{hi}$ $CD45^+$ CSCs within the tumor tissues. b) Quantification of IFN-γ, TNF-α, CXCL-9, and CXCL-10 secretion by ELISA. The data show mean ± SEM with ($n = 12$ mice/group for panel a and $n = 5$–11 mice/group for panels b-j). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

proliferation of effector memory $CD44^{hi}CD62L^-$ $CD8^+$ T cells (Tem), compared with central memory $CD44^{hi}CD62L^+$ $CD8^+$ T cells (Tcm) (Fig. 5c,d). The ratio of frequencies of intratumoral $CD8^+$ Tem to Tcm was 44 for the A-S-N-Nanodisc group, indicating a more cytolytic phenotype [57]. In terms of $CD4^+$ T cell responses, there was a trend for increased intratumoral $CD4^+$ T cells for the A-S-N-Nanodisc group with a significant increase in Ki67+ staining, indicating robust proliferation of $CD4^+$ T cells (Fig. 5e). A-S-N-Nanodisc vaccination triggered robust induction and proliferation of $CD44^{hi}CD62L^-$ $CD4^+$ Tem cells, compared with $CD44^{hi}CD62L^+$ $CD4^+$ Tcm cells (Fig. 5f, g). Similar to $CD8^+$ T cell response after A-S-N-Nanodisc vaccination, the ratio of frequencies of intratumoral $CD4^+$ Tem to Tcm was 748. Importantly, A-S-N-Nanodisc vaccination also significantly decreased the frequency of Tregs ($Foxp3^+CD4^+$) in the tumor, compared with PBS ($P < 0.0001$, Fig. 5h) with a trend for decreased proliferation of Tregs. Overall, there was a significant 6-fold increase in the ratio of $CD8^+$ T cells to Tregs for mice vaccinated with the A-S-N-Nanodisc (Fig. 5i). Taken together, these results show that A-S-N-Nanodisc vaccination induced robust immune activation, characterized by strong tumor-infiltration and proliferation of CD8+ and CD4+ Tem cells with reduced Tregs, thus indicating a transition from "cold" to "hot" tumor.

3.6. Depletion of CSCs in tumor

Having shown CSC antigen-specific T cell responses and a significant inhibition of D5 tumor growth, we proceeded to assess the CSC frequency in the tumor subjected to treatment targeting CSCs. In D5 tumor-bearing mice, cells with the highest ALDH expression in D5 tumors also expressed CD45 without CD3 expression (Supplementary Fig. 9). Backgating showed this $CD45^+CD3^-ALDH^{hi}$ CSC population has a different size and granularity than traditional $CD45^+$ and $CD45^+CD3^+$ lymphocytes (Supplementary Fig. 10). Mice treated with A-S-N-Nanodisc showed a significant decrease in $CD45^+CD3^-ALDH^{hi}$ CSCs with only 2.23% of cells in the tumor, compared with CSC frequency for free peptides + CpG group at 4.57% and PBS at 5.80% (Fig. 5j). Taken together, these data show that CSC-targeted nanodisc vaccination could reduce the frequency of CSCs in the tumor.

3.7. Safety profile of nanodisc vaccination

Normal stem cells are the building blocks of all organs, tissues, blood, and the immune system, and cells that express ALDH, Sox2, and Nanog are required in adults for tissue homeostasis to regenerate to replace lost or damaged cells [9,58]. Depletion of normal stem cells would cause many adverse events. Hence, we examined whether nanodisc vaccination against ALDH, Sox2, and Nanog caused any toxicity in the host. We performed vaccinations on days 1 and 8 in tumor-bearing mice, followed by assessment of safety profiles (Fig. 6). All the treatment groups had normal complete blood count and serum chemistry on day 15 (Fig. 6a). All major organs collected on day 15 showed no abnormal morphology (Fig. 6b). The body weight of mice showed no significant differences up to day 24 among the treatment groups (Fig. 6c). Lastly, to ensure we did not deplete normal stem cells after nanodisc vaccination, we assessed the frequency of mammary epithelial stem cells, which express stemness factors, such as ALDH, Sox2, and Nanog [59,60], and can be characterized as ALDH^{hi}. There was no significant difference in the frequency of ALDH^{hi} mammary epithelial stem cells among the different treatment groups and PBS control (Fig. 6d). These data indicate that vaccination against CSC antigens employed in our studies did not induce any overt toxicity among normal stem cells or cause autoimmune-mediated damage in major organs. We speculate a few possible reasons for this: (1) CSCs may have higher expression of ALDH, Sox2, and Nanog than normal adult stem cells; (2) MHC-I/CSC antigen complex may be expressed at a higher level on the surface of CSCs than on normal stem cells; (3) Sox2 and Nanog are expressed mainly in embryonic stem cells [31] whereas normal stem cells may only express them transiently [32,33]. We will examine these issues in the future; however, it is beyond the scope of this current study to delineate the differences between CSCs and normal adult stem cells. Overall, these results indicate that nanodisc-based vaccination with CSC antigens is safe and well-tolerated.

4. Conclusions

In this study, we report a novel and powerful immunotherapeutic strategy to target CSCs. We identified new CSC antigen peptides derived from the transcription factors Sox2 and Nanog. Nanodiscs formulated with epitopes from Sox2, Nanog, and ALDH served as a platform for a CSC-targeted immunotherapy. Immunogenic sequences were identified based on predicted MHC-I binding affinities and *in vivo* testing using a strongly adjuvanted CSC antigen peptide cocktail. Once each CSC antigen peptide was loaded onto nanodiscs, we evaluated their therapeutic efficacy in the D5 murine melanoma model and evaluated antigen specificity, cytokine production, and tumor-infiltrating immune cell populations. We have demonstrated that (i) the A-S-N-Nanodisc containing all 6 CSC antigen peptides (ALDH-A1, ALDH-A3, Sox2$_{105–121}$, Sox2$_{162–180}$, Nanog$_{91–108}$, and Nanog$_{235–248}$) can be administered to mice in a single dose; (ii) multiple CSC targets improves the therapeutic efficacy over a subset; and (iii) the A-S-N-Nanodisc remodels the TME by increasing the infiltration of activated, proliferating CD8$^+$ T cells and

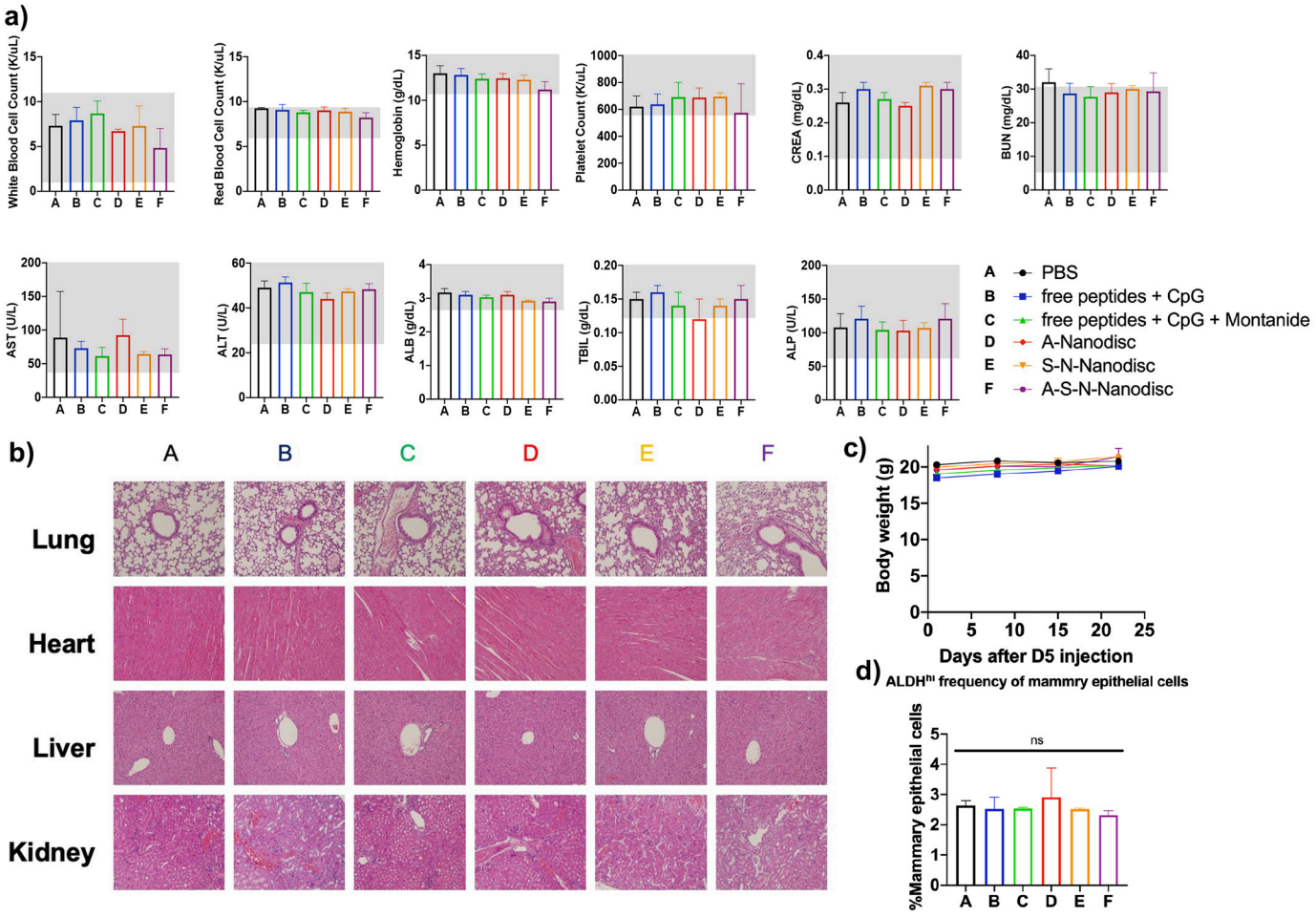

Fig. 6. Safety, toxicology, and histology after nanodisc vaccination. a) CBC and serum biochemistry was assayed on day 15 after vaccination on days 0 and 7. b) On day 15, major organs were harvested for H&E staining and histological analysis. The tissue sections have been examined by a licensed pathologist in a blinded manner. c) Body weight over the course of treatment. d) Quantification of ALDH^{hi} mammary epithelial stem cells (normal stem cells). The data show mean ± SEM with $n = 5$ mice/group. ns, not significant.

reducing the frequency of immunosuppressive Tregs. Although this study was focused on a melanoma model, CSCs are present in a wide variety of cancer types where Sox2, Nanog, and ALDH may also be expressed, for which our A-S-N-Nanodisc may provide a new pathway to target CSCs [10–15].

Despite the exciting results showing the potential of the CSC nanodisc immunotherapy, there are still challenges to overcome before clinical translation, including (1) incomplete elimination of tumors, (2) therapeutic efficacy on well-established tumors, and (3) off-target effects. While the A-S-N-Nanodisc exerted significant anti-tumor efficacy, the monotherapy failed to eliminate tumors. This may be attributed to the fact that nanodiscs elicit immune responses to target CSCs which constitute only a small fraction of the cells found in the tumor. Eliminating CSCs will decrease the frequency of differentiated non-CSCs due to a smaller population of precursor cells, but this immunotherapy does not target the majority of tumor cells as they do not express ALHD, Sox2, or Nanog. Additionally, tumor cells have shown plasticity to de-differentiate into a stem-like state and proliferate based on epigenetic (intrinsic plasticity) and TME features (extrinsic plasticity) [61,62]. Lastly, our nanodisc vaccine may be unable to eliminate all CSCs due to the immunosuppressive TME. We speculate that one potential approach to achieve complete tumor regression is to use a combination therapy such as co-administration of our A-S-N-Nanodisc to target CSCs and a chemotherapeutic to target differentiated tumor cells. Another potential approach is to use a combination therapy of our A-S-N-Nanodisc with an immune checkpoint blockade to prevent T cell exhaustion. Additionally, it is more clinically relevant to treat well-established tumors. In this manuscript, vaccination began on day 1 post tumor inoculation. Using a combination therapy of chemotherapy, immune checkpoint blockade, or targeted therapies with the A-S-N-Nanodisc could potentially improve the therapeutic efficacy in the clinic. Finally, there are safety concerns associated with targeting Sox2, Nanog, and ALDH, which are present in both CSCs and normal stem cells. While their expression is minimal or transient in normal stem cells, there is potential for normal stem cells to be targeted. This could have negative downstream effects on tissue development and regeneration. In the current study, we did not observe any adverse behavior or a decrease in mammary epithelial stem cells after vaccination. However, further research must be done to understand the effect of A-S-N-Nanodiscs on normal stem cells and ensure its safety *in vivo*. Ultimately, more work needs to be done to better understand safety and off-target effects, immunotoxicity, and how nanodisc immunotherapy affects non-CSCs.

Overall, the immunotherapeutic approach utilizing sHDL nanodiscs, with an established, scalable manufacturing procedure and acceptable safety profiles from prior phase I trials [63], provides a promising approach to target CSCs through minimally invasive vaccination against CSC antigens [63]. The approach outlined here may be applied to melanoma and potentially other cancer types and serve as a critical component in combination therapy to eliminate CSCs and non-CSCs in a tumor.

Credit Author Statement

M.E.A., Q.L., and J.J.M. designed the experiments. H.D. and Y.Q. contributed to the design of specific experiments. M.E.A. developed the nanodisc and soluble vaccines. M.E.A., H.D., and Y.Q. performed the experiments. H.D. contributed technical expertise, including flow cytometry analysis and flow panel design. K.L. and Y. X. aided with specific experiments. A.H.N. and Y.Q. contributed to the initial screening of immunogenic sequences. M.E.A., Y. X., and Y.Q. contributed to the ELISPOT assay. M.E.A., H.D., A.S., M.S.W., and A.E.C. Q.L., and J.J.M. analyzed and interpreted the data. M.E.A. and J.J.M. wrote the paper.

Declaration of Competing Interest

A patent application for nanodisc vaccines has been filed, with M.E. A., H.D., A.H.N., A.S., and J.J.M. as inventors. J.J.M. and A.S. are co-founders of EVOQ Therapeutics, LLC. that develops the nanodisc technology for immunotherapeutic applications.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2022.09.061.

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