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ORIGINAL ARTICLE

Concomitant gemcitabine therapy negatively affects DC vaccine-induced CD8$^+$ T-cell and B-cell responses but improves clinical efficacy in a murine pancreatic carcinoma model

Christian Bauer · Alexander Sterzik · Franz Bauernfeind · Peter Duewell · Claudius Conrad · Rosemarie Kiefl · Stefan Endres · Andreas Eigler · Max Schnurr · Marc Dauer

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Abstract
Background Multiple studies have shown that dendritic cell (DC)-based vaccines can induce antitumor immunity. Previously, we reported that gemcitabine enhances the efficacy of DC vaccination in a mouse model of pancreatic carcinoma. The present study aimed at investigating the influence of gemcitabine on vaccine-induced anti-tumoral immune responses in a syngeneic pancreatic cancer model.
Materials and methods Subcutaneous or orthotopic pancreatic tumors were induced in C57BL/6 mice using Panc02 cells expressing the model antigen OVA. Bone marrow-derived DC were loaded with soluble OVA protein (OVA-DC). Animals received gemcitabine twice weekly. OVA-specific CD8$^+$ T-cells and antibody titers were monitored by FACS analysis and ELISA, respectively.
Results Gemcitabine enhanced clinical efficacy of the OVA-DC vaccine. Interestingly, gemcitabine significantly suppressed the vaccine-induced frequency of antigen-specific CD8$^+$ T-cells and antibody titers. DC migration to draining lymph nodes and antigen cross-presentation were unaffected. Despite reduced numbers of tumor-reactive T-cells in peripheral blood, in vivo cytotoxicity assays revealed that cytotoxic T-cell (CTL)-mediated killing was preserved. In vitro assays revealed sensitization of tumor cells to CTL-mediated lysis by gemcitabine. In addition, gemcitabine facilitated recruitment of CD8$^+$ T-cells into tumors in DC-vaccinated mice. T- and B-cell suppression by gemcitabine could be avoided by starting chemotherapy after two cycles of DC vaccination.
Conclusions Gemcitabine enhances therapeutic efficacy of DC vaccination despite its negative influence on vaccine-induced T-cell proliferation. Quantitative analysis of tumor-reactive T-cells in peripheral blood may thus not predict vaccination success in the setting of concomitant chemotherapy.

Keywords Vaccination · Dendritic cells · Pancreatic carcinoma · Chemotherapy · Gemcitabine · Survival

Christian Bauer and Alexander Sterzik, and Max Schnurr and Marc Dauer have contributed equally to this article.

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C. Bauer · A. Sterzik · F. Bauernfeind · P. Duewell · R. Kiefl · M. Schnurr
Section of Gastroenterology, Medizinische Klinik und Poliklinik IV, University of Munich, Munich, Germany

C. Conrad
Massachusetts General Hospital, Harvard University, Boston, MA, USA

S. Endres
Division of Clinical Pharmacology, Medizinische Klinik und Poliklinik IV, University of Munich, Munich, Germany

A. Eigler
Department of Internal Medicine I, Klinikum Dritter Orden, Munich, Germany

M. Dauer (✉)
Department of Medicine II, Kliniken St. Elisabeth, Müller-Gnadenegg-Weg 4, 86633 Neuburg an der Donau, Germany
e-mail: marc.dauer@kliniken-st-elisabeth.de

Abbreviations

CFSE	Carboxyfluorescein succinimidyl ester
CTL	Cytotoxic T-cell
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FoxP3	Forkheadbox P3
Gem	Gemcitabine
GM-CSF	Granulocyte macrophage colony-stimulating factor
IFN-γ	Interferon-γ
i.v.	Intravenous
i.p.	Intraperitoneal
ICS	Intracellular staining
LPS	Lipopolysaccharide
MHC-I	Major histocompatibility complex I
MDSC	Myeloid-derived suppressor cell
OD	Optical density
OVA	Ovalbumine
OVA-DC	OVA protein-loaded DC
p15E	Retroviral protein expressed by Panc02 cells
s.c.	Subcutaneous
SIINFEKL	Immunodominant MHC-I epitope of the ovalbumine protein
TNF-α	Tumor necrosis factor-α
T_{reg}	Regulatory $CD4^+$ $Foxp3^+$ T-cell
TRP2	Tryosinase-related peptide 2
U-DC	Unloaded but LPS-stimulated DC

Introduction

Chemotherapy with gemcitabine is currently considered standard treatment for patients with advanced pancreatic carcinoma, yet resulting in an only moderate increase in survival time compared to 5-fluorouracil treatment [1]. Phase-III trials using gemcitabine in combination with other chemotherapeutic drugs have shown only limited additional benefits [2]. Recently, a combination therapy using oxaliplatin, irinotecan, fluorouracil and leucovorin (FOLFIRINOX) was shown to be superior to gemcitabine in patients with metastatic pancreatic adenocarcinoma. However, therapy was associated with a high incidence of side effects, limiting its use to patients with excellent performance status [3].

There is a need for novel strategies for the treatment of patients with advanced pancreatic cancer, such as immunotherapy. It has been shown that tumor infiltration with $CD8^+$ cytotoxic T-cells (CTL) and $CD4^+$ T helper (Th) cells represent independent favorable prognostic factors for patients with pancreatic adenocarcinoma [4]. Furthermore, functional tumor-reactive T-cells capable of tumor rejection in vitro and in vivo have been isolated from the blood of pancreatic carcinoma patients [5]. These tumor-reactive T-cell responses can be enhanced by vaccination with tumor antigen-loaded dendritic cells (DCs) [6]. DC vaccination has been shown to induce tumor regression in some cancer patients [7]. Recently, we have shown that antitumor immune responses induced by DC vaccination correlated with better clinical outcome in patients with advanced pancreatic cancer [6].

However, tumor escape mechanisms can render cancer cells insensitive toward CTL-mediated lysis. Combination with other treatment strategies, such as irradiation or chemotherapy, may decrease immune resistance of cancer cells [8]. Our group demonstrated that combination of DC vaccination with gemcitabine increased survival in a murine pancreatic carcinoma model [9]. Gemcitabine has been demonstrated to augment efficacy of other therapeutic strategies such as in vivo CD40 activation [10–12]. Novak et al. [13] were able to show that gemcitabine increases antigen uptake from apoptotic tumor cells by local DCs. Suzuki et al. [14, 15] demonstrated that a population of $CD11b^+Gr\text{-}1^+$ cells with immune suppressive characteristics, termed myeloid-derived suppressor cells (MDSC), is selectively reduced by gemcitabine. These findings indicate that gemcitabine-based chemotherapy may synergize with immunotherapy under certain circumstances.

Little is known about the immunological interplay of DC vaccination and chemotherapy. In this study, we aimed at investigating the influence of gemcitabine on immune responses induced by DC vaccination in a syngeneic pancreatic carcinoma model and to correlate these findings with therapeutic outcome. Furthermore, we aimed to investigate specific treatment variables such as the mode of vaccine application and timing of chemotherapy administration. The purpose of these studies was to facilitate the development of future clinical trials investigating the role of DC vaccination in the treatment of pancreatic cancer.

Materials and methods

Mice

Female 8–10-week-old C57BL/6 mice were purchased from Harlan Laboratories (Borchen, Germany). Animal studies were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany). OT-1 mice were kindly provided by Prof. Thomas Brocker (Department of Immunology, University of Munich, Germany). $CD8^+$ T-cells of transgenic OT-1 mice possess a T-cell receptor specific for the ovalbumin (OVA) epitope SIINFEKL presented on MHC-I haplotype $H2\text{-}K^b$ [16, 17].

Cell lines

The murine pancreatic adenocarcinoma cell line Panc02 is derived from a 3-methylcholanthrene-induced tumor in a C57BL/6 female mouse [18, 19]. Panc02 cells expressing the model antigen Ovalbumin (PancOVA) were generated as described and selected in media containing G418 (Sigma, Hamburg, Germany) [20]. Original and OVA-transfected cell lines showed identical growth kinetics.

Peptides

H2-K^b-restricted peptides were synthesized by Jerini Peptide Technologies (Berlin, Germany) according to data from the literature: $OVA_{257\text{-}264}$ with peptide sequence SIINFEKL [21], $TRP2_{181\text{-}188}$ with sequence VYDFFVWL [22] and $p15E_{604\text{-}611}$ with sequence KSPWFTTL [23]. The p15E protein is part of the murine leukemia virus (MuLV) envelope protein that is expressed by Panc02 and thus represents a tumor-associated antigen. The tyrosinase-related-protein-2 (TRP2) epitope was used as negative control peptide for T-cell stimulation assays.

Reagents

Fluorochrome-labeled antibodies against CD11b (clone M1/70), CD11c (clone HL3), CD8 (clone 53-6.7), CD4 (clone GK1.5), CD86 (clone B7-2), Gr-1 (clone RB6-85C) and NK1.1 (clone PK136) were purchased from BD Biosciences (San Jose, CA, USA). Anti-CD25 (clone PC61.5) and anti-foxp3 (clone FJK-16s) were from eBioscience (San Diego, CA, USA). Antibodies against IFN-γ (clone XMG1.2) were purchased from Caltag (Burlingame, CA, USA). Gemcitabine (Gemzar) was purchased from Lilly (Indianapolis, Indiana). Granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) were purchased from PeproTech (London, UK). LPS, OVA protein and tumor necrosis factor-α (TNF-α) were from Sigma-Aldrich (Steinheim, Germany).

Generation and antigen-loading of bone marrow-derived DCs

Bone marrow-derived DCs were prepared as described [24]. Immature DCs were loaded with 1 mg/mL OVA protein. After stimulation with 300 ng/ml LPS, cells were harvested. Percentage and maturation status of DCs were examined by flow cytometry.

Tumor inoculation, monitoring of tumor growth and therapy

For subcutaneous tumor cell inoculation, mice were anesthetized with isoflurane (Abbott, Illinois, USA) and 10^6 PancOVA cells resuspended in a volume of 100 µl PBS were injected into the flank. Tumor growth was determined by caliper. Mice were killed when tumor size exceeded 150 mm^2. For orthotopic tumor induction, mice were anesthetized and the left flank was opened under sterile conditions. The spleen was mobilized to access the pancreas. A total of 2×10^5 PancOVA cells were injected in a volume of 20 µl PBS into the pancreas. Peritoneum and skin were occluded using sutures. For experiments in tumor-free mice, gemcitabine doses of 25, 50 or 75 mg/kg body weight were used as indicated. Gemcitabine was administered intraperitoneally twice weekly (at days 2 and 5 after DC vaccination) at a dose of 50 mg/kg for subcutaneous tumor experiments or 75 mg/kg body weight for orthotopic tumor experiments.

Isolation of immune cell populations

Mice were killed by cervical dislocation. Subcutaneous tumors, tumor-draining inguinal lymph nodes and spleens were removed for further ex vivo analysis. Tumors were dissected using razor blades and digested with 1.5 mg/ml collagenase D and 50 U/ml DNAse (both from Roche) for 1 h at 37 °C.

Intracellular IFN-γ staining and MHC-I pentamer staining

Splenocytes or blood leukocytes were plated in 96-well plates. Samples were stimulated with SIINFEKL, p15E or TRP2 peptide (negative control). Four hours after incubation at 37 °C, 0.1 µg/ml brefeldin A was added to the samples. Four h later, plates were washed and cells were stained with anti-CD8 mAb, then with anti-IFN-γ after permeabilization with 0.5 % saponin for 15 min. The percentage of $CD8^+$ T-cells expressing IFN-γ was determined by flow cytometry. Staining with Pro5-OVA257-264-H2-K^b-PE pentamer was performed for 15 min at room temperature in the dark. After washing, cells were stained with anti-CD8 mAb and the percentage of antigen-specific $CD8^+$ T-cells was analyzed by flow cytometry (FACSCalibur, Becton–Dickinson, San Jose, CA, USA).

Detection of OVA-specific antibodies by ELISA

Ninety-six-well plates were coated overnight with 10 µg/mL OVA in PBS and blocked 1 h with 1 % BSA in PBS. After incubation of serum samples for 1 h, plates were washed with PBS/Tween 20 and goat anti-mouse IgG, IgG1, or IgG2a, conjugated to horse-radish peroxidase (HRP, Southern Biotechnology Laboratories, Birmingham, AL, USA), was added at 1 µg/mL for 1 h. Plates were washed again and the assay was developed by *o*-phenylenediamine (Sigma-Aldrich). Reaction was

stopped by addition of 1 M H_2SO_4 and optical density (OD) was measured. Measurements were performed in triplicates.

In vivo cytotoxicity assay

Splenocytes from C57BL/6 mice were divided into two populations and loaded with either 2 μg/ml SIINFEKL peptide for one h at 37 °C or no peptide. Cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) at a concentration of 2 μM for peptide-pulsed cells and 0.2 μM for unpulsed cells. After extensive washing, both populations were mixed at a ratio of 1:1 and 10^7 cells were injected via the tail vein. 20 h after injection, spleen and inguinal lymph nodes were removed and analyzed by flow cytometry for the presence of $CFSE^{high}$ versus $CFSE^{low}$ cells. Specific lysis in vaccinated mice was calculated: $\%\,\text{specific lysis} = 1 - \{(\text{CFSE}^{\text{high}}\text{vacc}/\text{CFSE}^{\text{low}}\text{vacc})/(\text{CFSE}^{\text{high}}\text{con}/\text{CFSE}^{\text{low}}\text{con})\}$.

OT-1 in vivo proliferation assay

Spleen and lymph nodes of OT-1 mice were removed, pooled into a single-cell suspension and labeled with 5 μM CFSE in PBS/0.01 % BSA for 20 min. One week after i.p. OVA-DC vaccination, 10^7 CFSE-labeled OT-1 cells were injected i.v. in a volume of 100 μl PBS. Seven days after adoptive transfer, proliferation of CFSE-labeled $CD8^+$ OT-1 cells was determined by flow cytometry.

^{51}Chromium-release cytotoxicity assay

Panc02 or PancOVA cells were used as target cells. Tumor cells were incubated for 10 h in the absence or presence of 10 nM or 100 nM gemcitabine. Target cells were incubated with $Na_2^{51}CrO_4$ (Hartmann Analytic, Braunschweig, Germany) (100 μCi/10^6 target cells) at 37 °C for 1 h. Cells were washed three times and 5×10^3 target cells/well were cocultured with effector cells in 96-well round-bottomed plates in a volume of 200 μl in different ratios. After 4-h incubation at 37 °C, 50 μl of supernatant was harvested and radioactivity determined by a gamma counter (Wallac Oy, Turku, Finland). Samples were processed in triplicates. Maximum release was defined as the mean cpm released from triplicates of 5×10^3 cells incubated in medium containing 2.5 % Triton X (Sigma, Munich, Germany). Spontaneous release was defined as the mean cpm released from labeled target cells in the absence of effector cells. Specific lysis was calculated according to the formula: specific ^{51}Cr release = {(cpm of sample − cpm of spontaneous release)/(cpm of maximum release − cpm of spontaneous release)} × 100 %.

Statistics

All data are presented as mean ± SEM. Student's *t* test was applied to reveal significant differences. A value of $P < 0.05$ was accepted as the level of significance. Tumor growth kinetics was tested for differences in linear regression curves of tumor growth using an analysis of variance (ANOVA) test model. Survival curves were analyzed using the Cox proportional hazards model. Statistical analyses were performed using Prism software (version 5.0f, GraphPad Software, La Jolla, CA, USA).

Results

Gemcitabine suppresses DC vaccine-induced T- and B-cell responses

The induction of SIINFEKL-specific $CD8^+$ T-cells in mice vaccinated with OVA-DC was analyzed by pentamer staining and IFN-γ ICS assay. Immune responses correlated significantly with the number of DCs administered (data not shown). Different vaccination routes were tested for their ability to mount an immune response (Fig. 1a). Intravenous (i.v.) and intraperitoneal (i.p.) injections were superior to subcutaneous (s.c.) injections of DCs in regards to $CD8^+$ T-cell priming. For anti-OVA antibody responses, no significant differences were observed for all three vaccination routes. To study the effect of gemcitabine on DC-induced OVA-specific immune responses, mice were immunized two times in weekly intervals with 2×10^6 OVA-DC either s.c., i.p. or i.v. Animals received 75 mg/kg body weight gemcitabine i.p. at days 2 and 5 after DC vaccination. Gemcitabine significantly reduced the frequency of OVA-specific T-cells and antibody titers for all three vaccination routes (Fig. 1b, c). Dose reduction in gemcitabine to 25 mg/kg body weight per mouse did not diminish the level of immune suppression (Suppl. Fig. 1a-d, online resource). As the i.p. route for DC administration was more effective than the s.c. route, we decided to vaccinate mice i.p. in subsequent experiments.

To test the influence of gemcitabine on DC-induced recall T-cell responses in a vaccination setting, mice that had been vaccinated with OVA-DC 6 months earlier were tested for the presence of antigen-specific memory T-cells (Fig. 1d). Animals were divided into three groups with similar OVA-specific $CD8^+$ T-cell frequencies. Two groups were re-vaccinated with OVA-DC. One of these two groups received concomitant gemcitabine therapy at a dose of 50 mg/kg body weight. Mice of the group that was not re-vaccinated received gemcitabine alone. Immunomonitoring by IFN-γ ICS assay was performed 7 days after re-vaccination. A single re-vaccination with OVA-DC led to

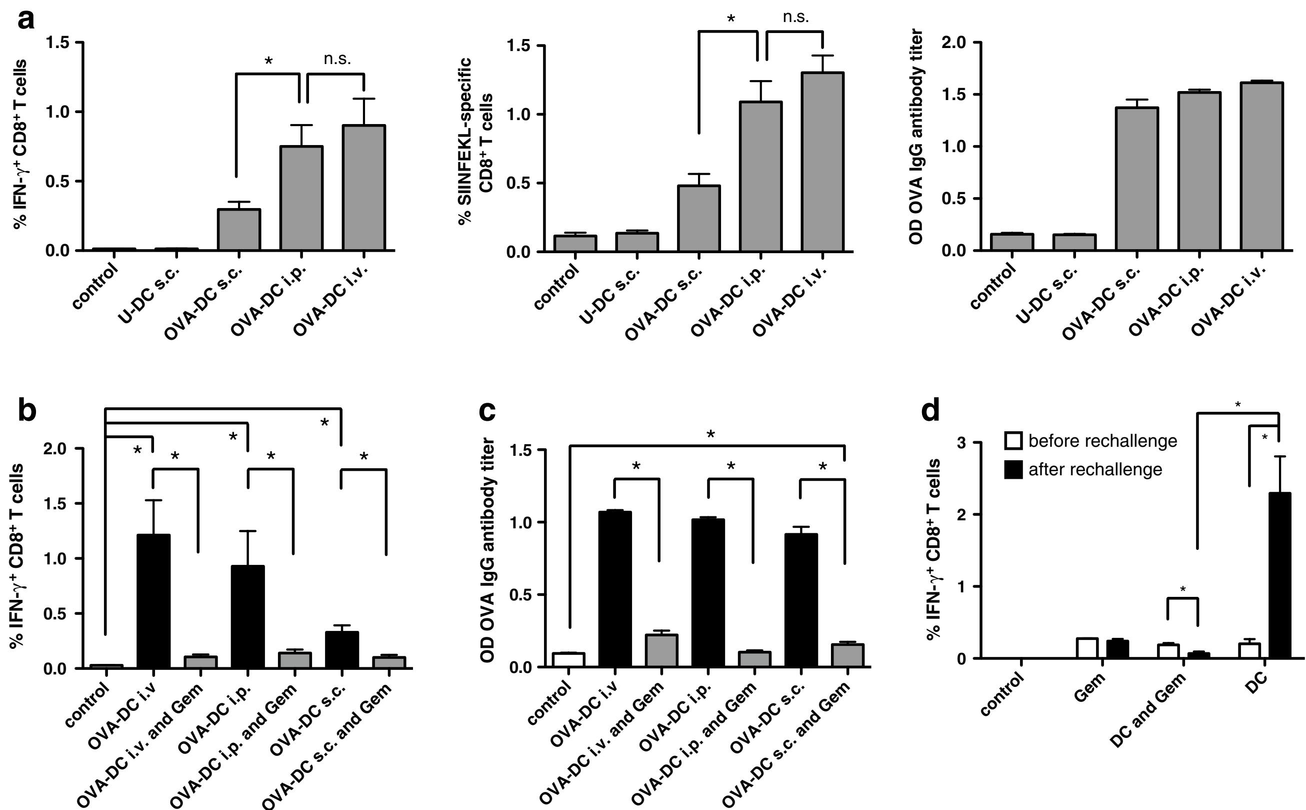

Fig. 1 Gemcitabine impairs OVA-DC vaccination-induced antigen-specific T- and B-cell responses. **a** Mice were vaccinated s.c., i.p. or i.v. with 2×10^6 OVA-DC two times in weekly intervals. Control mice were vaccinated s.c. with LPS-activated DCs that had not been loaded with OVA (U-DC). Immunomonitoring was performed 7 days after the second vaccination from peripheral blood by IFN-γ ICS (*left*) and pentamer staining (*middle*). Induction of Ag-specific B-cell responses was determined by anti-OVA antibody ELISA (*right*). Data represent one of the two independent experiments ($n = 8$ mice per group). $*P < 0.05$. **b** Mice were vaccinated twice by s.c., i.p. or i.v. OVA-DC injection in weekly intervals. Subgroups received gemcitabine i.p. twice weekly for a total of four times. The frequency of OVA-specific $CD8^+$ T-cells was assessed by IFN-γ ICS. **c** OVA-specific B-cell responses after DC vaccination and concomitant gemcitabine treatment were analyzed by ELISA. Data represent one of the three independent experiments ($n = 5$ per group). **d** Mice having received OVA-DC vaccination 6 months earlier were split into three groups and rechallenged once with gemcitabine alone, OVA-DC with concomitant gemcitabine therapy or OVA-DC alone. Frequency of OVA-specific CTL was analyzed by IFN-γ ICS. The *graph* represents one of the two independent experiments ($n = 3$ per group)

the induction of a profound T-cell response (2.29 ± 0.63 % compared to 0.21 ± 0.11 % before re-vaccination, $P = 0.02$). Gemcitabine significantly inhibited induction of a recall response. Gemcitabine alone had no influence on the frequency of antigen-specific T-cells. This finding indicates that gemcitabine might act particularly on T-cells during the proliferative phase of an ongoing immune response.

Characterization of DC vaccine-induced anti-tumoral immune responses in the subcutaneous PancOVA tumor model

Previously, we reported a synergistic therapeutic effect of combined gemcitabine and DC vaccination in the subcutaneous Panc02 model [9]. To characterize DC vaccine-induced immune responses against tumor-associated antigens, we injected Panc02 cells expressing the model antigen ovalbumin (PancOVA) into the flank of mice that received or did not receive OVA-DC starting 7 days after tumor inoculation. The frequencies of $CD8^+$ T-cells specific for the OVA epitope SIINFEKL or a tumor-specific but vaccine-unrelated epitope, p15E, were measured in peripheral blood. As expected, mice of the OVA-DC plus gemcitabine group had a significantly better therapeutic outcome than mice in the groups receiving either OVA-DC or gemcitabine alone (Fig. 2a, $P < 0.001$, OVA-DC vs. OVA-DC and Gem). Vaccination with DCs that were not loaded with OVA showed no therapeutic efficacy, indicating that the vaccine response was antigen-specific in the OVA-DC group ($P < 0.001$, OVA-DC vs. U-DC). Survival analysis using a Cox proportional hazards model confirmed differences between U-DC- and OVA-DC-treated mice ($P < 0.01$) as well as between mice treated with

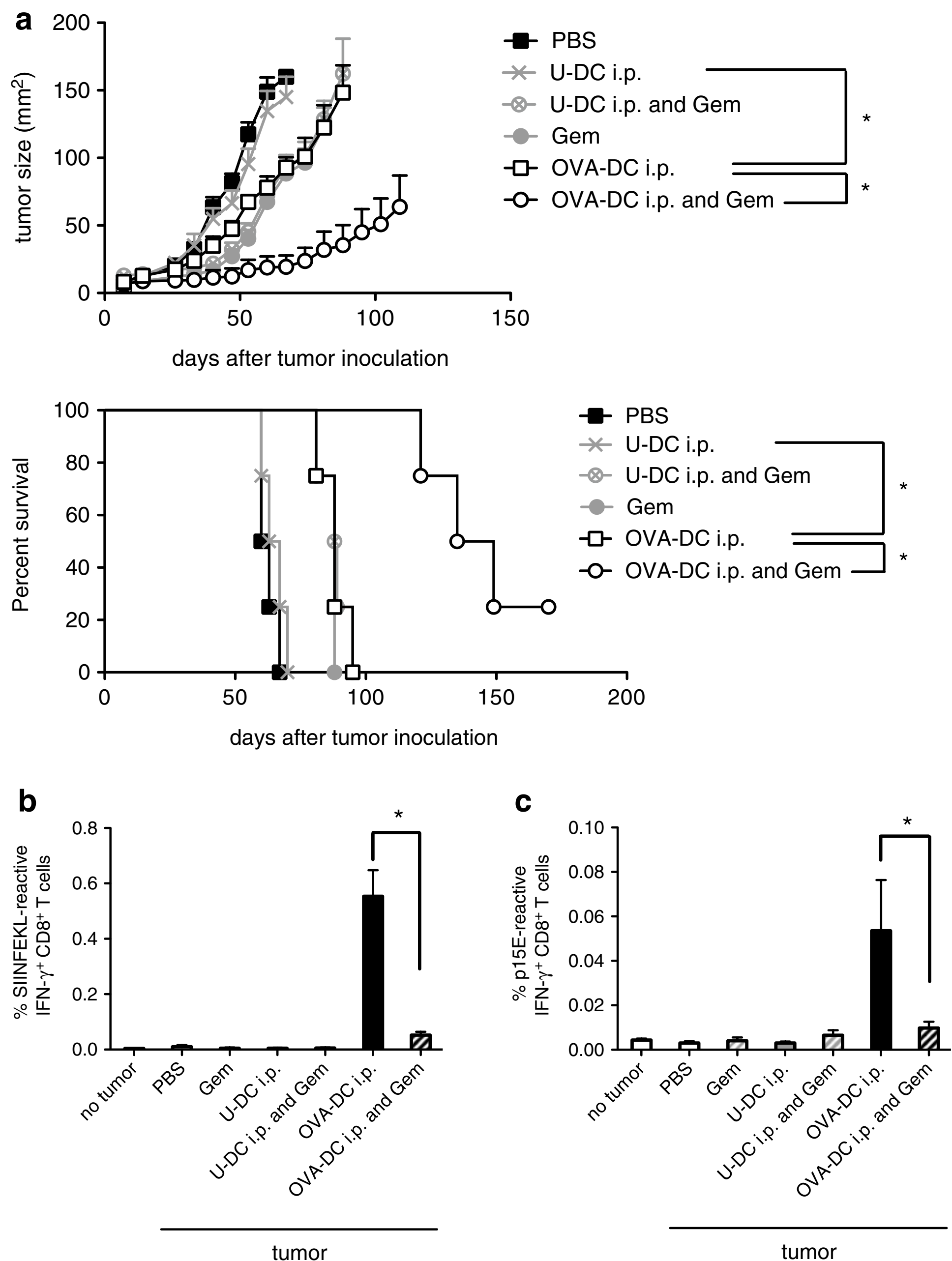

Fig. 2 Characterization of antitumor immune responses and treatment efficacy induced by gemcitabine-based chemoimmunotherapy in the subcutaneous PancOVA tumor model. **a** Therapy was started 1 week after induction of s.c. PancOVA tumors by administering either OVA-DC, LPS-activated unloaded DC (U-DC) or PBS with a total of six vaccinations. Subgroups received concomitant gemcitabine therapy on days 2 and 5 after DC injection. The *graph* depicts mean tumor burden in mm^2 + SEM (*top*, $n = 4$ mice per group). Kaplan–Meier survival curves demonstrate treatment efficacy (*bottom*). Mice that had developed tumors >150 mm^2 were killed. One of the two independent experiments with similar results is shown. **b**, **c** Tumor-specific CD8$^+$ T-cell responses were assessed on day 35 after tumor inoculation by IFN-γ ICS after ex vivo stimulation with SIINFEKL or p15E peptide, respectively. Data are representative of two independent experiments ($n = 4$ per group)

OVA-DC alone and mice treated with OVA-DC and gemcitabine ($P < 0.01$). As previously observed, frequency of OVA-specific CD8$^+$ T-cells was significantly lower in PancOVA tumor-bearing mice as compared to mice without tumors, indicative of tumor-induced immune suppression (Figs. 1, 2b) [20]. Ex vivo analysis found 0.55 ± 0.09 % OVA-specific CD8$^+$ T-cells in mice that had received OVA-DC four times (on day 35 after tumor inoculation). Concomitant gemcitabine treatment led to a further reduction in OVA-specific CD8$^+$ T-cells to 0.05 ± 0.01 % ($P < 0.01$ compared to OVA-DC alone).

Furthermore, stimulation of leukocytes with p15E peptide demonstrated the presence of p15E-reactive CD8$^+$ T-cells in tumor-bearing mice treated with OVA-DC but not with gemcitabine alone (Fig. 2c). Again, concomitant gemcitabine treatment significantly suppressed the induction of p15E-specific CD8$^+$ T-cells. Noteworthy, unloaded DC did not induce p15E-reactive CD8$^+$ T-cells. The presence of p15E-reactive CD8$^+$ T-cells found in OVA-DC-vaccinated animals indicates that DC vaccination is capable of not only inducing a T-cell response against vaccine-related antigens but also to unrelated tumor-associated antigens, a phenomenon termed "epitope-spreading."

Efficacy of OVA-DC and gemcitabine in an orthotopic pancreatic cancer model

To assess the efficacy of our therapy regimen in a tumor model more closely resembling the biology found in humans, we used the orthotopic PancOVA model. Mice were implanted with orthotopic PancOVA tumors and treated with combinations of DC-OVA i.p. and/or gemcitabine. To control for vaccine antigen-unspecific immune effects induced by DC vaccination, a group of mice receiving unloaded DC (U-DC) and a group of mice receiving PBS sham injections were included (Fig. 3a). Untreated mice (PBS) had to be killed within 65 days due to progressive tumor burden. Gemcitabine treatment alone (Gem) improved survival ($P < 0.001$, Gem vs. PBS); however, the absolute effect on median survival was relatively small. Of note, treatment with U-DC also demonstrated a limited beneficial effect on survival (median survival 84 days, $P < 0.001$ compared to untreated mice). Mice treated with OVA-DC, however, showed highly efficient tumor control ($P = 0.0015$ compared to the U-DC group) with nine out of 13 mice rejecting their tumors leading to long-term survival beyond 150 days. Equally efficient tumor control was observed in the combined treatment group ($P < 0.001$, OVA-DC and Gem vs. Gem alone).

In the U-DC group, frequencies of antigen-specific $CD8^+$ T-cells were approximately 0.2 % for SIINFEKL and 0.05 % for p15E, which was significantly higher as in untreated mice (Fig. 3b, c). Thus, U-DC facilitated a tumor-specific CTL response, although at lower levels as compared to OVA-DC. OVA-DC led to development of 0.55 % SIINFEKL-reactive $CD8^+$ T-cells, whereas concomitant gemcitabine reduced the frequency to 0.12 % ($P < 0.001$). Similarly, the frequency of p15E-reactive $CD8^+$ T-cells was reduced by gemcitabine treatment.

Gemcitabine does not impact DC function but sensitizes tumor cells toward CTL-mediated cytotoxicity

To assess the possibility that gemcitabine affects the function of DCs after injection in tumor-bearing hosts, we examined the influence of gemcitabine on DC migration to draining lymph nodes and antigen presentation in vivo (Fig. 4a–c). Migration and in vivo survival of DCs were tested by s.c. injection of 2×10^6 CFSE-labeled, OVA-DC into the foot pad of mice ($n = 5$ per group). At day 7,

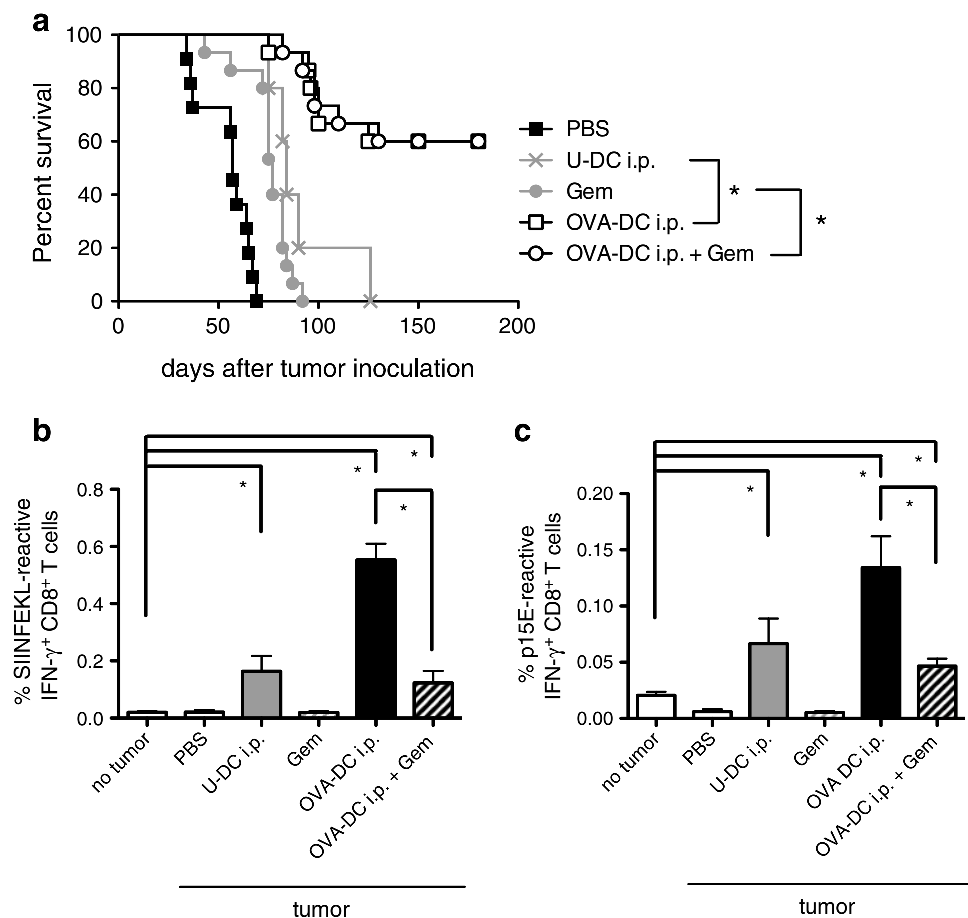

Fig. 3 Treatment of mice with orthotopic PancOVA tumors with DC vaccination and gemcitabine prolongs survival despite reduced numbers of tumor-reactive $CD8^+$ T-cells. **a** PancOVA tumors were implanted orthotopically in the pancreas. Treatment with gemcitabine, unloaded DC (U-DC), OVA-DC or OVA-DC + gemcitabine was started on day 7. Treatment efficacy is blotted as Kaplan–Meier survival curves. Data are pooled from two independent experiments (total of $n = 15$ mice in the OVA-DC and/or gemcitabine groups). **b**, **c** Frequencies of SIINFEKL- and p15E-reactive $CD8^+$ T-cells were determined by IFN-γ ICS 35 days after tumor inoculation. Data are representative of two independent experiments ($n = 7$ per group). $^*P < 0.05$

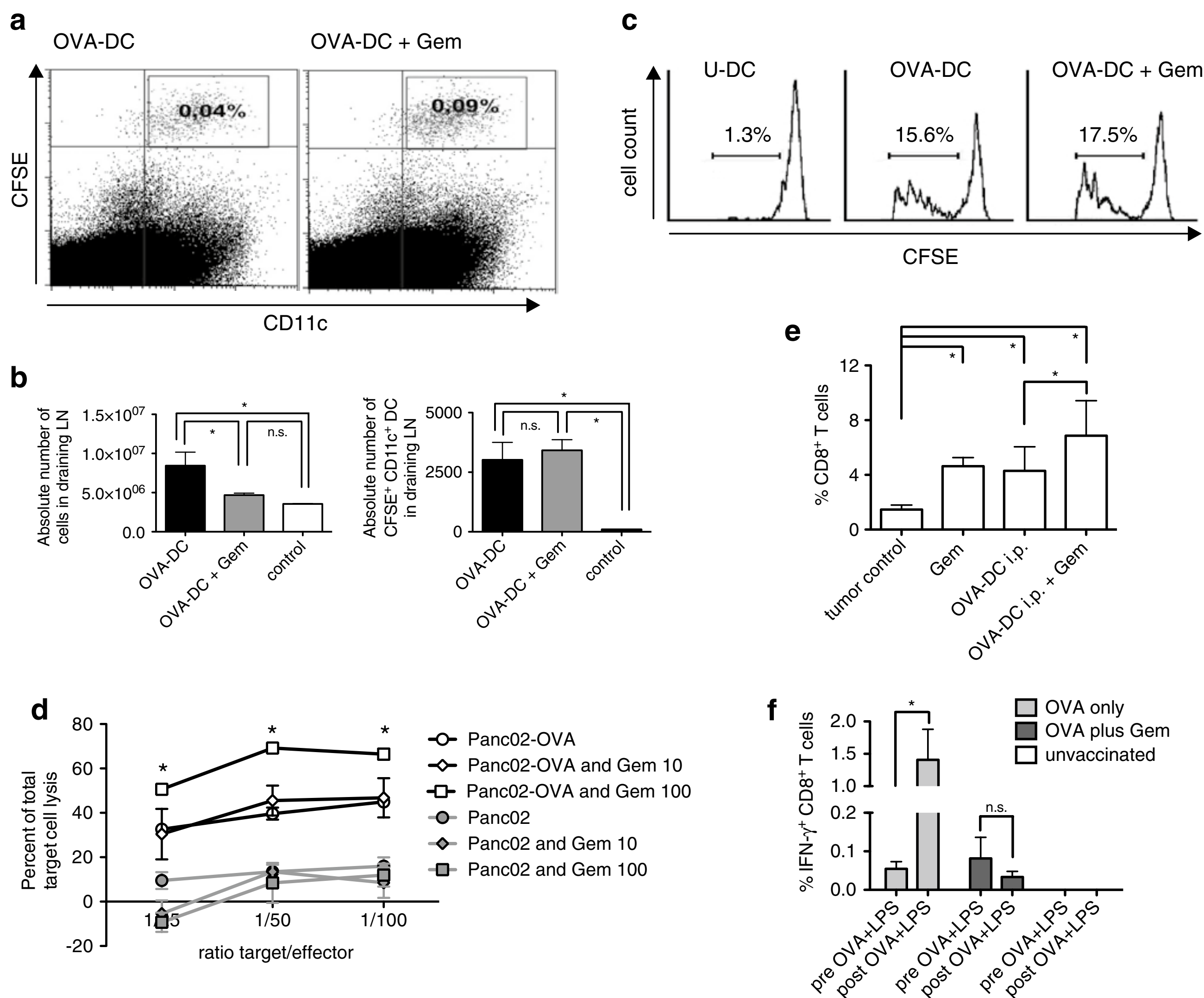

Fig. 4 Gemcitabine does not impair DC function but sensitizes tumor cells to CTL-mediated killing. **a** Mice received s.c. injections of CFSE-labeled DCs into the foot pad and DC migration was assessed 7 days later in popliteal lymph nodes by flow cytometry. Representative FACS blots are shown. **b** Absolute numbers of total cells (*left*) and CFSE-labeled DCs that had migrated to draining lymph nodes (*right*) were determined in mice treated with PBS (*control*), OVA-DC or OVA-DC + Gem. **c** Mice were vaccinated with OVA-DC i.p. with or without concomitant gemcitabine administered at days 2 and 5. Control mice were vaccinated with unloaded DC (U-DC). CFSE-labeled OT-1 T-cells were adoptively transferred at day 7 and proliferation was analyzed 3 days later ($n = 5$ per group). **d** Gemcitabine increased sensitivity of PancOVA cells toward OVA-specific CTL-mediated lysis. PancOVA or Panc02 cells were treated with gemcitabine at concentrations of 10, 100 nM or left untreated and were co-cultured with OT-1 effector CTL. Tumor cell lysis was assessed in a chrome-release assay. Depicted is one of the four independent experiments performed as triplicates ± SEM. **e** Mice with s.c. PancOVA tumors were treated with four rounds of OVA-DC vaccination with or without Gem. Numbers of tumor infiltrating $CD8^+$ T-cells were determined by flow cytometry ($n = 4$ per group). **f** After stratification according to frequency of OVA-reactive $CD8^+$ T-cells in peripheral blood, pre-vaccinated mice ($n = 3$ per group) were rechallenged with 0.5 mg OVA protein and 1 µg LPS per mouse, given intravenously. Gemcitabine was administered 24 and 72 h after the rechallenge in respective subgroups. 48 h after the second gemcitabine administration, IFN-γ ICS was performed. $*P < 0.05$

mice were killed and popliteal lymph nodes were analyzed. Interestingly, the frequency of $CFSE^+$ $CD11c^+$ $CD11b^+$ cells was increased in mice treated with 50 mg/kg body weight gemcitabine (Fig. 4a). However, taking into consideration that the cellular content in the draining lymph node almost doubled in the OVA-DC group as compared to the combined group (Fig. 4b, left), gemcitabine treatment did not affect the absolute number of $CFSE^+$ $CD11c^+$ $CD11b^+$ DCs in draining lymph nodes (Fig. 4b, right).

Next, we tested the influence of gemcitabine on antigen presentation and T-cell stimulatory capacity of DCs in vivo by analyzing the proliferation of adoptively transferred CFSE-labeled $CD8^+$ OT-1 T-cells in vaccinated mice (Fig. 4c). OT-1 T-cell proliferation was not affected by

50 mg/kg gemcitabine, indicating that antigen-presenting function, similar to DC migration to draining lymph nodes, was not negatively influenced.

The effect of gemcitabine on pancreatic carcinoma cells was assessed in regard to their sensitivity toward CTL-mediated lysis. Panc02 and PancOVA cells were treated with or without gemcitabine and lysis by OVA-specific CTL from OT-1 mice was studied in vitro using a ^{51}Cr-release assay (Fig. 4d). At an E/T ratio of 100:1, we observed 45 ± 2 % specific lysis as compared to only 16 ± 4 % of Panc02 wild-type cells. Gemcitabine (100 nM) significantly sensitized PancOVA cells toward CTL-mediated lysis with a specific lysis reaching 66 ± 2 % ($P < 0.001$). Gemcitabine alone at a dose of 100 nM was below the cytotoxic threshold in this assay.

Gemcitabine facilitates recruitment of CD8$^+$ T-cells to tumors, but inhibits DC-induced proliferative T-cell responses

To test whether gemcitabine alters tumor microenvironment in the PancOVA model, frequencies of various immune cell populations in spleen, draining lymph nodes and tumor tissue were analyzed. Interestingly, intratumoral infiltration with CD8$^+$ T-cells (Fig. 4e), CD4$^+$ T-cells and NK cells (Supp. Fig. 2a and 2b, online resource) was increased by DC vaccination as well as gemcitabine when compared to untreated control tumors. Of note, CD8$^+$ T-cell infiltration was additively increased by concomitant DC-OVA and gemcitabine therapy ($P = 0.03$).

Absolute numbers of splenic B-cells, CD4$^+$ T-cells and CD8$^+$ T-cells were higher in tumor-bearing as compared to tumor-free mice (Supp. Fig. 2c). DC vaccination had no influence on splenic immune cell populations. However, splenic B-cells as well as CD4$^+$ and CD8$^+$ T-cells were significantly reduced by gemcitabine as compared to untreated controls. Gemcitabine showed a trend to reduce CD11b$^+$ Gr-1$^+$ MDSC numbers in the spleen. The suppressive effect, however, was moderate and missed statistical significance. OVA-DC vaccination also reduced MDSC numbers, but again statistical significance was missed (Supp. Fig. 2d, $P = 0.075$). As previously reported [20], the frequency of Foxp3$^+$ T_{reg} cells among all CD4$^+$ T-cells was significantly increased in spleens and tumor-draining lymph nodes of tumor-bearing mice (Supp. Fig. 2e and 2f). Neither DC vaccination nor gemcitabine had a significant influence on T_{reg} frequency. No significant differences in the percentage of CD4$^+$ T-cells, CD8$^+$ T-cells or B-cells were observed between tumor-draining lymph nodes of tumor-free and tumor-bearing mice (Supp. Fig. 2 g).

Suppression of DC-induced T-cell responses by gemcitabine was further investigated by testing the influence of gemcitabine on T-cell recall responses (Fig. 4f) to eliminate potential influences of gemcitabine on OVA-DC. Similar to the experimental setup shown in Fig. 1d, mice that had been vaccinated with OVA-DC 6 months earlier were stratified into subgroups according to the frequency of OVA-reactive CD8$^+$ T-cells in peripheral blood (0.055 ± 0.019 vs. 0.082 ± 0.058 % IFN-γ$^+$ CD8$^+$ T-cells). Mice were i.v. rechallenged with OVA protein and LPS. 24 and 72 h after the rechallenge gemcitabine at 50 mg/kg body weight was administered or not. Two days after, the second gemcitabine dose IFN-γ ICS was performed. Single re-vaccination induced a significant boost in the T-cell response ($P = 0.046$). Similar to the suppressive effect demonstrated in Fig. 1d, gemcitabine inhibited induction of a recall response, indicative of a detrimental effect on proliferating T-cells.

Timing of gemcitabine chemotherapy determines efficacy of DC vaccination

As T-cells are vulnerable toward chemotherapeutic drugs mainly in the exponential phase of proliferation, we hypothesized that modification of the vaccination scheme with a delayed start of gemcitabine therapy could lead to improved immunological outcome. This hypothesis was first tested in mice without tumors. Gemcitabine at a dose of 50 mg/kg body weight was either combined with OVA-DC vaccination from the beginning ("concomitant Gem") or started after two rounds of vaccination ("delayed Gem"). A control group received OVA-DC only. Treatment regimens are summarized in Fig. 5a. As observed before, "concomitant Gem" significantly suppressed vaccine-induced CD8$^+$ T-cell and antibody responses (Fig. 5b, c). In contrast, no impairment of the vaccine-induced immune response was seen in the "delayed Gem" group. To test whether the "delayed Gem" strategy improved T-cell effector function, we assessed in vivo cytolytic activity using adoptively transferred peptide-loaded splenocytes as target cells (Fig. 5d). Mice treated with OVA-DC showed highly efficient lysis of target cells, whereas concomitant gemcitabine impaired cytotoxic function, correlating with reduced numbers of antigen-specific CTL found in peripheral blood (Fig. 5b). Mice in the "delayed Gem" group exhibited significantly higher cytotoxic function compared to the "concomitant Gem" group ($P = 0.02$). These data indicate that delaying gemcitabine therapy to a time point when T-cell responses have already been established could be beneficial.

Therapeutic outcome of "delayed Gem" was examined in the s.c. PancOVA model. As expected, gemcitabine concomitant to DC therapy led to better tumor control than gemcitabine alone (Fig. 5e). The difference between the two groups became evident 35 days after tumor induction, indicating that the DC-induced immune response required

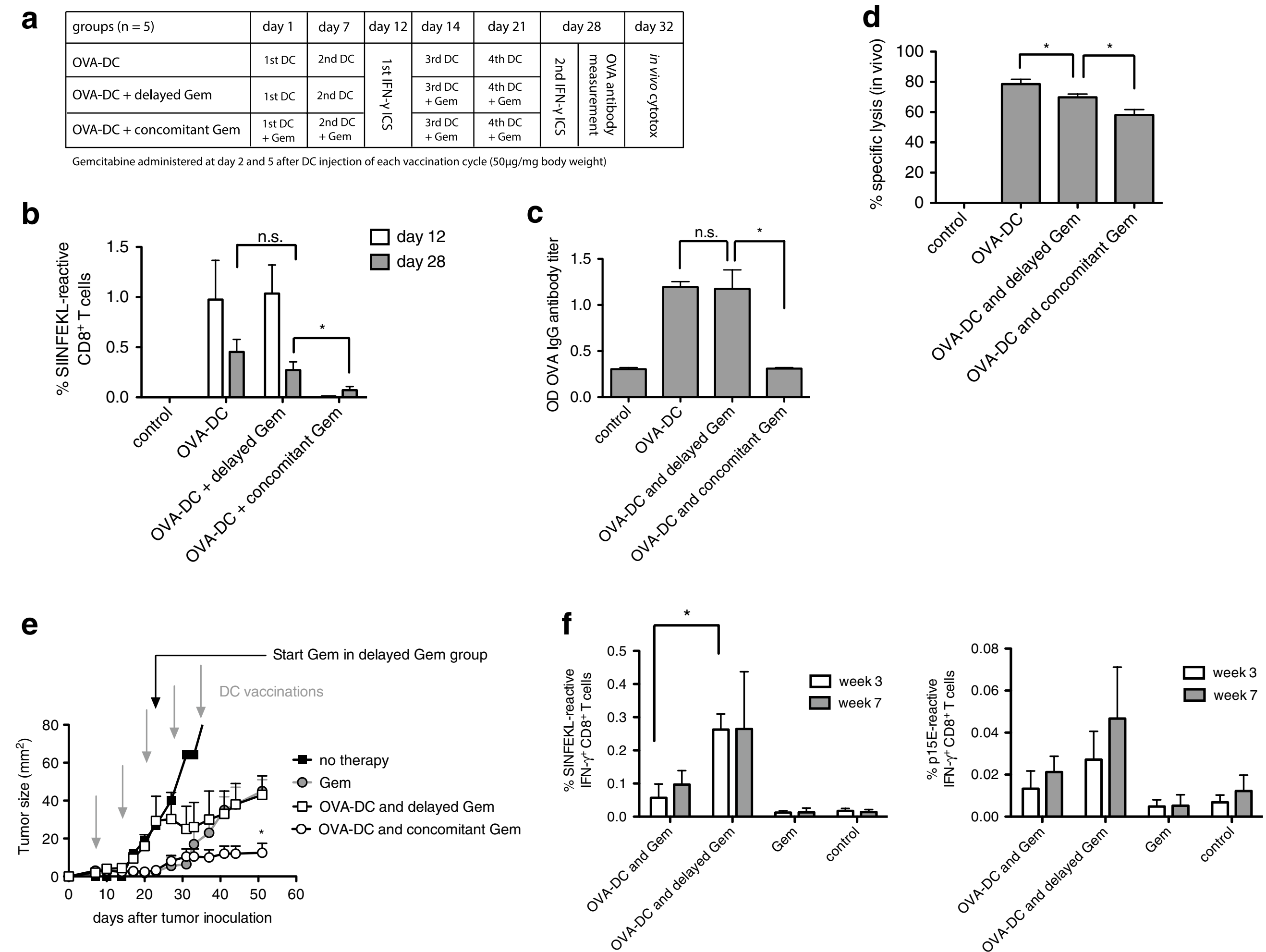

Fig. 5 Delayed gemcitabine administration avoids chemotherapy-induced B- and T-cell suppression. **a** Treatment and immune monitoring scheme. OVA-DC-vaccinated mice were divided into three groups: OVA-DC with concomitant gemcitabine treatment (OVA-DC and concomitant Gem), delayed start of chemotherapy (OVA-DC and delayed Gem) and no chemotherapy (OVA-DC). **b** OVA-specific CD8$^+$ T-cell responses were measured after the second DC vaccination (before the start of gemcitabine in "OVA-DC and delayed Gem" group) and at the end of the experimental protocol after four vaccinations by IFN-γ ICS assay. **c** Anti-OVA IgG antibody titers were assessed after four cycles of DC treatment. **d** In vivo OVA-specific CTL-mediated cytotoxicity was examined by measurement of target-cell lysis 20 h after adoptive transfer of CFSE-labeled, SIINFEKL-loaded splenocytes. **e** Mice were injected s.c. with 10^6 PancOVA cells and treated with Gem alone or OVA-DC ± Gem. Gray arrows indicate DC vaccinations; a *black arrow* indicates the start of gemcitabine therapy in the "OVA-DC and delayed Gem" group. **f** OVA- and p15E-specific CD8$^+$ T-cell responses were determined before the start (*white bars*) and after the start of chemotherapy (*gray bars*) in the "OVA-DC and delayed Gem" group. Figure 4e, f represents one of two independent experiments ($n = 7$ per group)

time to be beneficial. Mice receiving delayed gemcitabine treatment initially had quicker tumor progression than mice in the groups that had received gemcitabine from the beginning of the treatment. However, after three rounds of vaccination, tumor progression was stabilized. Measurement of SIINFEKL- and p15E-reactive CD8$^+$ T-cell frequencies was performed 3 and 7 weeks after tumor inoculation (Fig. 5f). As observed in mice without tumors, SIIINFEKL-reactive and p15E-reactive CD8$^+$ T-cell frequencies were higher in mice treated with OVA-DC and delayed gemcitabine when compared to mice receiving OVA-DC and gemcitabine concomitantly.

Discussion

Interplay of chemotherapy and immunotherapy has attracted much attention since the demonstration that certain chemotherapeutic regimens stimulate endogenous immune responses against tumors by triggering an immunogenic form of cancer cell death [25, 26]. Over the last years, data accumulated showing that effects of cytotoxic drugs are not limited to cancer cells, but also affect stromal cells and immune cells. Recently, Kang et al. [27] suggested that chemotherapeutic regimens can convert the tumor microenvironment into a highly permissive state for

vaccination-induced antitumor immunity, demonstrating a role for DCs and $CD8^+$ T-cells. Kim et al. [28] correlated therapeutic outcome with changes in the immunological microenvironment and T-cell responses in a colon cancer model. However, the influence of chemotherapy on DC vaccine-induced immunity in pancreatic cancer is poorly understood. Here, we show in a pancreatic cancer model that gemcitabine-based chemoimmunotherapy is feasible and highly effective, despite its apparent negative effect on DC vaccine-induced adaptive immune responses. Furthermore, we provide a mechanistic explanation for superior efficacy as compared to either treatment alone and propose a strategy for minimizing the suppressive effects of gemcitabine on vaccine-induced adaptive immune responses.

Gemcitabine has been the mainstay of pancreatic cancer therapy since the study of Burris et al. in the late 1990s [1]. For long, combination of a presumably immunosuppressive therapy like gemcitabine with an immunostimulatory one such as vaccination has been considered counter-intuitive. However, clinical studies demonstrated that gemcitabine can be administered to patients with pancreatic cancer without relevant loss of T-cell and DC function [12]. In fact, several studies have suggested an immunomodulatory function of gemcitabine. Gemcitabine was shown to inhibit Th2- and augment Th1-type immune responses in cancer patients, which is critical for an efficient CTL response [12]. Nowak et al. [13, 29] investigated effects of gemcitabine on immune responses induced by CD40 ligation in a murine model of combined chemoimmunotherapy, finding a mixed pattern of enhanced T-cell but suppressed B-cell responses. Furthermore, gemcitabine has been described to reduce the number of MDSC, a population of immature myeloid cells suppressing T-cell activation, in tumor-bearing animals [14, 15, 30, 31]. This is particularly interesting, as pancreatic cancer leads to MDSC expansion and accumulation in tumor tissue [32, 33]. However, others have found that gemcitabine can trigger cathepsin B release in MDSC thereby promoting tumor growth via activation of the Nlrp3 inflammasome [34]. Ghansah et al. [35] recently found that gemcitabine specifically targets granulocytic MDSC. Reduced numbers of MDSC, however, were only associated with increased survival when gemcitabine therapy was combined with DC vaccination, indicating that the beneficial effects of gemcitabine might be at least in part immune-mediated. These data are concordant with our finding of superior tumor control with the combined treatment and preserved cytotoxicity despite chemotherapy-induced numerical suppression of the DC-induced T-cells. Our study also shows a trend toward reduced MDSC numbers by gemcitabine as well as DC vaccination; however, this was not statistically significant. Shevchenko et al.[36] found that low-dose gemcitabine depleted intratumoral T_{reg} and improved survival in the Panc02 model. One explanation might be that T_{reg} are particularly sensitive toward chemotherapy, including gemcitabine [37], possibly due to the higher turnover of these cells as compared to other $CD4^+$ T-cell populations. Concordant with earlier findings [20, 38], we found an increased frequency of T_{reg} in spleen and tumor-draining lymph nodes of PancOVA tumor-bearing mice. However, we could not detect a reduction in T_{reg} frequencies in tumor-draining lymph nodes or spleens of mice treated with either gemcitabine and/or DCs. As numbers do not necessarily reflect T_{reg} function, a more detailed analysis of T_{reg} phenotype, such as CD103 expression, and suppressive activity should be addressed in further studies.

We have previously described efficacy of combining DC vaccination and gemcitabine in a murine pancreatic carcinoma model in regards to tumor control [9]. As immunological mechanisms leading to tumor control were not investigated due to the lack of a defined tumor antigen for immunomonitoring, we modified the tumor model by using OVA-expressing tumor cells. Data presented here indicate that in a setting of DC vaccination concomitant gemcitabine therapy suppresses induction of a vaccine-specific adaptive immune response. Similarly, the response against a vaccine-unrelated tumor antigen, the tumor cell epitope p15E, which was induced as a secondary effect of the vaccine termed "epitope-spreading," was also suppressed. However, despite the numerical reduction in tumor antigen-specific CTL in peripheral blood, therapeutic efficacy of DC vaccination was improved by gemcitabine. A possible explanation could be more efficient recruitment of T-cells to the tumor site, as combined treatment resulted in increased numbers of intratumoral $CD8^+$ T-cells. In addition, the immunosuppressive effect of gemcitabine was probably balanced by mechanisms augmenting efficacy of immunotherapy, such as sensitization of tumor cells to CTL-mediated lysis. In fact, CTL-mediated killing of target cells in vivo was only mildly impaired by gemcitabine treatment, indicating that lower numbers of tumor-reactive $CD8^+$ T-cells were equally effective when administered under favorable conditions induced by chemotherapy. Our own group described increased cytotoxic activity of CTL when tumor cells were pretreated with gemcitabine [39]. This phenomenon, also termed chemosensitization, has been suggested to be mediated by upregulation of antigenic surface molecules on tumor cells [40, 41]. Recently, Takahara reported that gemcitabine enhances WT1 expression in pancreatic carcinoma cells thereby sensitizing these cells against WT1-specific T-cells [42]. In our study, we could confirm a CTL-sensitizing effect of gemcitabine on PancOVA cells, corroborating data from Ishizaki et al. [43].

An interesting finding of our study is that the negative impact of gemcitabine treatment on vaccine-induced adaptive immune responses can be influenced by the timing

when chemotherapy is started. Once the DC vaccine had established an adaptive immune response, addition of gemcitabine in subsequent cycles did not negatively impact the numbers of antigen-specific $CD8^+$ T-cells or antibody titers. Concerning therapeutic outcome, an "OVA-DC and delayed Gem" strategy was non-inferior to gemcitabine treatment alone despite lower doses of Gem. Thus, in future clinical trials exploring the efficacy of DC vaccines, e.g., in an adjuvant setting after resection of the primary pancreatic tumor, vaccination should precede gemcitabine-based chemotherapy in order to facilitate the generation of an effective tumor-directed immune response and to control disease recurrence. On the other hand, in a palliative situation, concomitant therapy might be the better option to avoid tumor progression until the immune response has been established.

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Conflict of interest All authors declare that no conflicts of interest exist.

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