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ORIGINAL RESEARCH

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An adjuvant-containing cDC1-targeted recombinant fusion vaccine conveys strong protection against murine melanoma growth and metastasis

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ABSTRACT

Type 1 conventional dendritic cells (cDC1) efficiently cross-present antigens that prime cytotoxic $CD8^+$ T cells. cDC1 therefore constitute conceivable targets in cancer vaccine development. We generated recombinant fusion cancer vaccines that aimed to concomitantly deliver tumor antigen and adjuvant to $CD103^+$ migratory cDC1, following intranasal administration. The fusion vaccine constructs comprised a cDC1-targeting anti-CD103 single chain antibody (aCD103) and a cholera toxin A1 (CTA1) subunit adjuvant, fused with MHC class I and II- or class II-restricted tumor cell antigens to generate a CTA1-I/II-aCD103 vaccine and a CTA1-II-aCD103 vaccine. The immunostimulatory and anti-tumor efficacy of these vaccines was evaluated in murine B16F1-ovalbumin (OVA) melanoma models in C57BL/6 J mice. The CTA1-I/II-aCD103 vaccine was most efficacious and triggered robust tumor antigen-specific $CD8^+$ T cell responses along with a Th17-polarized $CD4^+$ T cell response. This vaccine construct reduced the local growth of implanted B16F1-OVA melanomas and efficiently prevented hematogenous lung metastasis after prophylactic and therapeutic vaccination. Anti-tumor effects of the CTA1-I/II-aCD103 vaccine were antigen-specific and long-lasting. These results imply that adjuvant-containing recombinant fusion vaccines that target and activate cDC1 trigger effective anti-tumor immunity to control tumor growth and metastasis.

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Dendritic cells; cancer vaccine; metastatic melanoma; murine models; immunostimulatory

Introduction

Dendritic cells (DC) play a key role in anti-tumor defense by virtue of their supreme capacity to (i) take up antigen and migrate to draining lymph nodes for priming of $CD4^+$ and $CD8^+$ T cells, (ii) provide co-stimulation that regulates T cell priming and differentiation, and (iii) generate chemoattractants that recruit anti-tumor effector cells.[1] These features have inspired the development of immunotherapies that target DC aiming at achieving effective and durable T cell-mediated immunity against cancer.[2] In the clinical setting, such strategies have mostly comprised of the adoptive transfer of patient-derived DC, where autologous DC or precursor cells are treated *ex vivo* with cytokines to induce their differentiation and maturation.[3,4] In patients, such DC-targeted anti-tumor strategies may entail local and systemic T cell activation, but objective anti-tumor responses have hitherto been unimpressive.[5] The limited clinical benefit of DC-centered approaches has been attributed to insufficient antigen presentation or co-stimulation or reduced survival of DC in the malignant microenvironment.[6,7] The lack of efficient T cell priming of anti-tumor immunity calls for additional or improved DC-based strategies.

DC comprise three major populations including monocyte-derived DC, plasmacytoid DC, and conventional DC (cDC); the latter are subdivided into type 1 (cDC1) and type 2 (cDC2). The DC populations differ in transcription factor dependency, phenotype, and function.[1,8] cDC1 thus rely on the transcription factors interferon regulatory factor 8 (IRF8) and the basic leucine zipper transcription factor ATF-like 3 (Batf3) for their development, and comprise lymphoid tissue-resident $CD8\alpha^+$ cDC1s and $CD103^+$ migratory cDC1s.[8,9] cDC1 have been ascribed a pivotal role in priming anti-tumor immunity based on studies in *Batf3*$^{-/-}$ mice and other models of cDC1 deficiency.[10,11] Additionally, cDC1 gene signatures and/or the presence of cDC1 in tumors herald favorable prognosis in several histotypes of human cancer.[12] The mechanisms that explain the purported advantage of cDC1 in anti-tumor immunity are partly unknown but has been attributed to their unique ability to cross-present soluble antigen to $CD8^+$ T cells,[13] although recent studies propose that also their ability to prime $CD4^+$ T cells may contribute.[11,14] In addition, cDC1 may enter into tumor tissue and produce cytokines and chemoattractants of relevance to T cell immunity suggesting that cDC1 bolster anti-tumor immunity also beyond antigen presentation.[8,11]

Strategies to directly target tumor antigens to DC populations by use of antibodies have gained increasing attention in recent years. CDX-1401 is a vaccine construct in which the tumor antigen NY-ESO-1 was fused to an antibody against DEC-205, which is expressed by all subsets of DC. This

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vaccine construct triggered NY-EOS-1-specific immune responses in phase I and II trials in advanced cancer.[15,16] Another approach under investigation is to target tumor antigens to cells expressing the C-type lectin-like receptor (CLEC9A), which is more specific to cDC1.[17] In a humanized mouse model, tumor antigens fused with antibodies against CLEC9A primed $CD8^+$ T cells more efficiently than corresponding antigens fused to anti-DEC-205 antibodies, highlighting the superior efficacy of the cDC1 population in inducing cytotoxic T cell responses.[18,19] Notable, neither of these antibody-based DC vaccine constructs were fused with adjuvants.

For this study, we explored the potential anti-cancer efficacy of adjuvant-containing vaccine constructs that were developed to target cDC1. In an original fusion protein, we genetically linked the vaccine adjuvant cholera toxin A1 subunit (CTA1) to a dimer of the D-domain of *Staphylococcus aureus* protein A (DD).[20] Previous studies showed that the CTA1-DD fusion protein with incorporated peptides from infectious agents yielded strong and protective immunity against these infectious agents.[21] The DD-based fusion proteins were found to attach cDC via complement receptors.[22] In an extension of these studies, DD was replaced by a single-chain antibody against CD103 (scFvCD103, aCD103), which resulted in efficient targeting to the migratory $CD103^+$ cDC1 cell subset that, in turn, resulted in strong priming of $CD4^+$ and $CD8^+$ T cell responses.[23]

For the present study, we aimed at determining whether functional anti-tumor immunity was induced by cDC1-targeted fusion protein and utilized the B16F1-OVA model in which murine B16 melanoma cells were transfected with the ovalbumin gene. We generated constructs containing aCD103 and CTA1 fused with the MHC class I-restricted SIINFEKL epitope and/or the MHC class II-restricted p323 epitope of ovalbumin. These constructs were administered intranasally and evaluated for efficacy against local tumor growth and metastasis. We report that prophylactic and therapeutic immunization using CTA1-aCD103 vaccines, fused with tumor cell epitopes, markedly reduced melanoma growth and metastasis and also entailed strong anti-tumor immune responses in lung tissue. The observed anti-tumor effects were dependent on the presence of antigen-specific $CD8^+$ T cells with a significant contribution also by $CD4^+$ T cells, of which T_h17 cells predominated.

Materials and methods

Study design and approval

The aim of this study was to evaluate the anti-tumor efficacy *in vivo* of cDC1-targeting vaccines on experimental melanoma. CTA1-aCD103 fusion constructs were generated and fused to MHC class I and/or class II restricted tumor cell epitopes. Mice were immunized intranasally with these constructs before or after intravenous or subcutaneous challenge with melanoma cells. Experiments were performed according to institutional guidelines and were approved by the Research Animal Ethics Committee in Gothenburg, application no. 14836/2019.

Preparation of fusion vaccines

The CTA1-aCD103 constructs were prepared as follows: The variable light (VL) and variable heavy (VH) chains of anti-CD103 were cloned from the anti-CD103-producing hybridoma M290. These regions were amplified by RT-PCR from M290, and the amplified regions were linked using four GGGGS linker regions to obtain a CD103 single-chain antibody (anti-CD103 scFv, aCD103). Subsequently, a DNA sequence encoding functional CTA1 was fused upstream of aCD103 scFv. DNA sequences corresponding to the MHC class I-restricted OVA peptide p257-264 (SIINFEKL) and/or the MHC class II-restricted OVA peptide p323-339 (ISQAVHAAHAEINEAGR; here denoted p323) were inserted between the aCD103 and scFv CTA1 sequences. The sequence for a 6× HIS tag followed by a FLAG tag was inserted at the end of the sequence.

In some experiments, constructs based on a dimer of the D-domain of *Staphylococcus aureus* protein A (DD) fused to CTA1 was used. These include CTA1-SIINFEKL-p323-DD (carrying the same OVA-derived MHC class I and class II epitopes as specified above) and CTA1-3M2e-DD (carrying 3 copies of the influenza virus-specific M2e-peptide). These vaccines were produced by MIVAC Development AB (Gothenburg, Sweden) as previously described.[24]

All fusion proteins were expressed in *E. coli* and purified using His column affinity chromatography and size exclusion chromatography. The endotoxin levels were <100 EU/mg protein in all preparations. All fusion proteins contained an enzymatically active CTA1 unit as determined using the ADP-ribosylation assay as described.[25,26]

Culture of melanoma cell lines and analysis of MHC class I SIINFEKL expression

B16F1-OVA cells were kindly provided by Kerstin Hoffmann and Andreas Thiel (Berlin-Brandenburg Center for Regenerative Therapies, Germany). B16F10 cells were obtained from the cell culture laboratory at the Department of Virology, University of Gothenburg. Cells were cultured in Iscoves' Modified Dulbecco's Medium (IMDM) (GE Healthcare Life Sciences) supplemented with 10% fetal calf serum (PAA Laboratories GmbH) 100 µg/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma), 1 mM sodium pyruvate (Gibco) and 2 mM L-glutamine (Gibco). Cells were cultured for one to two weeks prior to inoculation into mice. To assess presentation of the SIINFEKL peptide, B16F1-OVA and B16F10 cells were incubated overnight with 20 ng/ml of IFN-γ (BD Biosciences) to boost MHC expression. Cells were then pulsed or not pulsed with 5 µM SIINFEKL peptide (Sigma) for 30 minutes at 37°C before staining with MHC I-SIINFEKL tetramer (Biolegend) for one hour at 4°C and analysis by flow cytometry (LSR Fortessa, BD) and FlowJo version 10.

In vitro cytotoxicity assay

T cell receptor (TCR) transgenic OT-1 mice (Charles River) were the source of $CD8^+$ T cells bearing a TCR specific for MHC I-SIINFEKL. Spleen and lymph nodes of OT-1 mice

were dissected and $CD8^+$ T cells were isolated from single-cell suspensions using the EasySep Mouse $CD8^+$ T Cell Isolation Kit (Stem Cell) according to the manufacturer's instructions. Purity was consistently >97%. The purified $CD8^+$ T cells were cultured in IMDM supplemented with 10% FCS, 1% PEST and 2 mM L-glutamine in the presence of 1 µg/ml of anti-CD3 (clone 145–2C11) and 2 µg/ml anti-CD28 (Clone-37.51, BD Pharmingen) for 24 hours. B16F1-OVA and B16F10 cells were labeled with Carboxyfluorescein succinimidyl ester (CFSC) (Thermofisher Scientific) for 15 minutes at 37°C according to the manufacturer's instructions. The CSFC-marked tumor cells were then co-cultured at various ratios with stimulated OT-1 T cells for 24 hours. The cells were then stained with live dead aqua (Thermofisher Scientific) and the frequency of dead CFSC-labeled tumor cells was determined by flow cytometry (LSR Fortessa, BD) and analyzed using FlowJo version 10.

Mice

Five- to seven-week-old female C57BL/6 J mice were purchased from Charles River. The mice had unlimited supply of food and water and were housed under specific-pathogen-free conditions at the Experimental Biomedicine (EBM) animal facility at Gothenburg university. Upon arrival to EBM, all mice underwent a one-week acclimatization period, prior to being used in experiments. The mice were randomly assigned to experimental groups. Treatments and tumor cell inoculations were performed in random order between groups. Mice were ear tagged and animals from different experimental groups were thus often hosted in the same cages. All experimentalists were aware of group allocations at all times. In most experiments, a sample size of five mice per group was utilized, as this number was found sufficient to yield significant results. One metastasis experiment and one solid tumor experiment was excluded in full, the reason being that no or very few mice got metastasis or solid tumors growing even in the unvaccinated setting in these two experiments. Even in these experiments, however, less or no tumors formed in the vaccinated animals (data not shown).

Administration of vaccines

Mice were anesthetized and treated by intranasal injections with 5 µg of the CTA1-I/II-aCD103, CTA1-II-CD103, CTA1-I/II-DD, or CTA1-M2e-DD constructs, or PBS (control) in a volume of 20 µl. Mice were immunized once a week for two consecutive weeks. To compare local and systemic vaccine administration, mice were also injected intraperitoneally with 5 µg of the CTA1-I/II-aCD103 vaccine twice with one week in between.

Metastasis model

Mice were intravenously (i.v.) injected with 100 000 B16F1-OVA or B16F10 melanoma cells by tail vein injection. For mice vaccinated in a prophylactic setting, tumor challenge was performed one week after the administration of the second vaccine dose. To study vaccine memory, tumors were injected three months after the final vaccine dose. For mice vaccinated in a therapeutic setting, tumor challenge was performed three days prior to the first dose of vaccine. In all settings, mice were sacrificed approximately 17 days after tumor cell inoculation. The number of metastases in lungs was assessed macroscopically and visible pulmonary metastatic foci were counted under a light microscope.

Solid tumor growth model

C57BL/6 J mice were anesthetized and 100 000 B16F1-OVA cells were injected to flank subcutaneous tissue. After seven days, mice were randomly assigned to be vaccinated intranasally with the CTA1-I/II-aCD103 vaccine, or PBS. Vaccination was repeated one week later. Tumor size was measured thrice weekly and calculated as length × width. Mice were sacrificed when tumor size reached the ethical limit of >1.5 cm in diameter or at the pre-determined experiment endpoint three weeks after tumor cell inoculation.

Depletion of T cell subsets

$CD8^+$ or $CD4^+$ T cells were depleted from vaccinated mice prior to and after tumor cell inoculation using an anti-mouse CD8 monoclonal antibody (mAb) (clone 53–6.7; BioXcell) or an anti-mouse CD4 mAb (clone GK1.5; BioXcell). Mice received 400 µg of mAb by intraperitoneal injection two days prior to tumor cell injection and 200 µg of mAbs four and eight days post tumor cell challenge.

T cell transfer

Mice were vaccinated twice with the CTA1-I/II-aCD103 vaccine construct with an interval of seven days. One week after the final vaccination, mice were sacrificed, and lymph nodes were harvested. Single-cell suspensions of lymph nodes were prepared, and $CD8^+$ and $CD4^+$ T cells were purified using EasySep™ Mouse $CD8^+$ T Cell Isolation Kit and EasySep™ Mouse $CD4^+$ T Cell Isolation Kit, respectively (both from Stemcell Technology, Cambridge, UK). Naïve mice were given 2 million purified $CD8^+$ T cells or 2 million $CD4^+$ T cells by intraperitoneal injections. One day after the adoptive T cell transfer, mice were challenged with 100 000 intravenously injected B16F1-OVA cells. Lungs were harvested for analysis of metastases 17 days later.

Tissue processing

Lungs harvested from mice in the metastasis models were after macroscopic tumor counts cut into small pieces and enzymatically dissociated using a lung dissociation kit (Miltenyi Biotec) along with mechanical dissociation using a gentleMACS Dissociator (Miltenyi Biotec) according to the manufacturer's instructions. Single-cell suspensions from lungs were analyzed for cytokine production by ELISPOT and phenotypic analysis was performed by flow cytometry.

Elispot

To determine the presence of OVA-specific $CD8^+$ T cells in the lungs of vaccinated mice, 300 000 cells from single-cell

suspensions of lungs were stimulated with 1 μM SIINFEKL peptide (OVA 257–264, Invivogen). Interferon-γ (IFN-γ)-producing cells were enumerated using the mouse IFN-γ single-Color ELISPOT kit (Immunospot) according to the manufacturer's instructions. To determine the frequency of OVA-specific $CD4^+$ T cells, 300 000 cells from single-cell suspensions from lungs were stimulated with 1 μM of the p323 peptide (OVA 323–339, Invivogen). Formation of IFN-γ and interleukin-17 (IL-17) was determined using the mouse IFN-γ/IL-17 double-color ELISPOT kit (Immunospot) according to the manufacturer's instructions. The number of spots formed were read by an immunospot plate reader (Immunospot S6 analyzer, CTL) using single or double color preference.

Flow cytometry

Single-cell suspensions from lungs were incubated with live dead aqua for 40 minutes at 4°C and then stained with CD8- or a CD4-based panel of antibodies. For the CD8 panel, cells were stained with anti-CD3 BUV737 (clone 17A2, BD Horizon) and anti-CD8a APC (clone 53–6.7, BD Biosciences) antibodies, washed and then stained with MHCI-SIINFEKL tetramer-PE (Proimmune). For the CD4-based panel, cells were surface-stained with anti-CD3 BUV737 (Clone 17A2) and anti-CD4 AF700 (clone RM4-5, BD Pharmingen) antibodies, followed by fixation and permeabilization (Thermofisher Scientific) and intracellular staining with an intracellular panel of antibodies comprising anti-Tbet BV786 (clone 04–46, BD Horizon), anti-Gata3 PE (clone TWAJ, Invitrogen), anti-RORγt PE (clone Q31-378, BD Horizon), and anti-Foxp3 AF647 (clone150D, Biolegend). For analysis of CD4 and CD8 memory phenotypes, cells were stained with anti-CD3 BUV737, anti-CD4 AF700, anti-CD8a APC, anti-CD103 BV421 (2E7), anti-CD69 APCCy7 (H1.2F3), anti-CD44 FITC (IM7), and anti-CD62L BV605 (MEL-14) antibodies. Cells were acquired on a BD LSR Fortessa and analyzed using FlowJo v10.

Statistics

Statistical analyses were performed using GraphPad Prism software (version 9 or later). Two-tailed unpaired Student´s *t*-test was used for comparisons between two groups and one-way ANOVA, followed by Tukey's multiple comparison test, for comparisons between three or more groups. In figures, *p*-values are designated as follows: $*p < .05$, $**p < .01$, $***p < .001$, $****p < .0001$. All *p*-values are two-sided.

Results

OVA-expressing melanoma cells are targeted by antigen-specific $CD8^+$ T cells

We first determined the ability of the melanoma cell lines B16F1-OVA and B16F10 to present the OVA epitope SIINFEKL on MHC class I. B16F1-OVA cells, but not B16F10 cells, stained positively for MHC I-SIINFEKL (Figure S1a). Incubation with SIINFEKL peptides further increased MHC I-SIINFEKL staining of B16F1-OVA and B16F10 cells, showing that both cell types present this peptide and that MHC class I loading was not saturated by endogenous SIINFEKL in B16F1-OVA cells (Figure S1a). To confirm that B16F1-OVA cells were specifically targeted by OVA-specific $CD8^+$ T cells, anti-CD3/anti-CD28-activated SIINFEKL-specific T cells isolated from TCR-transgenic OT-1 mice were co-cultured overnight with B16F1-OVA or B16F10 target cells. The OT-1-derived T cells efficiently killed B16F1-OVA cells but not B16F10 cells (Figure 1a with gating strategies in Figure S1b).

CTA1-aCD103 vaccine constructs prevent melanoma metastasis

A cDC1-targeting CTA1-aCD103 vaccine construct, containing the CTA1 adjuvant and aCD103 expressed by migratory cDC1s, was produced by multiple rounds of cloning, as described in the methods section. The immunostimulatory and anti-metastatic efficacy of the vaccine was determined in the B16F1-OVA metastasis model and compared with the efficacy of a CTA1-DD vaccine, in which the DD-region has been shown to broadly target DC.[22] The CTA1-aCD103 and CTA1-DD vaccines contained the MHC class I OVA-epitope SIINFEKL and/or the class II OVA-epitope p323 or an irrelevant epitope (the influenza virus M2e peptide). Figure 1b schematically depicts components of the fusion vaccines and the scheme of vaccination. The vaccines were administered intranasally, which previously has been shown to trigger robust induction of vaccine-specific T cells in lungs.[27]

The OVA peptide-containing vaccines CTA1-I/II-aCD103 and CTA1-I/II-DD efficiently protected C57BL/6 J mice from B16F1-OVA lung melanoma metastasis, whereas the vaccine construct containing CTA1 fused with the influenza-derived M2e peptide did not (Figure 1c). These results thus imply anti-metastatic efficacy of tumor antigens fused to the CTA1 adjuvant upon targeting DC, including the cDC1 subset. The CTA1-I/II-aCD103 vaccine triggered significantly higher levels of SIINFEKL MHC class I tetramer-positive $CD8^+$ T cells in lungs than the CTA1-I/II-DD vaccine (Figure 1d, with gatings shown in Figure S1c). We thus utilized the CTA1-aCD103 construct in further experiments. To further confirm vaccine specificity, mice received the CTA1-I/II-aCD103 vaccine followed by intravenous challenge with B16 melanoma cells that did or did not express OVA epitopes. The protection against metastasis achieved by the CTA1-I/II-aCD103 vaccine was confined to OVA-expressing tumors (Figure 1e).

Both local and systemic administration of CTA1-I/II-aCD103 vaccine protect from metastasis

To compare the antimetastatic efficacy of local and systemic vaccination, mice were immunized twice intranasally or intraperitoneally with 5 μg of the CTA1-I/II-aCD103 fusion protein, one week prior to i.v. challenge with B16F1-OVA cells. Vaccination via either route was found to exhibit strong protection from metastasis formation (Figure S1d).

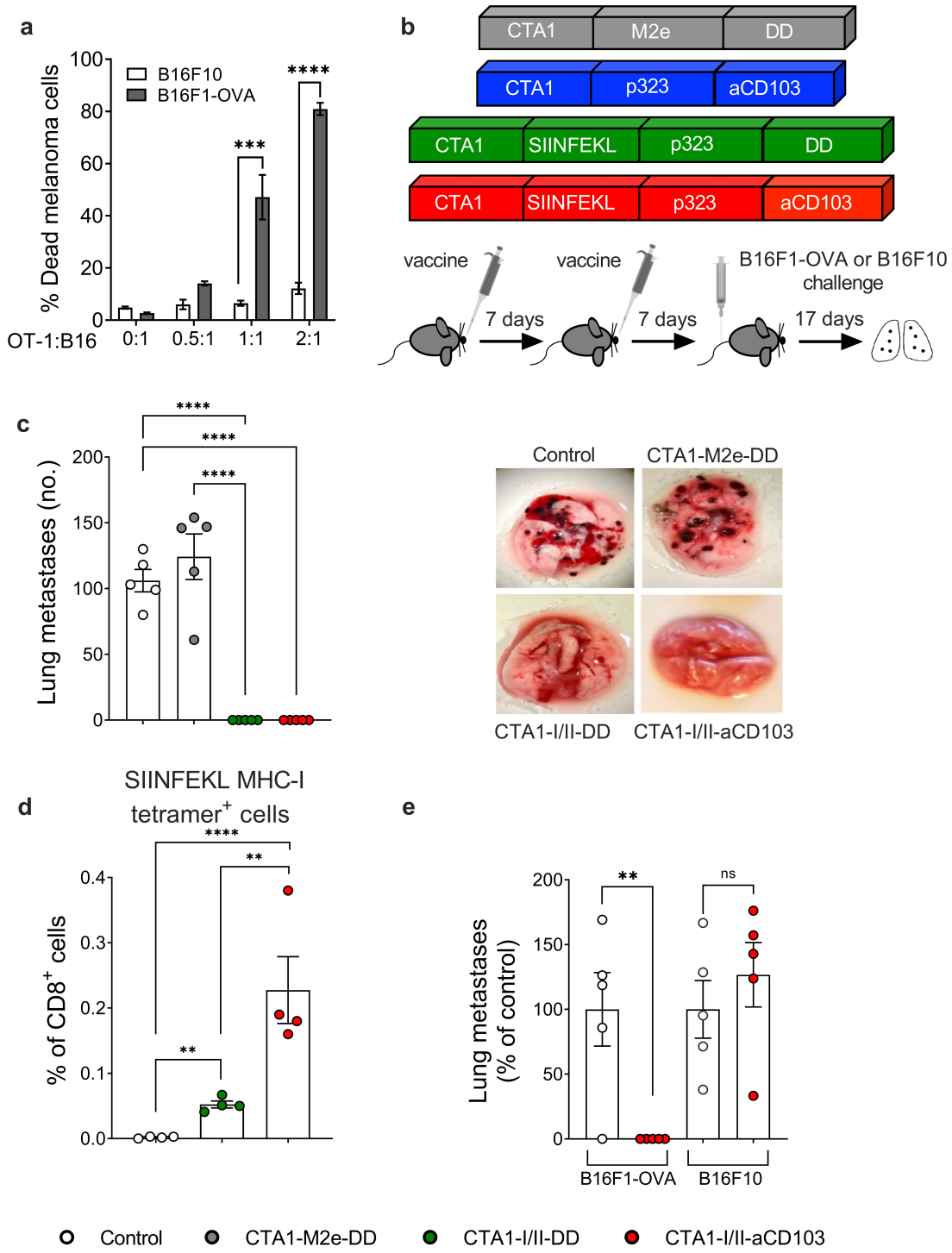

Figure 1. cDC1-targeted vaccines provide strong antigen-specific protection against OVA-expressing melanoma cells. (a) Results of microcytotoxity assays using anti-CD3/anti-CD28-activated OT-1 $CD8^+$ T effector cells and CFSE-labeled B16F1-OVA or B16F10 cells target cells at indicated effector to target cell ratios. Data are the mean T cell-mediated cytotoxicity ± s.e.m. (n = 3, Student's t-test). (b) Schematic representation of intranasal fusion vaccine constructs comprising the CTA1 adjuvant, a dimer of the D-domain of *Staphylococcus aureus* protein A (DD) or a single chain antibody against the cDC1 epitope CD103 (aCD103). The constructs were fused with the matrix protein 2 of influenza virus (M2e) or to MHC class I- and/or II-restricted epitopes expressed by B16F1-OVA melanoma cells (SIINFEKL and p323, respectively) to form: (i) an antigen-irrelevant vaccine carrying a pan-DC-targeting domain (CTA1-M2e-DD, gray), (ii) a vaccine with MHC class II OVA epitope carrying a CD103 antibody (CTA1-II-aCD103, blue), (iii) a vaccine with MHC class I and class II OVA epitopes carrying a pan-DC-targeting domain (CTA1-I/II-DD, green) and (iv) a vaccine with MHC class I and class II OVA epitopes carrying the CD103 antibody (CTA1-I/II-aCD103, red). The lower part shows the study design employed in this figure. (c) Mean number of lung metastases (± s.e.m.) formed 17 days after the intravenous inoculation of B16F1-OVA melanoma cells in mice receiving PBS (control) or indicated vaccine constructs prior to tumor cell inoculation. Statistics by ANOVA (n = 5 per group). The right part shows representative micrographs of lungs at the experimental endpoint. (d) Quantification of SIINFEKL MHC-I tetramer+ $CD8^+$ T cells by flow cytometry among single cell suspension from lungs of control mice and mice receiving the CTA1-I/II-DD vaccine or the CTA1-I/II-aCD103 vaccine as indicated in (b). Mean ± s.e.m. with statistics by ANOVA (n = 4 per group). (e) Mean number of lung metastases ± s.e.m. formed 17 days after the intravenous inoculation of B16F1-OVA or B16F10 melanoma cells in mice receiving PBS (control, open circles) or the CTA1-I/II-aCD103 vaccine (red circles) as described above in (b). (n = 5 per group, Student´s t-test).

Therapeutic vaccination reduces melanoma metastasis and growth of solid tumors

We asked if the CTA1-I/II-CD103 vaccine protected against metastasis also when administered in a therapeutic setting. Mice were vaccinated intranasally three and ten days after challenge with B16F1-OVA cells as outlined in Figure 2a. The class I/II vaccine construct significantly protected from metastasis also when administered therapeutically; however, in contrast to the complete protection against metastasis observed in the prophylactic setting (Figure 1c), low-grade metastasis was observed in 4/5 mice receiving the vaccine after tumor cell inoculation (Figure 2b).

We next determined effects of CTA1-I/II-aCD103 vaccination on solid melanoma growth. Mice received intranasal injections with the CTA1-I/II-aCD103 vaccine or PBS (control) one week after subcutaneous implantation of B16F1-OVA melanoma cells as outlined in Figure 2c. At this point of time, all mice had palpable tumors. All except one mouse responded by strikingly reduced tumor growth following therapeutic vaccination, and in 3/5 vaccinated mice tissue melanomas disappeared completely (Figure 2d). In contrast, tumors continued to grow in all five control mice (Figure 2d).

A CD4$^+$ T cell-targeting fusion vaccine partially protects against metastasis

To dissect the potential role of CD4$^+$ T cells for the efficacy of the CTA1-I/II-aCD103 vaccine construct, we compared its anti-metastatic efficacy with that of a CTA1-II-aCD103 fusion construct that only contained the CD4-restricted class II epitope p323. These vaccines were administered therapeutically, *i.e.* three and ten days after intravenous inoculation of B16F1-OVA cells. Both constructs significantly reduced lung metastasis but the class I/II vaccine was superiorly efficacious (Figure 3a). We observed a slight induction of CD8$^+$ T cells in lungs of mice receiving the CTA1-I/II-aCD103 vaccine (Figure 3b) and only this vaccine significantly induced SIINFEKL-specific T cells (Figure 3c). Furthermore, CD8$^+$ T cells from lungs of mice receiving the SIINFEKL-containing CTA1-I/II-aCD103 vaccine produced higher levels of IFN-γ in a SIINFEKL-specific ELISPOT assay compared with lung cells from control mice or CTA1-II-aCD103-vaccinated mice. However, also lung cells isolated from mice receiving the class II vaccine showed weak but significant production of IFN-γ in response to SIINFEKL (Figure 4d).

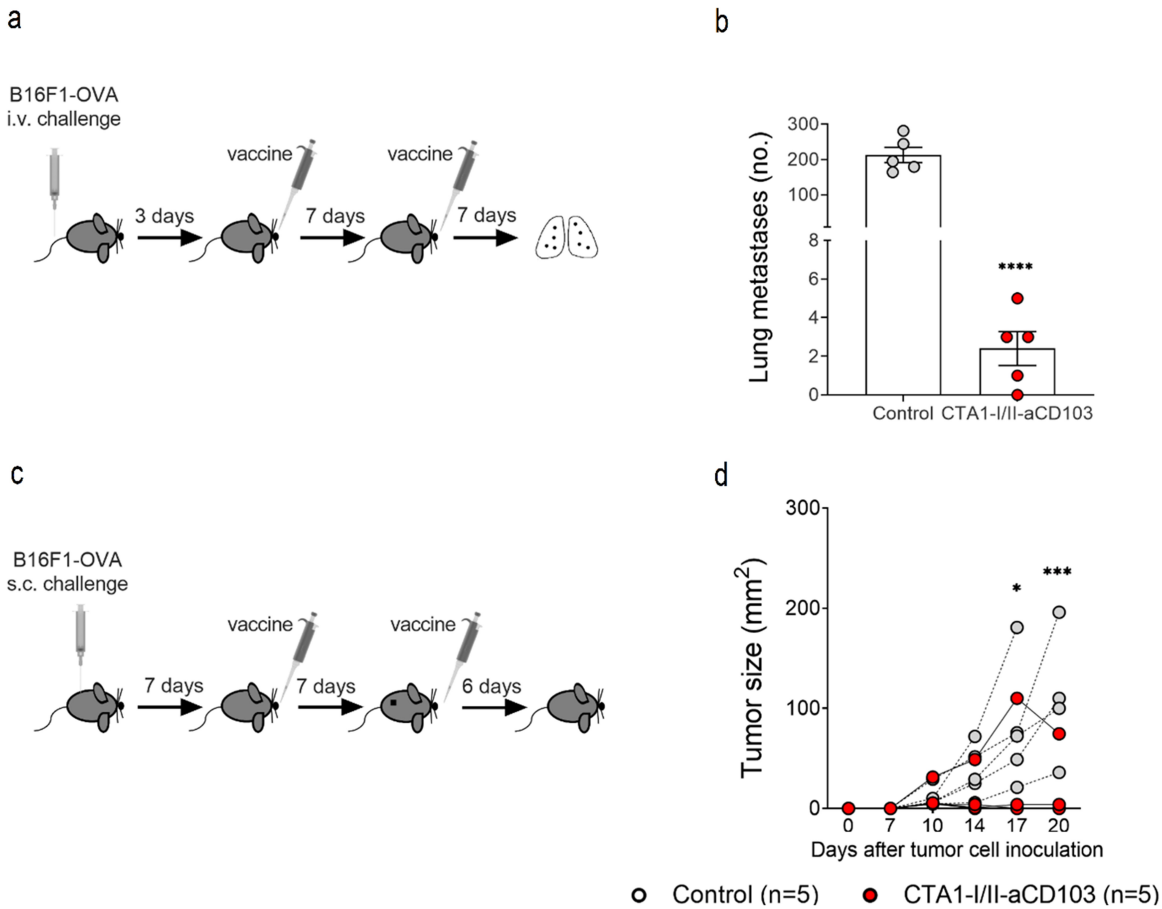

Figure 2. A cDC1-targeted vaccine reduces metastasis and tissue melanoma growth when administered in a therapeutic setting. (a) Study design in mice receiving therapeutic vaccination (at 3 and 10 days after the intravenous inoculation of B16F1-OVA melanoma cells) with the CTA1-SIINFEKL-p323-aCD103 (CTA1-I/II-aCD103) vaccine. (b) Results show the number of lung metastases (mean ± s.e.m.) formed 17 days after the intravenous inoculation of B16F1-OVA melanoma cells in mice receiving PBS (control, gray circles) or the CTA1-I/II-aCD103 vaccine (red circles) after tumor cell inoculation. Statistics by Student´s t-test (n = 5 mice per group). (c) Study design in mice carrying subcutaneously implanted B16F1-OVA melanomas. Mice received the CTA1-I/II-aCD103 vaccine at 7 and 14 days after tumor implantation. (d) Mice were subcutaneously implanted in their flank with B16F1-OVA melanomas and vaccinated as outlined in (c). Control mice (gray circles) received PBS and red circles depict mice receiving the CTA1-I/II-aCD103 vaccine intranasally. Growth of melanotic tumors was measured manually for up to 20 days or until the ethical tumor size limit was reached. Results show the growth of subcutaneous melanomas in individual mice (n = 5 mice per group) with statistics by ANOVA.

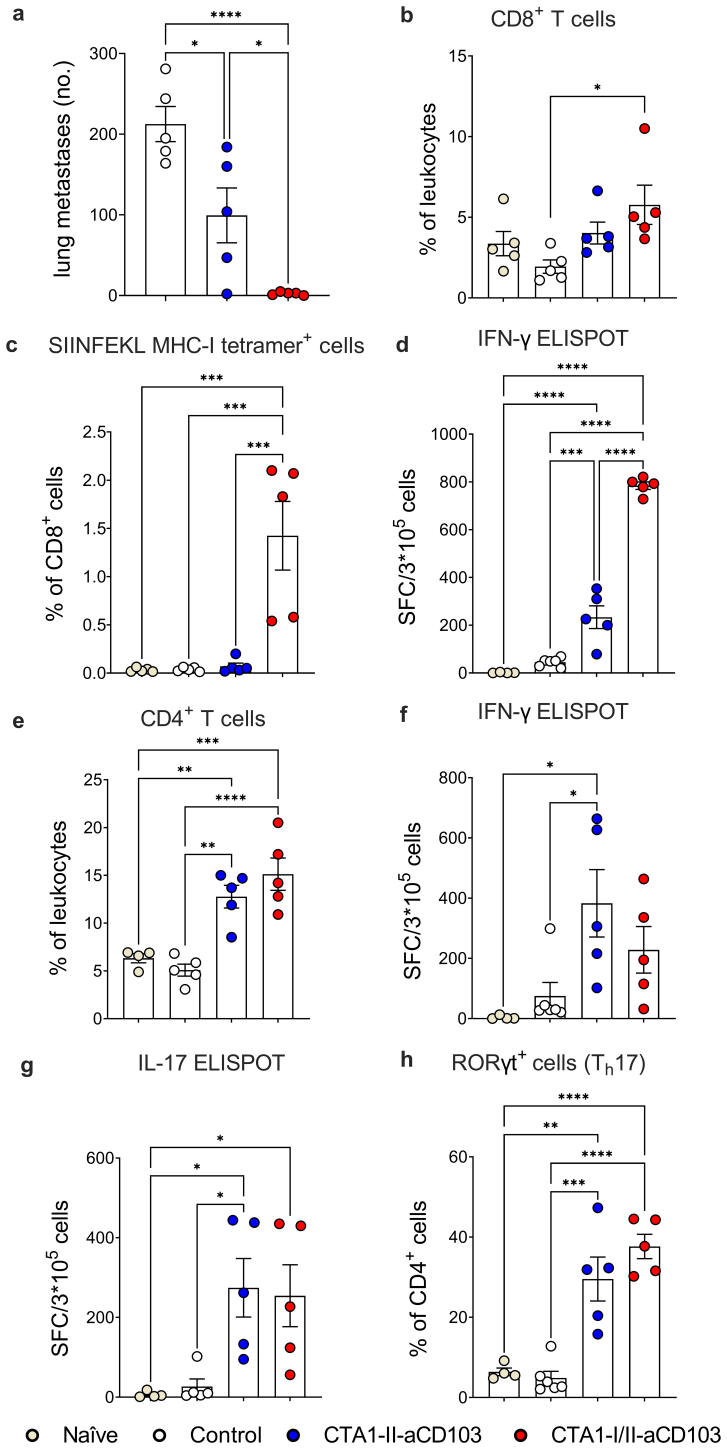

Figure 3. A CD4-biased cDC1 vaccine confers partial protection against metastasis. (a) Results show the number of lung metastases ± s.e.m. formed 17 days after the intravenous inoculation of B16F1-OVA melanoma cells in mice receiving PBS (control, open circles), the CD4-biased CTA1-p323-aCD103 vaccine (CTA1-II-aCD103, blue circles) or the CD4/CD8-biased CTA1-SIINFEKL-p323-aCD103 vaccine (CTA-I/II-aCD103, red circles) 3 and 10 days after tumor cell inoculation. Statistics by ANOVA (n = 5 mice per group). (b-d) show analyses of $CD8^+$ T cells in single-cell suspensions of lungs from naïve mice (gray circles), control tumor-bearing mice (open circles), CTA1-II-aCD103-vaccinated mice (blue circles) and CTA1-I/II-aCD103-vaccinated mice (red circles) at the experimental endpoint. Results in (b) show the % of $CD8^+$ T cells (flow cytometry); (c) shows the % of SIINFKEL tetramer MHC I^+ $CD8^+$ T cells (flow cytometry); and (d) shows the no. of SIINFEKL-specific IFN γ -producing $CD8^+$ T cells (ELISPOT). Statistics by ANOVA (n = 5 mice per group). (e-h) show corresponding analyses of $CD4^+$ T cells in lung suspensions, where (e) shows the % of $CD4^+$ T cells (flow cytometry); (f) shows the no. of p323-specific IFN γ-producing $CD4^+$ T cells (ELISPOT); (g) shows the no. of p323-specific IL 17-producing $CD4^+$ T cells (ELISPOT); and (h) shows frequency of RORγt cells (T_h17 cells) out of $CD4^+$ T cells (flow cytometry). Statistics by ANOVA (n = 5 mice per group).

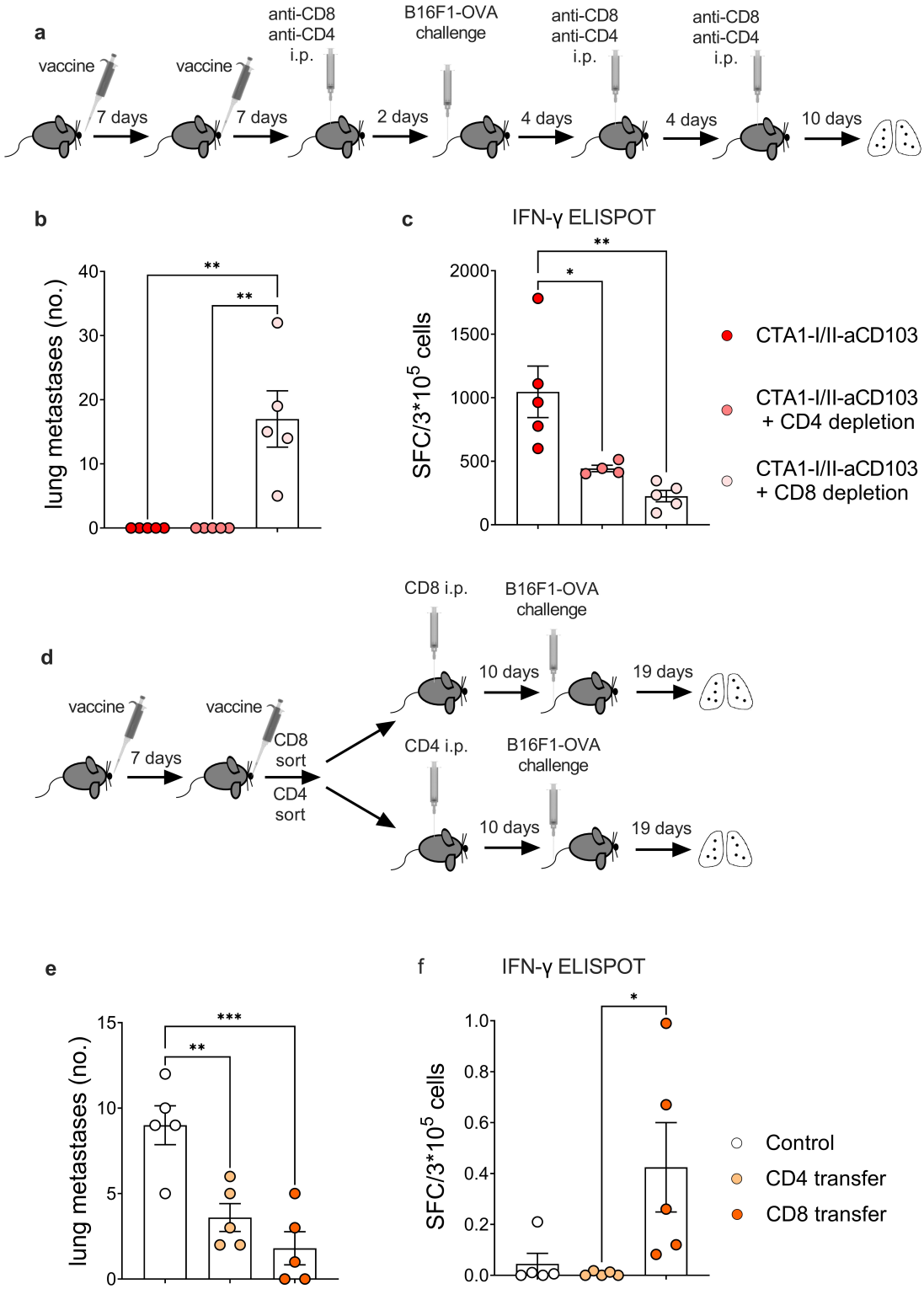

Figure 4. The anti-metastatic efficacy of the cDC1 vaccine is mediated by CD8$^+$ T cells. (a) Study design in mice receiving the CTA1-SIINFEKL-p323-aCD103 (CTA1-I/II-aCD103) vaccine followed by depletion of CD4$^+$ and CD8$^+$ T cells prior to intravenous inoculation of B16F1-OVA melanoma cells. (b) Results show the number of lung melanoma metastases ± s.e.m. formed in mice receiving the CTA1-I/II-aCD103 vaccine without T cell depletion (red circles), followed by depletion of CD4$^+$ T cells (light red circles) or followed by depletion of CD8$^+$ T cells as outlined in (A). (c) shows analysis of SIINFEKL-specific IFN-γ producing CD8$^+$ T cells (ELISPOT) in single cell suspensions of lungs from these mice (n = 5, ANOVA). (d) Study design in adoptive transfer of T cell subsets retrieved from vaccinated mice. CD8$^+$ and CD4$^+$ T cells were sorted from the lymph node of mice after administration of the CTA1-I/II-aCD103 vaccine. The sorted T cells were injected intraperitoneally to naïve mice followed by intravenous challenge with B16F1-OVA cells. (e) shows the number of lung melanoma metastases ± s.e.m. formed in mice receiving PBS (control, open circles), CD4$^+$ T cells (pink circles) or CD8$^+$ T cells (red circles). (f) shows analysis of SIINFEKL-specific IFN γ-producing T cells (mean ± s.e.m.; ELISPOT) in single cell suspensions of lungs from these animals at the experimental endpoint. Statistics by ANOVA, (n = 5 mice per group).

As expected, $CD4^+$ T cell responses were triggered in mice receiving the class I/II vaccine as well as the class II CTA1-aCD103 vaccines. Hence, both vaccines weakly enhanced the frequency of lung $CD4^+$ T cells (Figure 3e) and enhanced the frequency of OVA-specific $CD4^+$ T cells that produced IFN-γ and IL-17 in response to *ex vivo* stimulation with the p323 peptide (figure 3f-g). $CD4^+$ T cells in lungs of vaccinated mice were also analyzed for transcription factor expression profiles. Both vaccines significantly induced RORγt-expressing T_h17 cells in lung tissue (Figure 3h, Figure S2a). Only low levels of T_h1 ($Tbet^+$), T_h2 ($GATA3^+$) and T_{reg} ($Foxp3^+$) were observed in lung T cells from vaccinated mice (Figure S2b-d).

$CD8^+$ T cells are critical for vaccine efficacy with contribution also by $CD4^+$ T cells

To further decipher the relative contribution by $CD4^+$ and $CD8^+$ T cells for vaccine efficacy, mice were vaccinated with the CTA1-I/II-aCD103 vaccine followed by depletion of $CD4^+$ or $CD8^+$ T cells before and after intravenous injection of melanoma cells (Figure 4a). The efficacy of T cell depletion was confirmed in blood drawn from mice prior to tumor challenge (Figure S3). The results implied that $CD8^+$ T cells were critical for anti-metastatic efficacy. Hence, metastases were detected in the lungs of all vaccinated mice depleted of $CD8^+$ T cells whereas all mice with intact $CD8^+$ T cells were tumor-free, regardless of $CD4^+$ T cell depletion (Figure 4b). Analysis by SIINFEKL-specific ELISPOT in lung single-cell suspensions confirmed the presence of IFN γ-producing $CD8^+$ T cells in vaccinated $CD8^+$ T cell-intact animals and absence of this population in vaccinated $CD8^+$ T cell-depleted mice (Figure 4c). Of note, levels of SIINFEKL-specific IFN γ-producing $CD8^+$ T cells were significantly lower also in $CD4^+$ T cell-depleted mice (Figure 4c), suggesting that $CD4^+$ T cells may boost anti-tumor $CD8^+$ T cells in vaccinated mice.

In further experiments, $CD8^+$ T cells or $CD4^+$ T cells were sorted from spleen and lymph nodes of CTA1-I/II-aCD103-vaccinated mice. The sorted T cells were adoptively transferred to naïve mice one day prior to intravenous challenge with B16F1-OVA melanoma cells (Figure 4d). Adoptive transfer of $CD8^+$ as well as $CD4^+$ T cells from vaccinated mice significantly reduced melanoma metastasis (Figure 4e). Analysis of lung tissue confirmed the presence of SIINFEKL-specific IFN-γ producing $CD8^+$ T cells in mice receiving $CD8^+$ T cell transfer but not in mice receiving $CD4^+$ T cells (Figure 4f).

The CTA1-I/II-aCD103 vaccine triggers long-lasting memory

To study the duration of vaccine efficacy, mice received two doses of the CTA1-I/II-aCD103 vaccine and were then rested for three months prior to intravenous challenge with B16F1-OVA melanoma cells (Figure 5a). All vaccinated mice were protected against metastasis implying the development of long-lasting T cell memory after vaccination (Figure 5b). Endpoint analyses of lung cells showed the presence of SIINFEKL-responsive IFN γ-producing $CD8^+$ T cells and SIINFEKL-MHC class I tetramer-positive $CD8^+$ T cells only after vaccination with the CTA-I/II-aCD103 construct (Figure 5c,d). The SIINFEKL tetramer-positive $CD8^+$ T cells in the lungs of vaccinated mice were analyzed for memory populations and were found to contain a high fraction of $CD8^+CD44^+CD62L^-$ effector memory T cell along with a significant fraction of $CD8^+CD69^+CD103^+$ resident memory T cells. These memory cell subsets were lacking in non-vaccinated mice (Figure 5e,f, with gatings in Figure S4).

Discussion

The cDC subsets are pivotal inducers of antigen-specific immunity by virtue of their capacity to present antigen on MHC classes I and II to T cells. Several studies highlight a critical role of the cDC1 subset in tumor immunity as these cells uniquely cross-present soluble protein antigens to $CD8^+$ T cells, which are critical for the efficacy of anti-tumor immunity.[28,29] Human and murine cDC1 show phenotypic and gene transcriptional similarities and share functional characteristics, including cross-presentation of antigen to $CD8^+$ T cells.[29] Early-phase trials using cDC1-based strategies in advanced solid cancer have yielded encouraging results in terms of T cell activation and clinical efficacy.[30,31] For this study, we generated adjuvant-containing vaccine constructs aiming at targeting migratory $CD103^+$ cDC1s for efficient presentation of tumor antigens. However, also a subset of cDC2 cells express CD103[1,8,32] and may thus be targeted by these vaccines. These $CD103^+$ cDC2s are distinguished from cDC1s by CD11b-expression[1,8,32] cDC1 prime $CD4^+$ Th1 cells and $CD8^+$ T cells, the cDC2s subsets are linked to priming of CD4 T cell immunity, including T_h2, T_{fh} and T_h17 differentiation.[9,32]

In a previous study, we evaluated the relative contribution by cDC1 and cDC2 for T cell priming following administration of the CTA1-aCD103 vaccine construct. We observed that cDC1-deficient *Batf3*$^{-/-}$ mice did not mount $CD4^+$ or $CD8^+$ T cell immunity in response to these vaccine constructs. By contrast, mice deficient of $CD103^+$ cDC2 cells exhibited unperturbed adjuvant and immunogenic functions following vaccination, arguing that although aCD103-targeting may attach $CD103^+$ cDC2 cells, these are dispensable for the function of the vaccine.[23] These results thus imply that cDC1 cells are mediators of the efficacy of aCD103-CTA1 vaccine constructs and that both $CD4^+$ and $CD8^+$ T cell responses are effectively stimulated by vaccines targeting this DC subset.

Our study extends these findings by showing that CD103-targeted CTA1-based vaccine constructs also trigger anti-cancer immunity. We compared the immunostimulatory and anti-metastatic efficacy of CTA1-aCD103 and CTA1-DD vaccines with incorporated MHC class I- and class II-restricted tumor cell epitopes in a model of OVA-expressing melanoma (B16F1-OVA). While both vaccines efficiently protected against hematogenous metastasis, the aCD103-containing construct stimulated a stronger anti-SIINFEKL-specific $CD8^+$ T cell immunity, likely reflecting a more effective targeting of $CD103^+$ cDC1s.[23] These findings concur with earlier results of studies comparing $CD8^+$ T cell responses following vaccination with a pan-DC construct targeting DEC-205 with those achieved by a cDC1 construct targeting CLEC9A.[18,19]

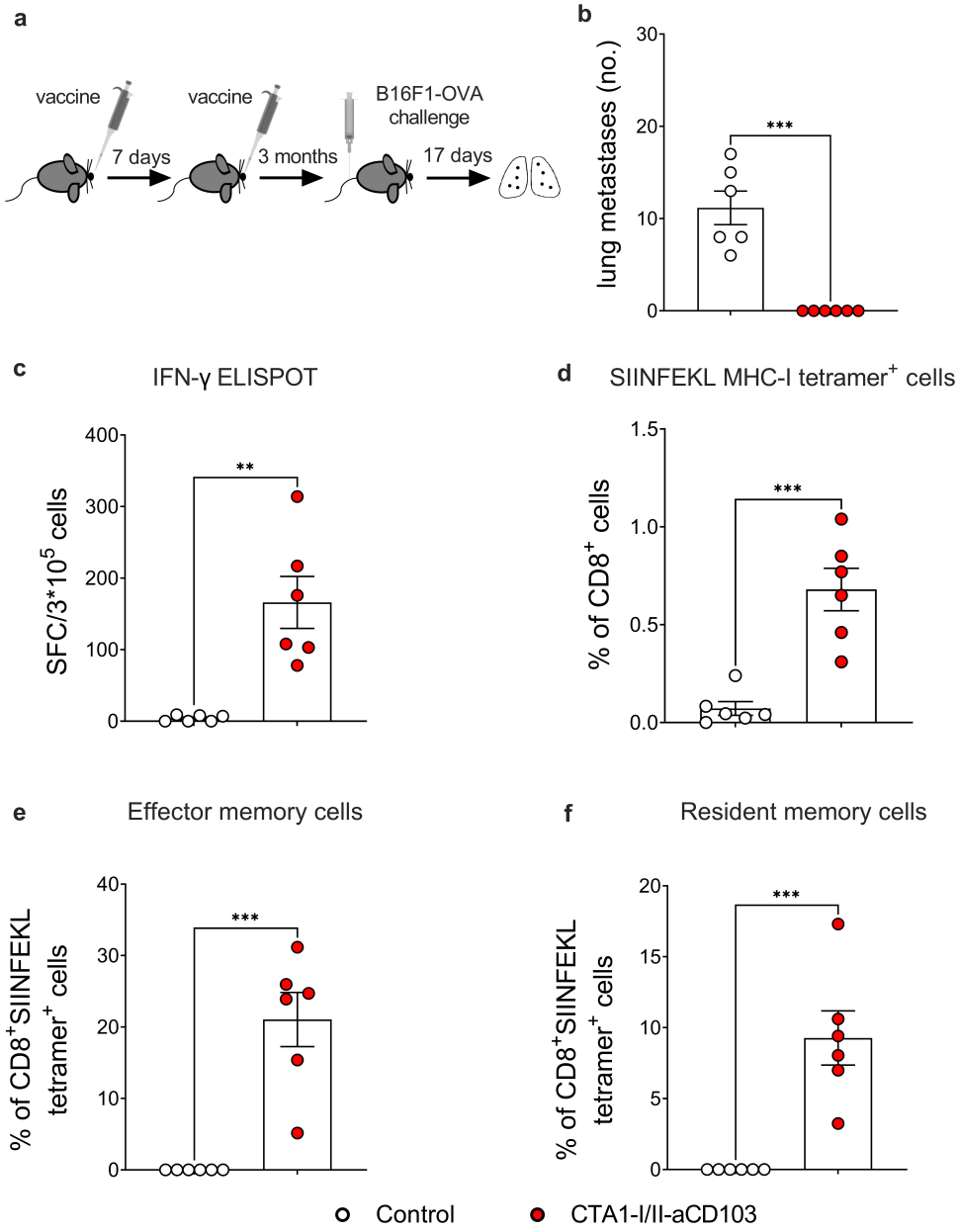

Figure 5. The CD4/CD8-biased cDC1 vaccine elicits long-lasting anti-tumor memory. (a) Study design for analyses of T cell memory after administration of the CT1A-SIINFEKL-p323-aCD103 (CTA1-I/II-aCD103) vaccine followed by a three month resting period, prior to intravenous challenge with B16F1-OVA melanoma cells. Results in (b) show the mean number of lung melanoma metastases ± s.e.m. formed in mice that had received PBS (control, open circles) or the CTA1-I/II-aCD103 vaccine (red circles) three months prior to intravenous challenge with melanoma cells as outlined in (a). Statistics by Student´s t-test (n = 6 mice). (c) shows analysis of SIINFEKL-specific IFN-γ producing $CD8^+$ T cells (ELISPOT) in single cell suspensions of lungs from these mice. (d) shows the quantification of SIINFEKL MHC-I $tetramer^+$ $CD8^+$ T cells by flow cytometry. (e) shows the frequency of cells with effector memory phenotype ($CD62L^-CD44^+$) and (f) the frequency of cells with resident memory phenotype ($CD69^+CD103^+$) among cells gated in (d) (n = 6 mice per group, Student´s t-test).

In the present study, vaccine constructs lacking OVA-peptides did not protect against B16F1-OVA metastasis, and none of the vaccine constructs protected against metastasis from B16F10 melanoma cells devoid of OVA. These findings imply antigen specificity of the observed anti-tumor responses. The induction of functional anti-tumor immunity was noted after prophylactic as well as therapeutic vaccination. Additionally, we found that intranasal immunization of mice with established solid B16F1-OVA melanomas entailed tumor growth arrest and/or decreasing tumor size. In most experiments the fusion vaccines were administrated intranasally. This route of administration was for other fusion vaccines shown to confer superior protection against challenge with airway infections, compared with systemic vaccine administration.[27] To test if the route of administration affected the antimetastatic efficacy of the CTA1-I/II-aCD103 vaccine, mice were immunized intranasally or intraperitoneally. The fusion vaccine was equally efficient in conferring protection from metastases following both routes of administration.

Mice receiving vaccine constructs exhibited strong anti-tumor immunity and harbored antigen-specific $CD8^+$ and $CD4^+$ T cells in lung tissue. The antigen-specific $CD8^+$ T cells produced enhanced levels of IFN-γ in response to the CD8-specific OVA peptide SIINFEKL whereas the expanded $CD4^+$

T cells were dominantly polarized toward T_h17 as indicated by RORγt expression and IL-17 production to recall antigen *in vitro*. Although the potential anti-tumor function of $CD4^+$ T_h17 cells is controversial,[33] these findings support the notion that T_h17 cells may confer protection against melanoma metastasis, which concurs with earlier studies showing that T_h17 cells may directly eliminate tumor cells and also recruit natural killer cells and $CD8^+$ T cells to the site of tumor growth.[34–37]

We utilized an aCD103-based vaccine that only contained the MHC class II-restricted OVA epitope p323 (CTA1-II-aCD103) with a view of determining the relative contribution by vaccine-induced $CD8^+$ and $CD4^+$ T cell subset for anti-metastatic efficacy. Although the efficacy of the class I/II vaccine was consistently superior, the vaccine only containing the class II OVA-epitope conferred significant protection against metastasis (Supplementary Fig. 5). To further clarify the contribution by $CD8^+$ and $CD4^+$ T cell subsets, we performed T cell subset depletion and T cell subset transfer experiments. The results consistently showed that the availability of $CD8^+$ T cells was critical for vaccine efficacy, but also that $CD4^+$ T cells significantly contributed in protection against metastasis. These findings are in agreement with previous studies showing that MHC class II epitopes, that stimulate $CD4^+$ T cells, augment tumor cell rejection mediated by $CD8^+$ T cells,[11,14] but also imply that $CD4^+$ T cells participate in anti-tumor immunity beyond conferring $CD8^+$ T cell help.

The anti-metastatic efficacy of the CTA1-I/II-aCD103 vaccine was long-lasting, and enhanced levels of effector memory T cells were detectable in lungs for at least 3 months after vaccination. While our results do not formally exclude contribution by $CD103^+$ cDC2 cells for the observed anti-tumor efficacy, the lack of T cell activation by the CTA1-aCD103 vaccine in *Batf3*$^{-/-}$ mice[23] suggest that the anti-tumor $CD4^+$ and $CD8^+$ T cell responses result from the targeting of $CD103^+$ cDC1. These findings thus support and extend earlier studies suggesting a pivotal role for cDC1 in the development of durable and functional T cell responses against tumor cell epitopes.[38–40]

In this study we show that CTA1-aCD103 fusion vaccines containing epitopes of the model antigen OVA protect against metastasis and reduced solid tumor growth of OVA-expressing B16-melanoma cells. Previous vaccine studies show that the OVA model antigen triggers stronger immunity compared with neoantigens,[41,42] where regulatory T cells and other means of inhibition may limit priming efficacy. Hence, the protective effect of a neoantigen-containing CTA1-aCD103 vaccine remains to be determined, as well as protective effects in other tumor models.

In conclusion, cDC1-targeted vaccine constructs fused to a potent adjuvant effectively stimulated tumor antigen-specific $CD8^+$ and $CD4^+$ T cell immunity in lungs, which protected against lung metastasis. Local and systemic administration of vaccine both protected against lung metastasis, and local vaccine administration efficiently reduced solid tumor growth. It remains to be investigated whether systemic vaccine administration may be superior in stimulating protective immunity against melanoma metastasis at other common loci than the lungs. Future studies should also address the ability of the CTA1-aCD103 fusion vaccine to trigger protective immunity against neoantigen and in other models of melanoma. This study may inspire further exploration of immunization using cDC1-targeting vaccines with a fused adjuvant component in cancer immunotherapy.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

All data needed to evaluate the conclusions in the paper are present in the main text and figures. Questions regarding availability of material should be addressed to author Nils Lycke at nils.lycke@microbio.gu.se.

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