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Author: Sluis, T.C. van der

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VACCINE-INDUCED TUMOR NECROSIS FACTOR PRODUCING T-CELLS SYNERGIZE WITH CISPLATIN TO PROMOTE TUMOR CELL DEATH

Tetje C. van der Sluis, Suzanne van Duikerem, Suzanna Huppelschoten, Ekaterina S. Jordanova, Elham Beyranvand Nejad, Arjen Sloots, Louis Boon, Vincent T.H.B.M. Smit, Marij J.P. Welters, Ferry Ossendorp, Bob van de Water, Ramon Arens, Sjoerd H. van der Burg and Cornelis J.M. Melief

ABSTRACT

Purpose

Cancer immunotherapy, such as vaccination, is an increasingly successful treatment modality, but its interaction with chemotherapy remains largely undefined. Therefore we explored the mechanism of synergy between vaccination with synthetic long peptides (SLP) of human papillomavirus type 16 (HPV16) and cisplatin in a preclinical tumor model for HPV16.

Experimental Design

SLP vaccination in this preclinical tumor model allowed the elucidation of novel mechanisms of synergy between chemo- and immunotherapy. By analyzing the tumor immune infiltrate we focused on the local intratumoral effects of chemotherapy, vaccination or the combination.

Results

Of several chemotherapeutic agents, cisplatin synergized best with SLP vaccination in tumor eradication, without requirement for the maximum tolerated dose. Upon SLP vaccination, tumors were highly infiltrated with HPV-specific, tumor necrosis factor alpha (TNF α)- and interferon-gamma (IFN- γ)- producing T-cells. Upon combined treatment, tumor cell proliferation was significantly decreased compared to single treated and untreated tumors. Furthermore, we showed that TNF α strongly enhanced cisplatin-induced apoptotic tumor cell death in a JNK dependent manner. This is consistent with upregulation of pro-apoptotic molecules and with enhanced cell death *in vivo* upon combined SLP vaccination and cisplatin treatment. *In vivo* neutralization of TNF α significantly reduced the anti-tumor responses induced by the combined treatment.

Conclusion

Taken together, our data show that peptide vaccination with cisplatin treatment leads to decreased tumor cell proliferation and TNF α -induced enhanced cisplatin-mediated killing of tumor cells, together resulting in superior tumor eradication.

INTRODUCTION

Cancer immunotherapy based on the activation of the patient's own immune system, in particular T-cells, has recently shown remarkable successes. Characteristically, clinical responses to active immunotherapy are often delayed compared with conventional debulking reagents (1-3), begging the question whether fast-acting chemotherapeutics can be combined with immunotherapy. In several clinical trials synergy between chemo- and immunotherapy was indeed observed (4, 5). Some chemotherapeutic agents can induce immunogenic cell death, featuring release of proinflammatory factors from dying cells with the potential to mature dendritic cells, thereby activating CD8⁺ T-cells (6-8). In addition, chemotherapeutics can convert the tumor microenvironment into a site permissive for vaccination by enhancement of antigen density (9). In about 50% of the patients with cervical cancer, the causative virus type is high risk HPV16 (10). The two viral oncoproteins E6 and E7 are required for maintenance of the transformed cell state (11), creating ideal targets for therapeutic vaccination (12). Previous results from our group show that vaccination with SLP vaccines, directed against HPV16 E6 and E7, can effectively cause tumor regression in preclinical mouse tumor models and eradication of lesions caused by HPV16 in patients with pre-malignant disease (13-15). Complete remission of grade 3 HPV16+ vulvar intraepithelial neoplasia correlated with strong and broad SLP vaccine-induced T-cell immunity. However, in end-stage cervical cancer this SLP vaccine causes less robust HPV-specific T-cell responses without an apparent effect on survival (16, 17). We now show in preclinical models that therapeutic HPV16 E7 SLP vaccination has remarkable synergy with defined chemotherapeutic agents through mechanisms that are clearly distinct from those previously reported. The DNA crosslinking agent cisplatin displayed the strongest synergy with vaccination. The mechanisms of action are abundant infiltration with polyfunctional vaccine-induced CD8 T-cells that synergize with cisplatin by TNF α -mediated increased sensitivity of tumor cells to cisplatin-mediated killing, associated with reduced tumor cell proliferation after combination therapy. Notably, combination of therapeutic SLP vaccination with cisplatin leads to superior tumor eradication without added toxicity.

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MATERIAL AND METHODS

Mice

Female C57BL/6 mice were purchased from Charles River (L'Arbresle, France) and housed in the animal facility of the LUMC. The congenic strains Thy1.1 (CD90.1) and Ly5.1 (CD45.1) and ovalbumin-specific T-cell receptor (TCR) transgenic OT-I mice, all on a C57BL/6 background, were bred in house. All mice were housed in Individually-Ventilated-Cage (IVC) systems under specific pathogen-free conditions and used at 8–10 weeks of age. Experiments were approved by the Animal Experiments Committee of the LUMC, in line with the guidelines of the European Committee.

Tumor cell lines and culture conditions

Tumor cell line TC-1 (a kind gift from T.C. Wu, John Hopkins University, Baltimore, MD) was generated by retroviral transduction of lung fibroblasts of C57BL/6 origin with HPV16 E6

and E7 and c-H-*ras* oncogenes (18) and maintained as previously described (14). Tumor cell line C3 was generated by transfection of B6 Mouse Embryonic Cells (MEC) with the complete HPV16 genome and maintained as previously described (19). The methylcholanthrene-induced fibrosarcoma cell line MCA205 was kindly provided by prof. T. Tüting (University of Bonn, Germany). All cell lines were tested by PCR for rodent viruses with negative results. The dendritic cell (DC) line D1 was provided by P. Ricciardi-Castagnoli (University of Milano-Bicocca, Milan, Italy) (20). Human cervical adenocarcinoma cell line HeLa, human cervical carcinoma SiHa and MCA205 cells were cultured in RPMI 1640 (Gibco), supplemented with 8% FCS (Greiner), 100 IU/ml penicillin/streptomycin (Gibco) and 2 mM glutamine (Gibco). *Mycoplasma* tests routinely performed for all cell lines by PCR were negative.

Tumor (-treatment) experiments

On day 0, C57BL/6 mice were subcutaneously inoculated with 1×10^5 TC-1 tumor cells or 5×10^5 C3 tumor cells in 200 μ l PBS and 0.2% BSA. When a palpable tumor was present (day 8 for TC-1 experiments, day 14 for C3 experiments), mice were split into groups with comparable tumor size and were treated (intraperitoneal, i.p.) with chemotherapy. Based on the results with cisplatin, doses of chemotherapy used were similar to the reported maximal tolerated dose (MTD), but provided in a split dose with an interval of one week, resulting in the following doses: Cisplatin (10 mg/kg and 4 mg/kg), doxorubicin (4 mg/kg), oxaliplatin (5 mg/kg), gemcitabine (100 mg/kg), carboplatin (40 mg/kg), paclitaxel (20 mg/kg) at day 8 and 9 or with topotecan (topo, 2 mg/kg) at days 8,9,10, and 11. At the same time of the first chemotherapy treatment, mice with and without chemotherapy were vaccinated in the contralateral flank with synthetic long HPV16 E7₄₃₋₇₇ peptide (GQAEPDRAHYNIVTFCCCKCDSTLRCLCVQSTHVDIR), dissolved in PBS and emulsified in Montanide ISA 51 on day 9 upon tumor challenge or with 4 mg/kg cisplatin in NaCl i.p. on day 15 upon tumor challenge or with the combination (Fig. 1A).

In vivo antibody treatment

For *in vivo* CD4⁺ and CD8⁺ T-cell depletion, mice were injected i.p. on day 7 and then every six days onwards with 100 μ g of the monoclonal antibodies (mAbs) GK1.5 and YTS-169, respectively. All mice used had a >99% depletion of the targeted T-cell population as measured by flow cytometry. For *in vivo* TNF α neutralization, mice were injected i.p. on day 7 and then twice weekly until day 23 with 450 μ g of monoclonal antibody MP6-XT22. mAbs were prepared and purified as described (21).

Adoptive transfer experiments

For adoptive transfers, 0.5×10^6 OT-I Thy1.1 or 2×10^6 OT-II Ly5.1 T-cells were negatively enriched via a CD8⁺ and CD4⁺ enrichment kit (BD), respectively, and together intravenous (i.v.) injected in Thy1.2/Ly5.2 recipient mice. Subsequently, the mice were vaccinated with two SLPs, one containing the SIINFEKL epitope (OVA₂₄₁₋₂₇₀) to stimulate OT-I cells and the other containing the (OVA₃₁₇₋₃₄₇) epitope to stimulate OT-II cells (22). One group also received cisplatin (4 mg/kg) via i.p. injection. On day 7, cisplatin treatment was repeated and on day

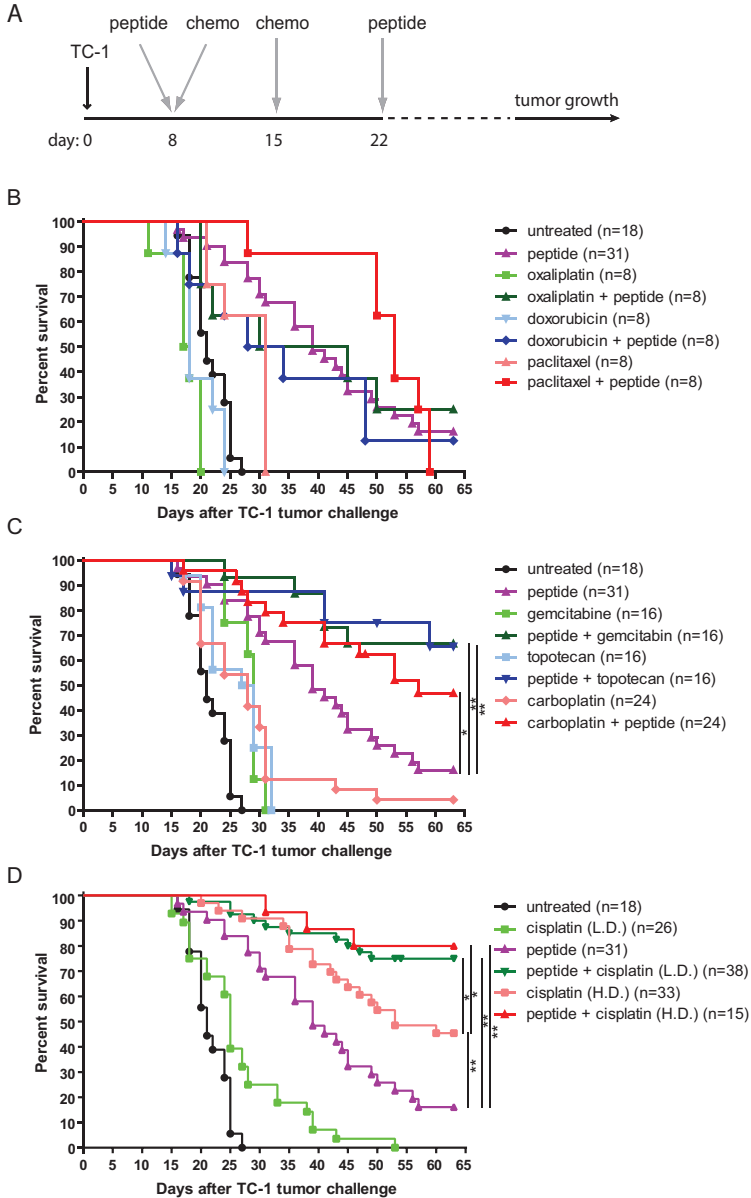


Figure 1. Cisplatin displays the strongest synergy with SLP vaccination.

Wild-type C57BL/6 mice were injected s.c. with TC-1 tumor cells in the flank. Eight days later, when tumors were palpable, mice were treated systemically with chemotherapeutics with or without HPV16 E7₄₃₋₇₇ peptide in Montanide in the opposing flank. Chemotherapy was repeated one week after initial treatment and vaccination was repeated 2 weeks after initial treatment. A) Schematic diagram of the therapy regimen. B-D) Kaplan-Meier survival plots for chemo(immune-) therapy with (A) oxaliplatin, paclitaxel, doxorubicin, (B) gemcitabine, topotecan, carboplatin, (D) High Dose (H.D.) and Low Dose (L.D.) cisplatin. Shown are pooled data from various experiments (number of mice is specified). Significant differences between peptide and the various combined treatments as well as between single treatment with H.D. treatment and combined cisplatin + peptide are indicated ($P < 0.05$ and $**P < 0.01$).

10 the mice were booster vaccinated with SLP in PBS with 20 μg adjuvant CpG. The presence of congenic marked T-cells in the spleens and lymph nodes on day 14 was analyzed by flow cytometry. Additionally, to determine the cytokine production, cells were incubated for 5 hours in the presence or absence of SIINFEKL peptide and brefeldin A after which an intracellular cytokine staining was performed as previously described (14).

For analysis of the capacity of T-cells to populate the tumor both donor (Thy1.1/Ly5.2 and Thy1.2/Ly5.1) and recipient mice (Ly5.2/Thy1.2) mice were injected with 1×10^5 TC-1 tumor cells on day -8. On day -5, CD8 depleting antibodies were injected i.p. in the recipient mice. All CD8⁺ T-cells were depleted but CD8⁺ T-cells re-appeared on day 5 as measured by flow cytometry. Both donor and recipient mice were injected on day 0 with SLP in Montanide. Donors with the congenic markers Thy1.1/Ly5.2 also received cisplatin (i.p.) on day 0. Two days after vaccination spleens and vaccine draining lymph nodes were isolated from both donor mice, 1:1 mixed and transferred to recipient mice. After 8 days the tumor, spleen and tumor draining lymph node (LN) were isolated from each mouse and analyzed for the presence of donor cells by flow cytometry. Relative infiltration was calculated by setting the % of CD8⁺ T-cells originated from the peptide treated mice on 100, the percentage of T-cells originated from the cisplatin + peptide treated mice as a percentage of that.

Analysis of tumor infiltrating immune populations

Isolated tumors from mice transcidentally perfused with PBS-EDTA were mechanically disrupted in small pieces and incubated for 15 minutes at 37°C in IMDM containing Liberase (Roche). Single-cell suspensions were next prepared by mincing the tumors through a 70- μm cell strainer (BD Biosciences). For cell surface staining, cells were resuspended in staining buffer (PBS + 2% FCS + 0.05% sodium azide) and incubated with H-2D^b tetramers containing HPV16 E7₄₉₋₅₇ peptide (RAHYNIVTF), 7-Aminoactinomycin D (for dead exclusion, invitrogen), CD19 (clone 1D3), CD8a (clone 53-6.7), CD3 (clone 145-2C11), CD45.2 (clone 104), Class II (clone M5/114.15.2), CD44 (clone IM7) for 30 minutes at 4°C. Fluorescent conjugated antibodies were purchased from BD Biosciences and eBioscience. For intracellular Ki-67 staining, surface stained cells were fixed and permeabilized with the FoxP3 staining buffer, and subsequently incubated with FITC labeled Ki-67 specific antibodies or isotype control (eBioscience).

Furthermore, single cell suspensions of tumor(-infiltrate) were incubated for 5 hours with 40.000 D1 cells pre-loaded with the SLP HPV16 E7₄₃₋₇₇ (10 $\mu\text{g}/\text{ml}$) in the presence of Brefeldin A (2 $\mu\text{g}/\text{ml}$) after which an intracellular cytokine staining was performed.

Microscopy

Isolated tumors from transcidentally perfused mice were embedded in Tissue-Tek and immediately frozen in 2-methylbutane cooled on dry-ice. Cryostat sections (4-5 μm), were cut at -20°C, dried overnight at 60°C and then frozen at -20°C.

After thawing samples were fixed with acetone, dried, washed in PBS, incubated with biotinylated CD4 mAb (clone RM4-5, Pharmingen), washed and stained with CD3 mAb (Alexa Fluor 647, clone 17A2, Biolegend) and streptavidin Alexa Fluor 546 (molecular probes®) in PBS

with mouse serum. Images (200x) were captured with a confocal laser scanning microscope (Zeiss LSM 510). For analysis of mitosis activity, slides were stained with hematoxylin-eosin (HE). Cell- and mitosis- counts were scored by two independent investigators. *In vivo* apoptosis was analyzed on cryostat sections by use of TUNEL technology (*in situ* cell death detection kit, POD, Roche, Mannheim, Germany) according to the manufacturer's instructions. TUNEL staining was visualized with the substrate DAB (3,3'-diaminobenzidine tetrahydrochloride) after which the samples were counterstained with Hematoxylin (Dako). Tissue sections treated without TdT were used as negative control.

CTL assay

Tumor-free C57BL/6 mice were immunized with synthetic HPV16 E7₄₃₋₇₇ peptide and 20 µg CpG. Eight days after boosting splenocytes were isolated, restimulated with the specific peptide for 7 days, and used as effectors in a CTL assay against TC-1 target cells. IFN-γ (Prospec) exposed or non-exposed TC-1 tumor cells were incubated with various concentrations of cisplatin and incubated overnight. The next day cells were washed, incubated for one hour with 1 µg/ml E7 peptide (RAHYNIVTF) and then used in a 4 hour ⁵¹Cr release assay. Target cells were then labelled with 100 µl ⁵¹Cr for 1 hour, washed and plated into a 96-well round-bottom plate at a density of 2.000 tumor cells/well together with T-cells (of which 4% was E7-specific as measured by tetramer staining) at the indicated E/T ratios. After 4 hours, cells were harvested, and the percentage ⁵¹Cr release was measured using a gamma counter.

Cell viability assays

For the various assays, TC-1 was seeded at 8000 cells per well with 20 ng (250 international units) mouse TNFα (Prospec) and 2 µg/ml cisplatin. Concentrations of topotecan, carboplatin and paclitaxel are indicated in the figure. C3 was seeded at 10.000 cells per well with 4 ng/ml TNFα and 6 µg/ml cisplatin, MCA205 was seeded at 7000 cells per well at concentrations of TNFα and cisplatin indicated in figure. HeLa cells were plated at 18.000 cells per well with 2 µg/ml cisplatin and 25 ng/ml hTNFα, 40 µg/ml Etanercept (Enbrel), or supernatant of PHA (0,5 µg/ml) stimulated PBMCs of a healthy donor. SiHa cells were seeded at 20.000 cells per well with 100 ng/ml TNFα and 12 µg/ml cisplatin. Real time apoptosis induction was quantified using a live cell apoptosis assay as previously described (23). Briefly, tumor cells were seeded into a GreinerBioOne 96-wells uClear plate and incubated for 24 hours. Then, cisplatin and/or TNFα were added to the cells. Binding of Annexin V-Cy3 conjugate to phosphatidyl serine present on the membranes of apoptotic cells was quantified in time by imaging with a BD Pathway 855 imager (Becton Dickinson). The total area of Annexin V-Cy3 fluorescence per image was quantified using Image Pro (Media Cybernetics, Bethesda, MD). Cell death induced by cisplatin with supernatant of peptide treated tumors was analyzed by Annexin-V staining using flow cytometry.

Cell viability was determined using a standard colorimetric MTT (3-4,5-dimethylthiazol-2-yl-2, 5-diphenyl-tetrazolium bromide) reduction assay (Trevigen) and performed via the setup used for the live cell apoptosis assay. Analysis was performed 22 hours after cisplatin and/or TNFα exposure. Control cells without agents were cultured in parallel using the same conditions

with comparable media changes. Absorbance was measured with a microplate reader (Bio-RAD) at a test wavelength of 570 nm, and a reference wavelength of 655 nm. For each treatment, cell viability was evaluated as a percentage using the following equation: (OD of treated sample/ mean OD of untreated sample) ×100.

Quantitative real-time PCR

TC-1 tumor cells were harvested and then seeded at 1.3×10^5 cells per well (6 wells plate). After 24 hours incubation TNF α and/or cisplatin were added. Twenty-four hours later cells were harvested and suspended in Trizol. Total RNA was isolated from cells using an RNeasy® Mini Kit (Qiagen) according to manufacturer's protocol. cDNA was synthesized using SuperScript™ III Reverse transcriptase (Invitrogen). Oligonucleotide primers are compiled in Table 1. Reactions were performed using iQ SybrGreen supermix (Biorad) on the my-iQ Real-Time PCR (Biorad) with an annealing temperature of 60°C. Cycle threshold (Ct) values were normalized to the expression levels of the GAPDH and β -Actin genes.

Statistical analysis

Survival for differentially treated mice was compared using the Kaplan-Meier method and the log-rank (Mantel-cox) test. Additional statistical methods are stated in the legends. All P values <0.05 were considered significant.

Table 1. RT-PCR primers.

Gene	Forward primer	Reverse primer
BCL-XL (Bcl2l1)*	TGGAGTAAACTGGGGTTCGCATCG	AGCCACCGTCATGCCCGTCAGG
Bfk (Bcl2l15)*	CCCCTATCCCACGCTGCTA	TCCTCTCCTGAGCATGGTTCA
Bruce*	ACATCCGACAAGCAACGGTTA	AGCACGGTGAAGGATTTCTGA
c-IAP2 (Birc3)*	TATTTGTGCAACAGGACATTAGGAGT	TCTTTCCTCCTGGAGTTTCCA
TNF α **	ACGGCATGGATCTCAAAGAC	GGTCACTGTCCCAGCATCTT
Ki-67**	CCTTTGCTGTCCCCGAAGA	GGCTTCTCATCTGTTGCTTCTT
Xaf1*	CTGTGGCTCTGCCTTTGAAGT	CGTCTCCTGTGGGAAGATCAG
GAPDH **	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG
B-actin **	GATGTCACGCACGATTTC	GGGCTATGCTCTCCCTCAC
PCNA **	GAACCTCACCAGCATGTCCA	ATTCACCCGACGGCATCTTT

Primers indicated with one star (*) were obtained from Eurogentec, primers indicated with two stars (**) were obtained from Sigma Aldrich, Zwijndrecht, The Netherlands.

RESULTS

Cisplatin displays the strongest synergy with SLP vaccination

To test which chemotherapeutics have favorable anti-tumor effects in combination with therapeutic SLP vaccination, the HPV16 SLP vaccine was combined with seven clinically relevant chemotherapeutic agents. Mice were inoculated with TC-1 tumor cells, expressing the HPV16 oncoproteins E6 and E7, and tumor size was longitudinally measured. When

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a palpable tumor was present on day eight post tumor challenge, treatment was started. Systemic treatment with chemotherapeutic agents alone, given twice with a one week interval (Fig. 1a), displayed a modest transient effect on tumor outgrowth (Fig. 1b,c,d and Supplementary Fig. S1a). SLP vaccines as a single therapy, given in Montanide to mimic the currently used clinical vaccine strategy in patients with HPV16+ tumors (13, 16, 17), induced only a temporal decrease in tumor size in most mice and complete tumor eradication in 16% of the mice. Strikingly, none of the chemotherapeutic treatments, at the doses used, significantly impaired the impact of peptide vaccination on tumor growth. Combined treatment consisting of oxaliplatin, doxorubicin or paclitaxel with SLP vaccination did not enhance overall survival when compared to SLP vaccination alone (Fig. 1b, Supplementary Fig. S1a). In contrast, the chemotherapeutics topotecan, gemcitabine, carboplatin or cisplatin clearly synergized with vaccination, showing significantly better survival in groups of mice treated with the chemo-immunotherapy combination, compared to either treatment alone (Fig. 1c,d, Supplementary Fig. S1a). In particular cisplatin synergized well with SLP vaccination, resulting in a survival of 75-80% of the mice. To avoid significant weight loss (Supplementary Fig. S1b) a lower, less toxic, dose of cisplatin was used. Similar to the higher dose, the lower cisplatin dose combined with peptide vaccination completely eradicated tumors in approximately 75% of the mice (Fig. 1d, Supplementary Fig. S1a). There was indeed no significant difference between these two groups of chemo-immunotherapy treated mice ($p=0.47$), emphasizing the fact that in combination with immunotherapy, dosing of chemotherapy can be lower, avoiding unnecessary toxicity such as weight loss. Mice successfully treated with this combination remained tumor free for at least two months. When such tumor-free mice were re-challenged with TC-1 tumor cells, all were protected (data not shown). In a different murine tumor model for cervical cancer, a transformed B6 Mouse Embryonic Cell line with the complete HPV16 genome (C3), the combined SLP and cisplatin treatment again resulted in a stronger decrease in tumor size than single treatment (Supplementary Fig. S1c). Of note, after day 32, many of the shrunken C3 tumors grew out again, possibly via immune-escape.

In TC-1 tumor-bearing mice treated with both cisplatin and SLP vaccination, we observed that whilst elimination of CD4⁺ T-cells had no effect on treatment efficacy, CD8⁺ T-cell depletion completely abolished tumor control by the combined treatment (Supplementary Fig. S1d). Together these data show that clinically relevant chemotherapy can be safely combined with SLP vaccination. We selected the effective and non-toxic combination with lower dose cisplatin to further investigate the mechanisms of synergy.

Effect of cisplatin chemotherapy on T-cell numbers, function and homing capacity

To understand the mechanisms underlying the synergy observed with cisplatin plus SLP vaccination, we first asked whether chemotherapy can affect the antigen-specific expansion and differentiation of T-cells. To this end mice were injected intravenously with OT-I and OT-II TCR transgenic T-cells. On this same day these mice were vaccinated with two SLPs comprising the T-cell epitopes recognized by OT-I and OT-II T-cells, emulsified with Montanide (Fig. 2a).

Treatment with gemcitabine, carboplatin or cisplatin did not affect either the numbers of OT-I/OT-II T-cells or their cytokine production at the doses tested (Fig. 2b,c,d,e, and not shown).

Similar results were found in experiments in which the endogenous vaccine-induced HPV16-specific T-cell response in TC-1 tumor-bearing mice was measured. The percentages of circulating HPV16-specific tetramer-positive CD8⁺ T-cells in mice vaccinated with SLP alone were comparable to those in mice treated with SLP vaccination and cisplatin (Fig. 2f,g). Additionally, expression of the activation molecules CD25, CD69, CD137 and KLRG1 appeared unaffected on both CD4⁺ and CD8⁺ T-cells, at least for cisplatin treatment (Supplementary Fig. S2).

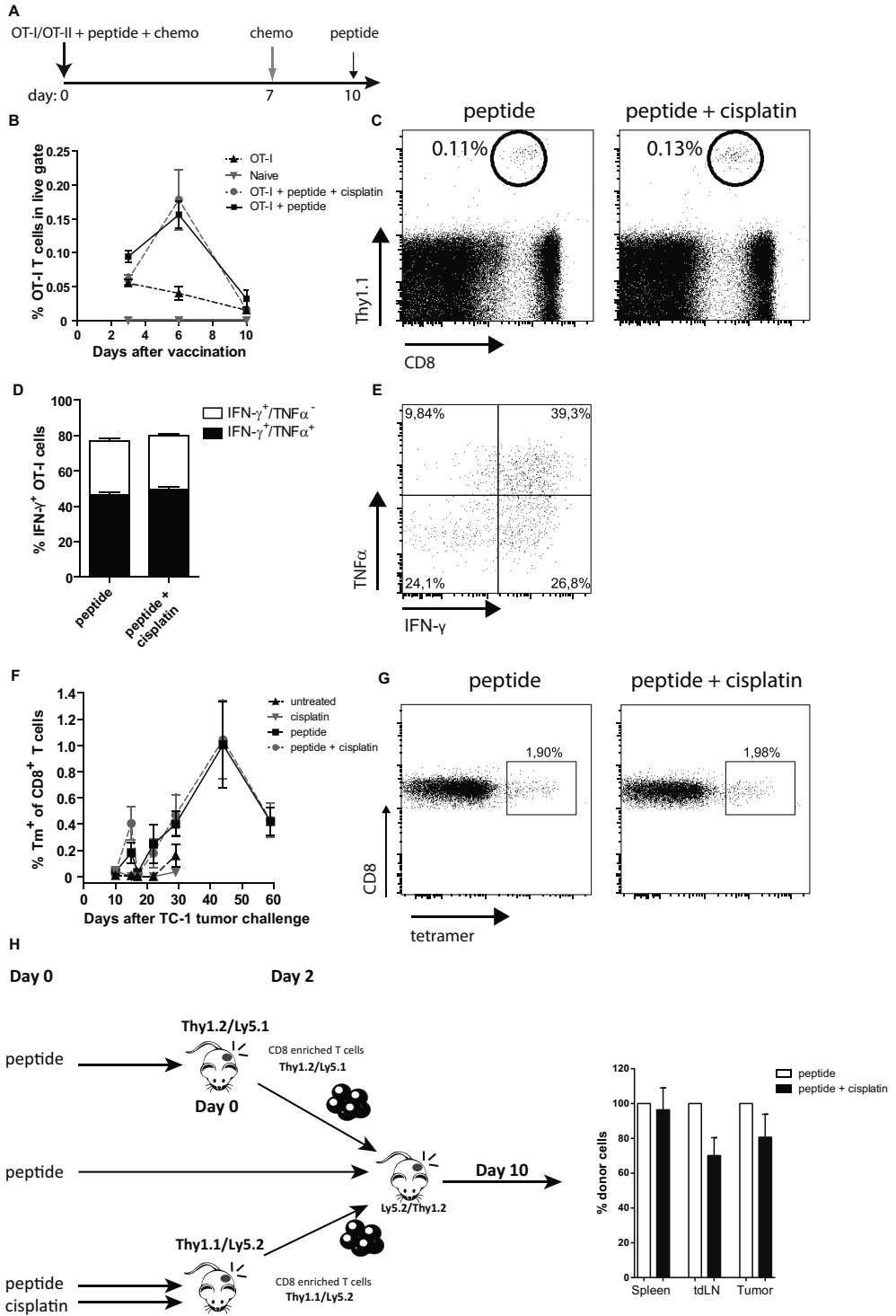
To explore whether cisplatin-exposed T-cells have differential homing capacity to the tumor microenvironment compared to unexposed T-cells, adoptive transfers of congenically marked endogenous T-cells from tumor-bearing mice, treated with SLP vaccine (Ly5.1) or with SLP vaccine and cisplatin (Thy1.1) were transferred into the same, CD8⁺ T-cell depleted host mice (Thy1.2/Ly5.2). Eight days later spleens, lymph nodes and tumors were harvested and the presence of adoptively transferred cells was analyzed, revealing that cisplatin-exposed T-cells were equally efficient in repopulating the spleen, lymph nodes and tumor as non-exposed T-cells (Fig. 2h). Taken together, we conclude that the synergy observed between some chemotherapeutic agents and vaccination is not related to overt changes in systemic T-cell immunity.

Vaccination strongly increases the number of intratumoral polyfunctional CTLs

Next, we focused on effects of combined chemo-immunotherapy on the tumor microenvironment. Tumor size significantly affects various immune parameters (24, 25). Therefore we employed a treatment protocol (Fig. 3a) in which co-treatment still effectively synergized (Supplementary Fig. S3), but analyzed the tumors at the start of the regression phase (Fig. 3b). Analysis of the immune infiltrate in the tumor microenvironment at the start of regression showed that the percentage of leukocytes significantly increased upon treatment with cisplatin, peptide and the combined treatment. The combination treatment resulted in a total of approximately 60% leukocytes in the tumor environment on day 19 (Fig. 3c,d). Given the significant tumor regression observed upon combined treatment, enhanced leukocyte infiltration at later time points is expected but has not been tested.

Although cisplatin was capable of inducing CD45⁺ leukocyte infiltration, very few of these cells were CD8⁺ T-cells (Fig. 4a-f). Interestingly, substantial numbers of CD8 T-cells were entering the tumor upon peptide vaccination or the combination treatment (7.8 times more CD8⁺ T-cells in the combined treatment group compared to untreated) (Fig. 4a). Confocal microscopy using antibodies to CD4 (red) and CD3 (blue) confirmed our observation that vaccination strongly increases the density of CD8⁺ T-cell infiltration (characterized as CD3⁺/CD4⁻ cells) in the tumor beds (Fig. 4b,c).

Further characterization of the tumor infiltrating CD8⁺ T-cell population revealed a high expression of Ki-67 (Fig. 4d) as a marker of proliferation, and showed that a very high percentage (~40%) specifically recognized the tumor antigen E7 (Fig. 4e). When isolated, many of the tumor infiltrating T-cells produced the proinflammatory cytokines IFN- γ and TNF α after *in vitro* exposure to E7 presenting antigen presenting cells (D1 cell line) (Fig. 4f).



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Together, these data show that although chemotherapy and vaccination can both enhance leukocyte infiltration of the tumors, IFN- γ - and TNF α -producing antigen-specific CD8 T-cells enter this tumor only upon specific vaccination. Since cisplatin addition to vaccination had no effect on these CD8 T-cells, we hypothesized that the enhanced anti-tumor responses observed upon combined treatment were not due to direct effects on the tumoricidal CD8 T-cells but rather due to differences in the tumor cells.

Combined treatment of vaccination with cisplatin decreases tumor cell proliferation

While the intratumoral T-cells expressed high levels of Ki-67, we noticed that the Ki-67 levels in tumor cells of the combined treatment group were considerably lower than in tumor cells from untreated, cisplatin or peptide treated mice, suggesting a decrease in tumor cell proliferation caused by the combined interaction of cisplatin and peptide (Fig. 5a). To corroborate this, the numbers of mitotic figures per high-power field in hematoxylin-eosin (HE) stained sections, a well-reproducible, strong prognosticator of disease in lymph node (LN)-negative patients (26), were compared between the untreated group and the various treatment groups. This analysis confirmed that upon either cisplatin or peptide vaccination alone the division rate of tumor cells declined but the lowest number of mitoses was observed upon combined cisplatin and vaccination treatment (Fig. 5b). Following *in vitro* treatment of tumor cells with TNF α , IFN- γ and/or cisplatin, both Ki-67 and Proliferating Cell Nuclear Antigen (PCNA) levels were not decreased (data not shown), suggesting that the decreased proliferation of tumor cells is not a result of direct exposure to either one of these T-cell produced cytokines. Together these data show that combined cisplatin and SLP vaccination decrease the proliferation of tumor cells more extensively *in vivo* than either treatment alone.

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► Figure 2. Cisplatin does not affect numbers, function or homing capacity of CD8 T-cells.

A-E) 0.5×10^6 Thy1.1 OT-I and 2×10^6 CD45.1 OT-II T-cells were injected i.v. in Thy1.2⁺/CD45.2⁺ recipient mice on day 0. Mice were vaccinated with 2 SLPs, one containing the CTL (SIINFEKL) epitope, the other containing the OVA CD4 helper epitope at day 0 and 10. One group also received i.p. cisplatin at day 0 and 7. A) Overview of experiment. B) The percentage of OT-I T-cells in blood measured on days 3, 6 and 10 (n=5). C, E, G) Representative flow cytometry plots. D) On day 14 splenocytes were isolated, stimulated with SIINFEKL peptide, and the response was measured by intracellular cytokine staining. Shown is a representative for three independent experiments. Mean + SEM is indicated. F) Wild-type C57BL/6 mice were injected with TC-1 tumor cells. When tumors were palpable (day 8), mice were treated with cisplatin (day 8 and 15) and/or HPV16 E7₄₃₋₇₇ peptide in Montanide (day 8 and 22). Quantification of % vaccine-specific cells within the CD8⁺ T-cell population in time in blood is shown as determined by H2-D^b E7₄₉₋₅₇ (RAHYNIVTF) tetramer staining. N=6 mice per group, representative for 3 individual experiments. Shown is the mean and SEM. H) On day -8 both donor (Thy1.1/Ly5.2 and Thy1.2/Ly5.1) and recipient (Ly5.2/Thy1.2) mice were injected with 1×10^5 TC-1 tumor cells. On day -5 CD8 depleting antibodies were injected i.p. in the recipient mice. All CD8 T-cells were depleted but CD8 T-cells reappeared on day 5. Both donors and recipient mice were injected on day 0 with SLP in Montanide. Donors with the congenic markers Thy1.1/Ly5.2 also received cisplatin (i.p.) on day 0. Two days after vaccination, spleens, and vaccine draining lymph nodes were isolated from both donor mice, and single cell suspensions of the two different donors were 1:1 mixed and transferred i.v. to recipient mice. On day 10, the tumor, spleen and tdLN were isolated and analyzed for the presence of donor cells. Percentage of cells from Thy1.2/Ly5.1 mice (peptide treated) within the organ was set as 100%. The black bars represent the percentage of cells from the Thy1.1/Ly5.2 mice, relative to that of the Thy1.2/Ly5.1 donor cells. Data are pooled from 5 individual experiments, each with ≥ 3 mice per group.

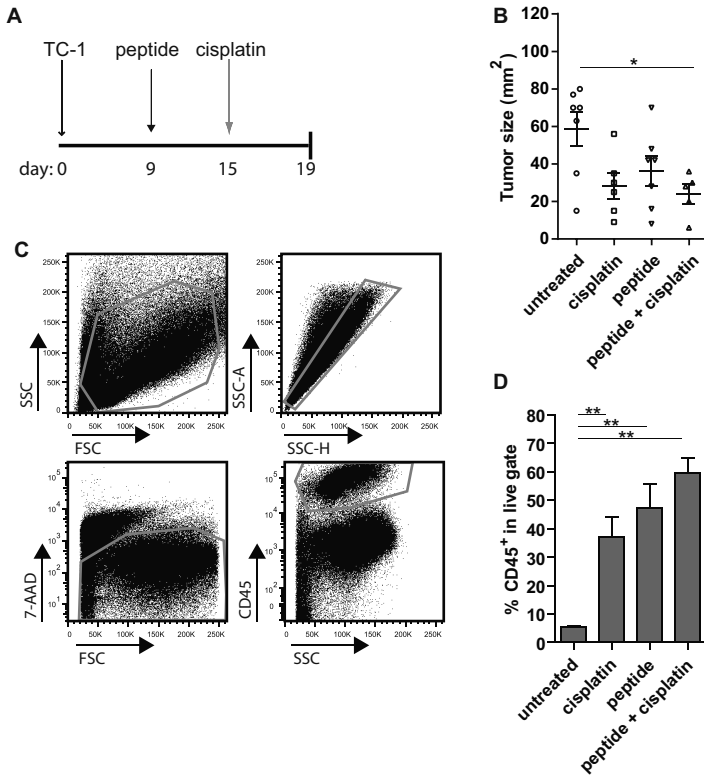


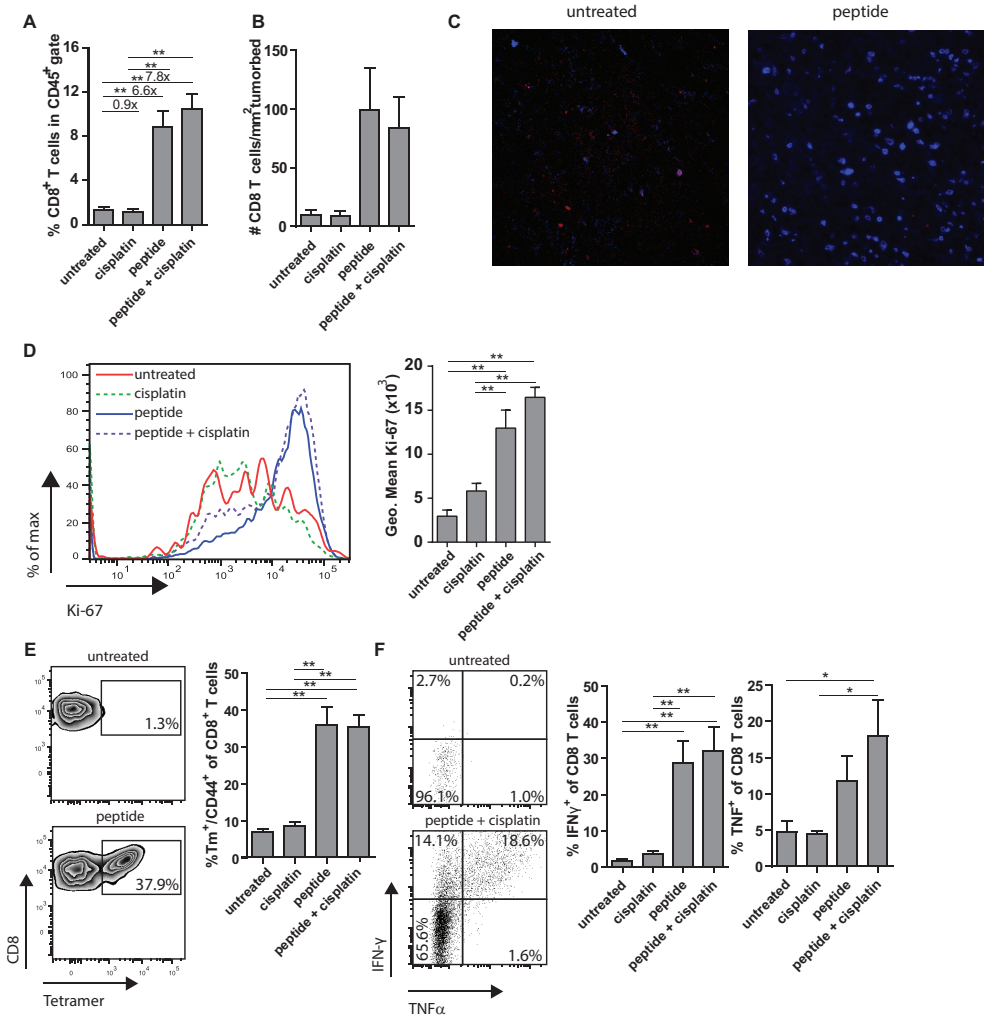
Figure 3. Strongly enhanced numbers of leukocytes within the tumor microenvironment after combined treatment of cisplatin and peptide vaccination.

Mice were inoculated on day 0 with 1×10^5 tumor cells. On day 9, when a palpable tumor of approximately 4-10 mm² was present, mice were treated with HPV16 E7₄₃₋₇₇ peptide in Montanide ISA-51. Six days later, on day 15, half of these mice received cisplatin treatment. Tumor infiltrate was analyzed on day 19. A) Schematic overview of experimental setup. B) Graph of tumor sizes in mm². Shown is the mean + SEM. Each dot represents the tumor size of a single mouse. Experiment is representative for three individual experiments. C-D) Tumor samples were isolated as described in material and methods and analyzed with multicolor flow cytometry. C) Representative depiction of gating strategy. D) Shown is the percentage of CD45 positive cells within the live gate on day 19. Shown data is representative for five individual experiments. Shown is the mean + SEM (*P < 0.05 and **P < 0.01 as determined by one-way ANOVA followed by Tukey's post-hoc analysis).

Combined treatment of vaccination with cisplatin enhances tumor cell death and is caused by the combined action of TNF α and cisplatin

Tumor regression and clearance ultimately depend on tumor cell death. We therefore analyzed *in vivo* cell death by the TUNEL assay, a technique that detects fragmented DNA resulting from apoptosis. Strikingly, we observed that the combination treatment significantly enhanced tumor cell death compared to the untreated and cisplatin treated tumors on day 17 already (supplementary Fig. S4a) and also on day 19 (Fig. 6a,b).

First, we hypothesized that cisplatin might enhance the susceptibility of tumor cells to T-cell killing. To test this, TC-1 tumor cells were pretreated with various doses of cisplatin.



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Figure 4. Peptide vaccination strongly increases the numbers of intratumoral, proliferating, polyfunctional CTLs TC-1 tumor bearing mice were treated on day 9 with HPV16 E7₄₃₋₇₇ peptide in Montanide in the opposing flank.

On day 15, half of these mice received cisplatin. The percentage of CD8 T-cells in the leukocyte gate on day 19 is depicted, numbers above the bars indicate the increase in CTLs (A). B-C) Confocal microscopy for CD4 (red) and CD3 (blue) was performed. CD8 T-cells are identified as blue cells. Intratumoral T-cell counts per mm². Data indicates the average scores for four mice per group, three images per tumor (B) and C) representative pictures from an untreated tumor and vaccine-treated tumor. D) Representative histogram plots (left) and quantification of Ki-67 expression by tumor infiltrating CD8 T-cells (right). E) Flow cytometry of single cell suspensions from tumors by tetramer staining. Representative plots for an untreated (upper) and peptide (lower) treated mouse (left). Quantification of the percentage Tm⁺ T-cells on day 19 (right). F) Single cell suspensions of tumors were co-cubated with HPV16 E7₄₃₋₇₇ peptide-pulsed D1 cells and stained intracellularly for TNFα and IFN-γ 5 hours later. Representative flow cytometry plots (left) and quantification (right) of nontreated tumor and a peptide/cisplatin treated tumors. Experiments were performed with six mice per group, data is representative for two experiments. Data is analyzed by one-way ANOVA followed by Tukey's post-hoc analysis and expressed as mean with (SEM) (*P < 0.05 and **P < 0.01).

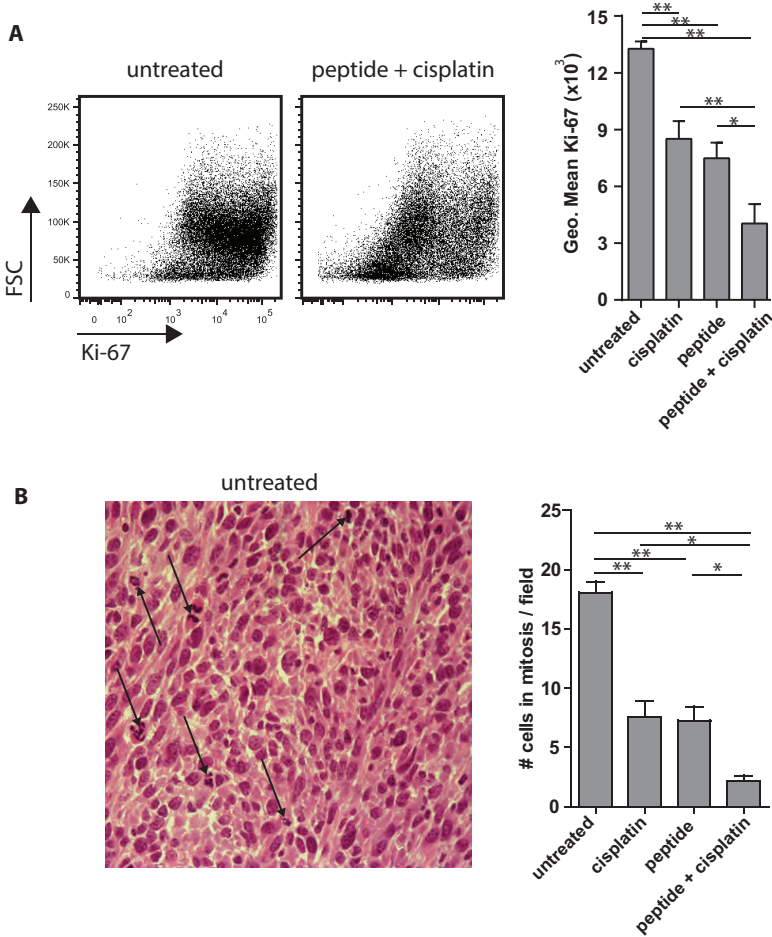


Figure 5. Combined treatment of vaccination with cisplatin decreases proliferation of tumor cells.

Mice were inoculated on day 0 with 1×10^5 tumor cells. On day 9, when a tumor of 4-10 mm² was present, mice were treated with HPV16 E7₄₃₋₇₇ peptide. Six days later, on day 15, half of these mice received cisplatin treatment. A) Single cell suspensions of six mice per group were stained for CD45, fixed, permeabilized and stained for Ki-67. Representative flow cytometry plots for untreated mice and peptide plus cisplatin treated mice (left), Ki-67 expression quantification (right). Data is analyzed by one-way ANOVA and expressed as mean with (SEM). B) HE-stained tumor sections were scored for mitotic activity. A representative photo from a non-treated tumor containing instances of mitotic activity (left) and mitotic activity scores in all indicated treatment groups (right). Four fields per section were scored for mitotic activity. Data indicates the average scores for four mice per group. Similar results were obtained in another independent experiment. Data is analyzed by ordinary one-way ANOVA followed by Tukey's post-hoc analysis (* $P < 0.05$ and ** $P < 0.01$).

The highest dose induced approximately 40% cell death and the lowest dose almost no cell death as measured by MTT assays (data not shown). After washing, the tumor cells were incubated with splenocytes containing 4% HPV16-specific CD8⁺ T-cells (data not shown). Although tumor cells were efficiently killed by these splenocytes in these short cytotoxicity assays, cisplatin-pretreatment did not enhance sensitivity towards CTL mediated killing

(Fig. 6c). Thus, the observed synergy between SLP vaccination and cisplatin cannot be explained by an increased sensitivity of cisplatin treated TC-1 tumor cells towards CTL mediated killing as measured by short chromium release assays. Therefore we hypothesized that the enhanced killing of tumor cells observed *in vivo* rather depends on prolonged and sustained cooperation between T-cell effector function and cisplatin activities on tumor cells.

Since we have seen that tumors from vaccinated mice are highly infiltrated with IFN- γ and TNF α producing T-cells, we hypothesized that the synergistic effect on tumor cell death was due to an enhancement of cisplatin-induced cell death by these cytokines. We therefore examined the effect of IFN- γ and TNF α on cisplatin-induced tumor cell death. By live apoptosis imaging it became apparent that co-incubation of cisplatin with various dosages of IFN- γ had no effect on cell death (Fig. 6d and data not shown). Strikingly, TNF α exposure strongly sensitized tumor cells to cisplatin-mediated killing (Fig. 6d, supplementary Fig. S4b). This observation was confirmed by performing an MTT assay with recombinant TNF α (Fig. 6e), and supernatant of overnight incubated single cell suspensions of peptide treated tumors (supplementary Fig. S4c).

To test whether TNF α sensitization of cisplatin-treated tumor cells is a general phenomenon, similar experiments were done with mouse tumor cell lines C3 and MCA 205 and human cervical cancer cell lines SiHa and HeLa, yielding comparable results (Fig. 6f, supplementary Fig. S4d,e). Similarly, the supernatant of activated PBMCs could sensitize HeLa tumor cells for cisplatin induced killing (Supplementary Fig. S4e and Fig. 6g). Since the addition of the TNF α inhibitor Etanercept fully abolished this synergy (Fig. 6g) we concluded that specifically the TNF α in the supernatant of these human T cells sensitized for cisplatin cell death. Also, we observed that TNF α could sensitize tumor cells for topotecan and carboplatin induced tumor cell death but not for paclitaxel induced apoptosis (Supplementary Fig. S4g)

Enhanced cell death of TC-1 cells was accompanied by a TNF α induced up-regulation of the anti-apoptotic molecule c-IAP2, and a cisplatin induced up-regulation of the pro-apoptotic gene Xaf1. Remarkably, treatment with both cisplatin and TNF α strongly enhanced expression of the pro-apoptotic molecule Bfk, suggesting a crucial role for this molecule in the observed synergy (Fig. 6h). Expression of the anti-apoptotic molecules Bruce and Bcl-xL was not altered by TNF α , cisplatin or combined exposure (data not shown).

TNF α binding to TNFR1 and TNFR2 can induce signaling leading to either cell survival or cell death. The activation status of the MAPK family member JNK determines cellular outcome (27). Additionally, it has been found that combined cisplatin/TNF α treatment of renal proximal tubular cells induces JNK activation, leading to a shift in apoptotic programming and, causing tubular cell apoptosis at much lower cisplatin levels than in the absence of TNF α (28). We hypothesized that a similar mechanism may play a role in the synergy between vaccine-induced T-cell responses and cisplatin treatment. When TC-1 tumor cells were pre-treated with the JNK inhibitor SP600125, cisplatin-TNF α induced cell death was largely inhibited (Fig. 6i), indicating that JNK activation is important in the TNF α -mediated sensitization of tumor cells to cisplatin induced killing. Next, we used TNF α neutralizing antibodies to examine the role for TNF α in the synergy between cisplatin and peptide vaccination observed *in vivo*. Strikingly, the use of TNF α neutralizing antibodies strongly decreased the synergy between cisplatin and peptide vaccination (Fig 6j).

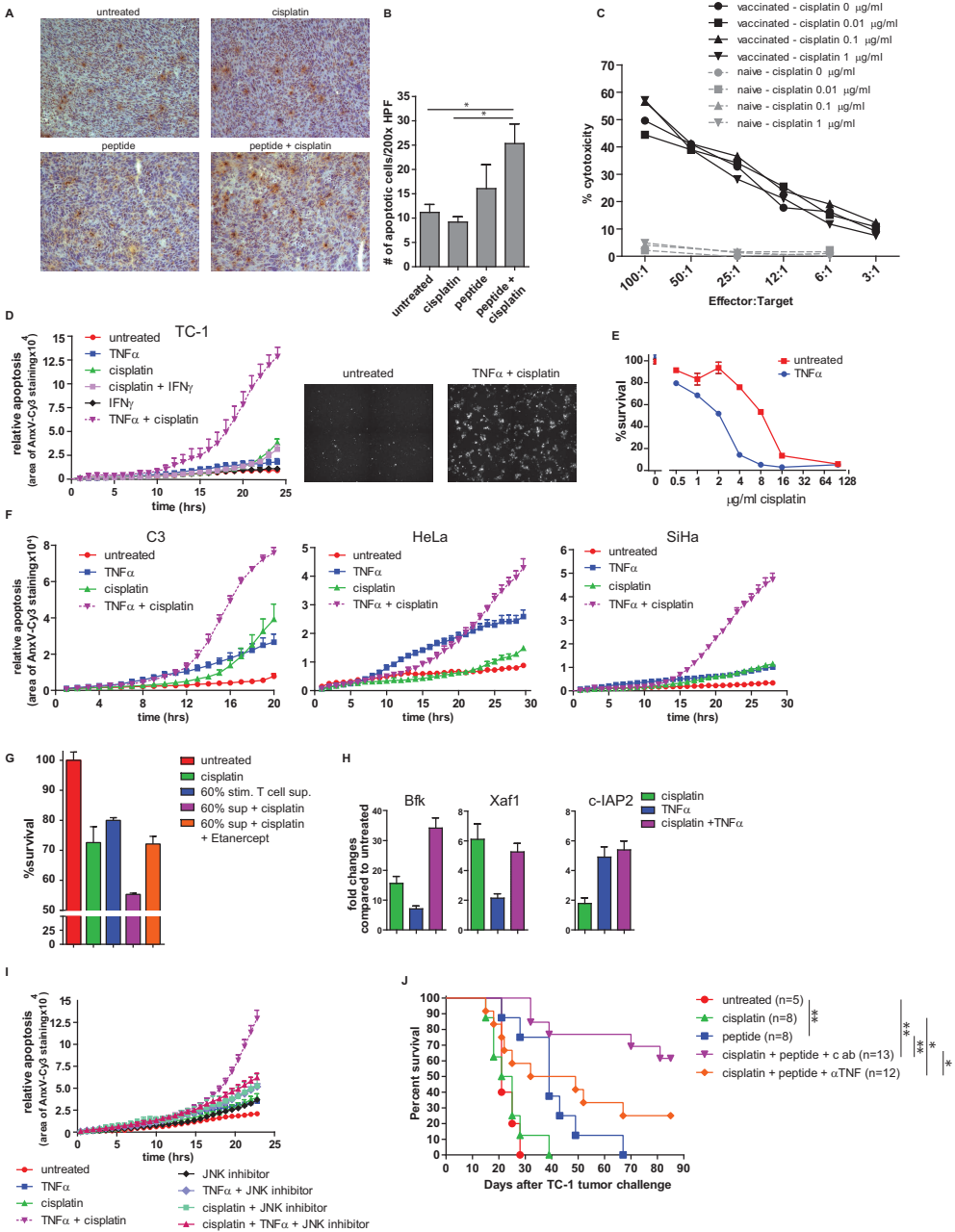


Figure 6. TNFα sensitizes for cisplatin mediated killing allowing synergistic apoptosis of tumor cells in a JNK dependent fashion.

Mice were inoculated on day 0 with 1×10^5 tumor cells. On day 9, when a tumor of 4-10 mm² was present, mice were treated with HPV16 E7₄₃₋₇₇ peptide. Six days later, on day 15, half of these mice received cisplatin treatment. Tumors were dissected, stained with TUNEL technique and analyzed while blinded for treatment. A) Representative photos from day 19 tumors. B) Graphical representation of TUNEL counts per 200x HPF for 4 sections per tumor (n=4 mice) at day 19 (right). Data is analyzed by one-way ANOVA followed by Tukey's post-hoc

Together these data show that TNF α sensitizes for cisplatin in a JNK dependent manner causing synergistic cell death. This is confirmed by the observation that tumors from the combined treatment group contain significantly more apoptotic tumor cells than tumors from single-treated mice. Also, we show that vaccination induces TNF α producing T cells in the tumor. When the mice, treated with peptide vaccination and cisplatin, receive TNF α neutralizing antibodies, the synergy between the two treatments is strongly decreased, emphasizing the important role for TNF α in the cisplatin + peptide induced antitumor response.

DISCUSSION

In the present study, we show that SLP vaccination combined with clinically relevant chemotherapeutic agents acts synergistically in tumor eradication. Combined treatment of low dose cisplatin with peptide significantly enhanced survival compared to single treatment with high dose cisplatin. Effective antitumor responses induced by the combined treatment were accompanied by enhanced leukocyte infiltration into the tumor, in particular by vaccine-specific CD8⁺ T-cells. Since our vaccination protocol includes subcutaneous antigen delivery in Montanide in the opposing flank, T-cells must have been actively attracted to the contra-lateral tumor site. In the local tumor environment these T-cells produce TNF α and IFN- γ , indicating that they are fully functional.

Reportedly, TNF α and IFN- γ can drive cancer cells into senescence, corresponding to a decreased Ki-67 expression (29). Although the cisplatin-treated TC-1 tumors infiltrated with vaccine-induced TNF α and IFN- γ producing T-cells show a decreased proliferation *in vivo*,

- analysis (*P < 0.05). C) Tumor-free C57BL/6 mice were immunized with HPV16 E7₄₃₋₇₇ peptide. Splenocytes were isolated, stimulated with peptide for 7 days, and used as effectors in a four hour ⁵¹Cr-release CTL assay against IFN- γ pretreated TC-1 target cells. 4% of the effectors were E7-specific (as determined by tetramer staining). TC-1 target cells were treated overnight with indicated doses cisplatin and then extensively washed. As a control T-cells from naive mice were used. Data represents nine individual experiments. D) TC-1 cells were *in vitro* exposed to cisplatin, IFN- γ and TNF α . Cell death was followed over time using Annexin-V staining and automated imaging on a BD pathway 855 imager (left). Still images of time laps movies were taken at 22 hours after cisplatin and TNF α addition (right). E) Dose response of TC-1 cell survival upon exposure to cisplatin in the presence (blue) or absence (red) of TNF α as measured by MTT assay. Representative result of 5 independent experiments. F) Annexin-V staining for tumor cells C3, HeLa and SiHa exposed *in vitro* to TNF α and cisplatin. G) HeLa cells were *in vitro* exposed to 2 μ g/ml cisplatin, 60% supernatant of PHA-stimulated PBMCs of a healthy donor and the TNF α inhibitor Etanercept (40 μ g/ml). The amount of viable cells was analyzed by MTT assay. Data shown is representative for three individual experiments. H) Gene expression of the pro-apoptotic genes Bfk and Xaf1 and the anti-apoptotic gene c-IAP2 in TC-1 cells 22 hours after exposure to cisplatin and TNF α , represented as fold change over untreated TC-1 cells. Representative for 3 independent experiments. I) TC-1 cells were incubated with 40 μ M of the JNK-specific inhibitor SP600125, 30 minutes prior to cisplatin/TNF α treatment and cell death was measured by Annexin-V staining and automated imaging on a BD pathway 855 imager. Representative for 3 independent experiments. J) Wild-type C57BL/6 mice were injected s.c. with TC-1 tumor cells in the flank. From day 7 and onwards mice received TNF α neutralizing antibodies via i.p. injection. On day 8, when tumors were palpable, mice were treated systemically with cisplatin and/or HPV16 E7₄₃₋₇₇ peptide in Montanide in the opposing flank. Chemotherapy was repeated one week after initial treatment and vaccination was repeated 2 weeks after initial treatment. Shown is a Kaplan-Meier survival plot for chemo (immune-) therapy with TNF neutralizing antibodies. Significance and number of mice per group is indicated (*P < 0.05 and **P < 0.01).

in vitro treatment of tumor cells with TNF α , IFN- γ and/or cisplatin, was not associated with decreased Ki-67 and PCNA levels (measured by qPCR, data not shown). This suggests that, at the doses used, TC-1 tumor cells were not driven into senescence by TNF α and IFN- γ . The two HPV16 oncoproteins E6 and E7 are expressed by TC-1 tumors (18). A recent study on mouse keratinocytes reported that the oncoprotein E7 can interfere with IFN- γ mediated JAK1/JAK2/STAT1/IRF-1 signalling pathways (30). Since STAT1 deficient β -cancer cells resisted TNF α and IFN- γ induced senescence (29) we hypothesize that E7 expression by TC-1 tumor cells protects them from complete senescence.

Over one third of patients treated with cisplatin develop renal injury within 10 days after a single dose of cisplatin. A molecule strongly associated with cisplatin-induced (nephro-) toxicity is TNF α (28, 31-33). Although this cytokine has been proposed as a strong trigger of cancer cell death (34), its life-threatening toxicity when administered systemically prohibits systemic clinical use (31, 35). Intra-tumoral administration of TNF α remains challenging for some primary tumors and especially for metastasis. In contrast to renal parenchymal cells (36), TC-1 tumor cells did not express TNF α after cisplatin treatment (data not shown). Strikingly, we show here that SLP vaccination induces large numbers of TNF α -producing T-cells that are likely further stimulated to produce TNF α by antigen encounter within the tumor. In turn this allows synergistic cell death of tumor cells when combined with cisplatin (Figure 6), and other (carboplatin, topotecan) but not all chemotherapeutics. Previously, it was observed that HPV16 E7-specific immune responses combined with cisplatin treatment can enhance apoptotic tumor cell death (37, 38). Here we confirm these observations and additionally show that TNF α has a key function in this enhanced cell death, indicated by the decreased synergy between cisplatin and vaccination in anti-tumor responses when TNF α was neutralized by systemic antibody treatment. Also, incubation of tumor cells with TNF α combined with low doses of cisplatin can enhance pro-apoptotic molecule expression in tumor cells and thus increases the level of cell death. Since cisplatin with TNF α mediated apoptosis is induced after a minimum of ten hours, this mechanism is likely not involved in short *in vitro* cytotoxicity assays as performed us (Fig. 6c) and others (37, 39). However, the synergy is relevant when TNF α producing T-cells and cisplatin are together with tumor cells for a longer period as occurs in the *in vivo* situation.

In addition to the previously described chemotherapy-enhanced sensitivity for granzyme B-mediated tumor cell death by intratumoral T-cells (39, 40), the synergy between TNF α and cisplatin is a novel mechanism involved in successful chemo-immunotherapy. We postulate that both mechanisms can be involved in previous studies in which synergy was observed between cisplatin and efficient T-cell based immunotherapy (9, 37, 38, 41-44).

In addition to cisplatin induced cell death, TNF α can enhance doxorubicin-induced apoptosis (45). However, it was recently shown that although doxorubicin enhances the RNA levels of TNF α by tumor cells, this cytokine is not involved in doxorubicin-induced anti-tumor responses in various tumor models (46). The levels of TNF α induced by the vaccine induced CD8⁺ T-cell infiltrated in the tumor beds are likely to be much higher than the levels produced by chemotherapy treated tumors, explaining why these T-cells can potentiate cisplatin mediated apoptosis, resulting in the remarkable synergistic anti-tumor activity of cisplatin and SLP vaccination seen in the current work. These data show that the introduction of different

forms of active T-cell-based immunotherapies into clinical practise calls for detailed analyses of the different forms of chemotherapy that best synergize with and support the immunotherapy. What is a scourge in the toxicity of cisplatin towards kidney tubular epithelial cells (28, 36) turns out to be a blessing in the enhanced demise of tumor cells helped along by the locally high levels of TNF α produced by vaccine-induced tumor-specific T-cells.

Other investigators have shown marked differences in the induction of immunogenic cell death by different chemotherapeutic compounds (47). This is obviously of major importance in the absence of additional robust stimulation of tumor-specific T-cell responses, such as possible by SLP vaccination. In the case of the current study, induction of a therapeutic T-cell immune response by chemotherapy itself is of no major consequence, because of the robust SLP vaccine that is capable of inducing a powerful therapeutic immune response by itself. Upon vaccination combined with cisplatin, the tumor micro-environment is highly infiltrated with leukocytes, including the HPV-specific, cytokine producing, tumoricidal T-cells. This work shows that together with TNF α produced by the abundant T-cells in the tumor, cisplatin enhances cell death and causes decreased proliferation of tumor cells. These data elucidate mechanisms by which therapeutic vaccination synergizes with cisplatin for efficient systemic anti-tumor T-cell responses, providing a strong rationale for implementation of this type of chemo-immunotherapy.

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