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VACCINE-INDUCED CD8 T CELL EXPANSION IS PAUSED BY TOPOTECAN YET STILL RESULTS IN SUPERIOR T CELL-MEDIATED TUMOR ERADICATION

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Work in progress

ABSTRACT

Purpose

The survival of cervical cancer patients treated with both cisplatin and topotecan is enhanced when compared to patients treated with only one of those chemotherapeutics. In mice, cisplatin and T cell-based immunotherapy have been shown to synergize resulting in strong anti-tumor responses. Here we explored whether T cell-based immunotherapy also synergizes with this dual chemotherapy treatment.

Experimental design

Mice bearing a human papilloma virus 16 (HPV16) E6 and E7 oncogene-positive TC-1 tumor were vaccinated with a HPV16 E7 long peptide and also received cisplatin and topotecan. Tumor sizes were measured and the T cells in the tumor microenvironment were analyzed. Furthermore, adoptive transfer of congenically marked T cells as well as vaccination experiments allowed dissection of the effect of chemotherapy on the kinetics and function of vaccine-induced T cell responses.

Results

While the expansion of vaccine-induced CD8 T cells was initially severely inhibited by topotecan, these cells recovered and robustly expanded in numbers. Also the contraction phase of the vaccine induced T cell response was prolonged by topotecan treatment. Similar effects on T cells were observed when TC-1 tumor bearing mice were vaccinated and treated with both cisplatin and topotecan. However, this resulted in CD8 T-cell dependent durable tumor rejections and 95% survival.

Conclusions

Taken together, our data show that although topotecan delays T cell expansion, triple treatment with cisplatin, topotecan and vaccination results in superior tumor eradication. These results make it worthwhile to further elucidate the processes involved in this synergy.

INTRODUCTION

The goal of cancer immunotherapy is to activate and boost tumor specific immune responses. Therapeutic cancer vaccination employs immunogenic delivery of tumor specific antigens to induce a tumor specific CD4+ and CD8+ T cell response. A significant proportion of human cancers are caused by viruses. An example of such a virus is high risk Human Papilloma Virus (HPV). High risk HPV infections are associated with several malignancies and virtually all cases of cervical cancer are caused by HPV (1). The virus encodes two oncogenes (E6 and E7) that are crucial for the induction and maintenance of cellular transformation and are constitutively expressed by malignant cells, making them ideal targets for therapeutic vaccination (2-4). The lack of central tolerance against viral oncogenes makes them as well as mutation-based neo-epitopes ideal targets for therapeutic vaccination (5).

Previously we have shown that therapeutic vaccination with overlapping synthetic long peptides (SLP) targeting the oncogenes E6 and E7 of HPV results in the induction of cytokine producing CD8 T cells and in clinical responses in mice and patients with (premalignant-) lesions (6-10). Interestingly, enhanced clinical responses in patients correlated with a stronger and broader HPV-specific T cell response (6, 8). However, when patients with advanced or recurrent gynecological lesions are vaccinated, no apparent effect on survival is observed (11, 12) and chemotherapy remains a valuable treatment option for this group of patients. Previously we have used a relevant mouse model for HPV induced cancer to test whether SLP vaccination could be combined with chemotherapy to improve the clinical effect. Although some chemotherapeutics tested had no effect on the anti-tumor effect of SLP vaccination, others, including topotecan and cisplatin, improved survival (7). In the case of cisplatin, vaccine-induced T cells were not directly affected by chemotherapy, but they augmented chemotherapeutic related tumor destruction via the release of TNFα, which was shown to act synergistically with cisplatin to promote tumor cell apoptosis (7).

The survival of cervical cancer patients treated with cisplatin in combination with the water-soluble derivative of camptothecin, topotecan, is enhanced compared to single treatment with cisplatin (13, 14), while the quality of life of the patients is similar (15). Therefore, we hypothesized that dual treatment with cisplatin and topotecan, combined with SLP vaccination could be an attractive novel treatment modality for cervical cancer patients.

To examine the effect of cisplatin and topotecan on the vaccine-induced tumor-specific T cell response we analyzed both the systemic as well as the intratumoral T cell response in multiple models. The effects of topotecan initially appeared detrimental for the SLP induced T cell response. However, this effect was only temporally and T cells expanded vigorously after topotecan treatment was stopped. Importantly, combination therapy of SLP vaccination, cisplatin and topotecan to treat TC-1 tumor bearing mice was highly effective and resulted in durable, complete tumor regressions in 95% of the mice. This indicates that such combination therapy might be beneficial for cancer patients.

MATERIALS AND METHODS

Mice

Six to eight week old female C57BL/6JRccHsd mice were purchased at Harlan Laboratories (Horst, Netherlands) and housed in the central animal facility of the Leiden University Medical Center (LUMC; Leiden, the Netherlands). The ovalbumin-specific T cell receptor (TCR) transgenic OT-I mice, on a C57BL/6 background, were bred in house. All mice were housed in individually ventilated cage (IVC) systems under specific pathogen-free conditions. Experiments were approved by the Animal Experiments Committee of the LUMC, in line with the guidelines of the European Commission.

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 the HPV16 E6 and E7 and c-H-*ras* oncogenes (16). The TC-1 tumor cell line and the colon adenocarcinoma cell line MC-38 were both cultured in Iscove's Modified Dulbecco's Medium (IMDM; BioWhittaker) supplemented with 8% fetal calf serum (FCS), 50 µmol/L 2-mercaptoethanol, and 100 IU/mL penicillin/streptomycin and 2 mmol/L glutamine. TC-1 culture medium was also supplemented 400 µg/ml Geneticin (G418; Life Technologies), nonessential amino acids (Life Technologies), and 1 mM sodium pyruvate (Life Technologies).

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Vaccination experiments

On day 0, C57BL/6JRccHsd mice were subcutaneously inoculated with 1×10^5 TC-1 tumor cells or 1×10^5 MC-38 tumor cells in 200-µL PBS and 0.2% BSA in the right flank. When a palpable tumor was present (day 8), mice were split into groups with comparable tumor size and treated with 150 µg synthetic long peptides (SLP) and /or chemotherapy. Cisplatin (4 mg/kg, intraperitoneal injection (i.p.)) was provided on day 8 and 15, topotecan (2 mg/kg, i.p) was provided on days 8, 9, 10, 11 and 15, 16, 17, 18) as previously described (7). TC-1 tumor bearing mice were vaccinated subcutaneously (s.c) in the left flank with synthetic long HPV16 $E7_{43-77}$ peptide (GQAEPDRAHYNIVTFCCKCDSTLRLCVQSTHVDIR) dissolved in PBS and 1:1 emulsified in Montanide (ISA-51, Seppic). MC-38 tumor bearing animals were treated with synthetic long *Reps1* peptide (ELFRAAQLANDVVLQIMELCA) dissolved in PBS with 30 µg CpG (ODN1826, InvivoGen). Analysis of the systemic T cell response was performed by flow cytometry, using HPV16 $E7_{49-57}$ peptide (RAHYNIVTF) loaded H-2D^b tetramers (HPV16 E7 vaccination experiments) or AQLANDVVL loaded H-2D^b tetramers (*Reps1*/MC-38 vaccination experiments) in combination with 7-aminoactinomycin D (for dead exclusion; Invitrogen), CD3 (clone 500A2) and CD8α (clone 53-6.7).

For *in vivo* CD8$^+$ T-cell depletion, mice were injected i.p. 100 µg of the monoclonal antibody 2.43. All mice used had a >99% depletion as measured by flow cytometry. Slope (*m*) was calculated using the following formula *m = (mean % vaccine-specific CD8 T cells of total of CD8$^+$T cells on peak of response – mean % vaccine-specific CD8 T cells of total of CD8$^+$T cells on next time point measured) / (day of peak of response – day next time point measured)*

Adoptive transfer experiments

For adoptive transfer of OT-I cells, 0.5×10^6 OT-I (Thy1.1 or Ly5.1) T cells were negatively enriched via a $CD8^+$ enrichment kit (BD). The OT-I cells were carboxyfluorescein diacetate succinimidyl ester (CFSE) labelled and the transgenic T cells were then intravenously (i.v.) injected in Ly5.2/Thy1.2-recipient mice. The next day, mice were vaccinated, with the SLP, containing the SIINFEKL epitope to stimulate OT-I cells ($OVA_{241-270}$) together with 20µg CpG emulsified in montanide ISA-51. Cisplatin (4 mg/kg, i.p.) was provided on day 0, topotecan (2 mg/kg, i.p.) on day 0, 1, 2. The kinetics of the OT-I cells were measured in blood by flow cytometry, using CD3 (clone 500A2), CD8α (clone 53-6.7), KLRG1 (clone 2F1), Vβ5.1/5.2 (clone MR9-4), CD90.1 (clone Ox-7) and CD127 (clone A7R34, biotin) in combination with streptavidin (Brilliant Violet 605). Samples were analyzed with a BD LSR II flow cytometer, and results were analyzed using FlowJo software (Tree Star). Slope (*m*) was calculated using the following formula *m = (mean % OT-1 cells of $CD8^+$T cells on peak of response – mean % OT-1 cells of $CD8^+$T cells on next time point measured) / (day of peak of response – day next time point measured)*

Analysis of tumor-infiltrating immune populations

For analysis of intratumoral immune subsets TC-1 tumor bearing mice were vaccinated on day 8, treated with cisplatin on day 14 and treated with topotecan on days 14, 15, 16. On days 17 and 21, mice were sacrificed and transcardially perfused with PBS-EDTA. Isolated tumors were disrupted in small pieces and incubated for 15 minutes at 37°C in IMDM—containing Liberase (Roche) after which the tumors were minced through a 70-µm cell strainer (BD Biosciences) to obtain single cell suspensions. After a short incubation with Fc-block and naïve mouse serum, cells were resuspended in staining buffer (PBS + 2% FSC + 0.05% sodium azide) and incubated with HPV16 $E7_{49-57}$ peptide (RAHYNIVTF) loaded H-$2D^b$ tetramers, 7-aminoactinomycin D (for dead exclusion; Invitrogen), CD19 (clone 1D3, to exclude samples containing > 5% B cells and therefor many PBMCs), CD8α (clone 53–6.7), CD3 (clone 145-2C11), CD45.2 (clone 104), class II (clone M5/114.15.2), 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).

Statistical analysis

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 $P < 0.05$ were considered significant.

RESULTS

Triple treatment of cisplatin, topotecan and SLP vaccination results in superior, CD8 dependent durable rejections of established tumors.

The survival of cervical cancer patients treated with cisplatin in combination topotecan, is enhanced compared to single treatment with cisplatin (13, 14). To test whether the addition of synthetic long peptide vaccination to this treatment regimen further improves survival, mice were subcutaneously inoculated with the HPV16 E6 and E7 expressing TC-1 tumor cell line and tumor size was measured in time. When a palpable tumor was present on day 8, treatment was started (Figure 1A). As previously observed, single treatment with cisplatin or topotecan only had a modest effect on survival. However, the combined treatment of these chemotherapeutics resulted in delayed tumor growth and improved survival (cisplatin vs. cisplatin + topotecan p=0.0102, Figure 1A). Single treatment with SLP induced a strong but temporary decrease in tumor size, however the addition of topotecan or cisplatin improved vaccine related survival (Figure 1A, B (7)). Strikingly, triple treatment with cisplatin, topotecan and SLP vaccination resulted in durable tumor rejection in nearly all mice (95% survival upon peptide with dual chemotherapy compared to 65% of the mice receiving vaccine with cisplatin and 25% for mice receiving vaccine with topotecan). Importantly, when CD8 T cells were depleted in animals treated with cisplatin, topotecan and vaccine, none of them survived, indicating that CD8 T cells are crucial for durable anti-tumor responses. However, these animals lived significantly longer than untreated mice, presumably due to the debulking effect of chemotherapy (Figure 1B). These data show that the combination of cisplatin, topotecan and SLP vaccination results in durable rejections of established tumors.

Given the importance of the CD8 T cells we analyzed the intratumoral T cells. Since tumor size can severely affect immune responses (17, 18) we utilized a treatment protocol in which we know that cisplatin also synergizes vaccination (7) and the tumors were analyzed at the peak of the vaccine-induced response in mice with similar tumor sizes (Figure 1C, supplemental Figure 1). A strikingly low number of CD8 T cells was found in the tumors of mice treated with vaccine + topotecan and of mice treated with vaccine + cisplatin + topotecan (Figure 1D), indicating that topotecan but not cisplatin affected the levels of intratumoral T cells. Interestingly, the vaccine-specific T cells intratumoral (characterized as HPV16 E7-specific tetramer-positive CD8 T cells) were more severely affected by topotecan as the total number of T cells (Figure 1E). Together these data show that peptide + cisplatin + topotecan results in superior tumor rejection. Although this effect depends on CD8 T cells, barely any CD8 T cells were detectable within the tumor microenvironment at the time of analysis when topotecan was part of the treatment regimen.

Topotecan causes a temporary block in T cell proliferation.

Given the importance of CD8 T cells in vaccinate + chemotherapy related regression we further examined the kinetics of HPV-specific T cell in tumors of vaccinated mice. Given the importance of T cells in the observed tumor rejections, we hypothesized that topotecan treatment might have affected T cell proliferation. To test this we analyzed the levels of the proliferation marker Ki-67 on these cells.

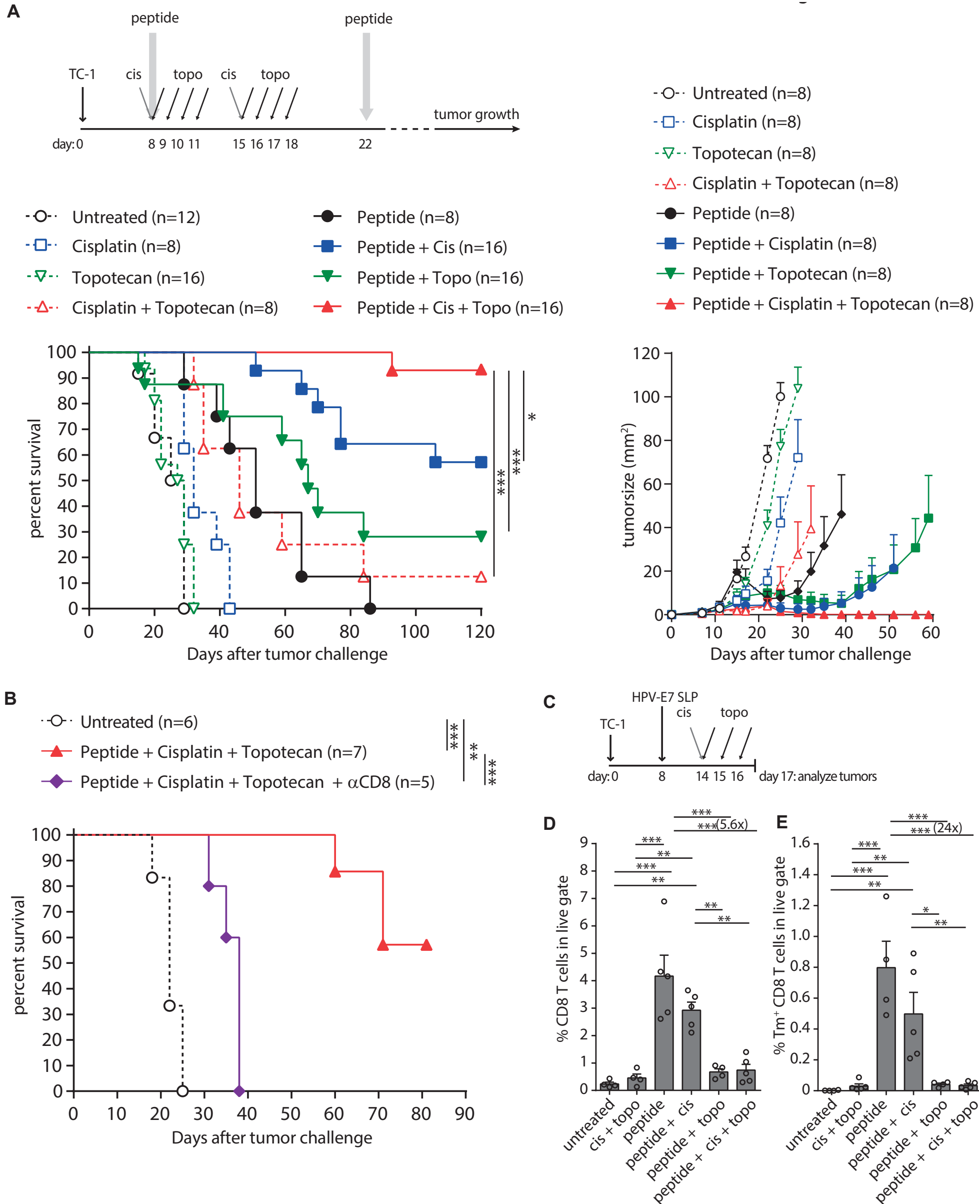

Figure 1. Although strong synergy between SLP vaccination, cisplatin and topotecan is CD8 T cell dependent, low numbers of SLP induced T cells identified in tumor.

A-B) Wild-type C57BL/6 mice were injected s.c. with $1x10^5$ TC-1 tumor cells. Eight days later, when tumors were palpable, mice were treated systemically with chemotherapeutics with or without HPV16 $E7_{43-77}$ peptide in Montanide in the opposing flank. Chemotherapy was repeated 1 week after initial treatment and vaccination was repeated 2 weeks after initial treatment. A) Schematic diagram of the therapy regimen is shown in the top left corner, Kaplan–Meier survival plot, pooled from multiple experiments, at the left bottom. Average tumor growth per group (until the first animal had to be killed for ethical reasons) from one experiment is shown on the right side. The number of mice per group is indicated. B) Mice were treated according to the setup as shown in A; however, one group (purple diamonds) received CD8 depleting antibodies from day 7 and then every six days.

Number of mice is indicated. C-E) To ensure similar tumor sizes on day 17, animals were treated with vaccine on day 8 and 6 days later with chemotherapy (cisplatin on day 14, topotecan on day 14-16). C) Schematic diagram of the therapy regimen. Tumors were dissected on day 17 and analyzed by flow cytometry. Shown is the (D) percentage of CD8 T cells and (E) vaccine-specific CD8 T cells (as determined by H2-D^b $E7_{49-57}$ (RAHYNIVTF) tetramer staining) within the tumor (live gate). Indicated is the fold decrease between "peptide" and "peptide + cisplatin + topotecan" group. Shown is the mean + SEM as determined by one-way ANOVA followed by Tukey *post hoc* analysis (*, $P < 0.05$ and **, $P < 0.01$, ***, $P < 0.001$).

We observed that the vaccine-induced CD8 T cells of animals receiving no chemotherapy displayed a high Ki-67 expression on day 9 after vaccination (day 17 of experiment) but a much lower Ki-67 expression four days later, indicating contraction of the T cell response (Figure 2A). This contraction was less pronounced when cisplatin was added to the treatment. The addition of topotecan, however, resulted in a much lower expression of Ki-67 on the vaccine-induced T cells on day 9 after vaccination. Strikingly, this expression significantly increased four days later, indicating that at this time the vaccine-activated T cells were vigorously proliferating and underwent an expansion period (Figure 2A, Supplemental figure 2A). Together these data indicate that topotecan affects predominantly fast-proliferating vaccine-induced T cells (Figure 1E, 2A), but also show that the antigen specific T cells can expand after topotecan treatment is stopped.

To confirm that topotecan affected T cell proliferation, CFSE labeled OVA specific transgenic OT-I T cells were *in vitro* stimulated with the corresponding peptide in the presence of increasing amounts chemotherapy. While no clear relationship between the cisplatin concentration and CFSE expression was observed, the topotecan concentration, in a dose-dependent manner, reciprocally corresponded with the level of proliferation (supplemental Figure 2B).

To further study the effect of topotecan on a T cell expansion we made use of congenically marked OT-1 T cells. This allowed us to fully control the number of T cells that can respond to vaccination and allows us to closely follow this response. We specifically asked how topotecan affects proliferation of T cells responding to vaccination and, at the same time compare how unstimulated, endogenous T cells are affected by this chemotherapy treatment. To this end, mice were injected i.v. with CFSE labeled OT-I TCR transgenic T cells on day -1. The next day (day 0), these animals were vaccinated with the SLP, comprising the epitope recognized by OT-I T cells, mixed with 20 μg Cpg and emulsified in IFA. On the same day, mice received systemic treatment with cisplatin (day 0) and topotecan (day 0, 1, 2). As previously observed, cisplatin treatment had no effect on the number of OT-I T cells and the response peaked on day 6 after vaccination (Figure 2B, (7)). In contrast, mice receiving topotecan had similar numbers of OT-I cells as non-vaccinated mice on day 3 and the T cell response peaked on day 9 (Figure 2B). However, the percentage of endogenous T cells on days 3 and 6 after vaccination in mice receiving topotecan were comparable (supplemental Figure 2C), indicating that topotecan specifically affects the adoptively transferred cells, probably because these T cells, during their response to specific antigen, were particularly vulnerable to this chemotherapeutic.

Next, we analyzed whether the kinetics of the Ki-67 expression of OT-I cells corresponded to their numbers and hypothesized that high Ki-67 levels would precede the peak of the T cell response. Indeed the OT-I cells of mice receiving vaccination without chemotherapy displayed

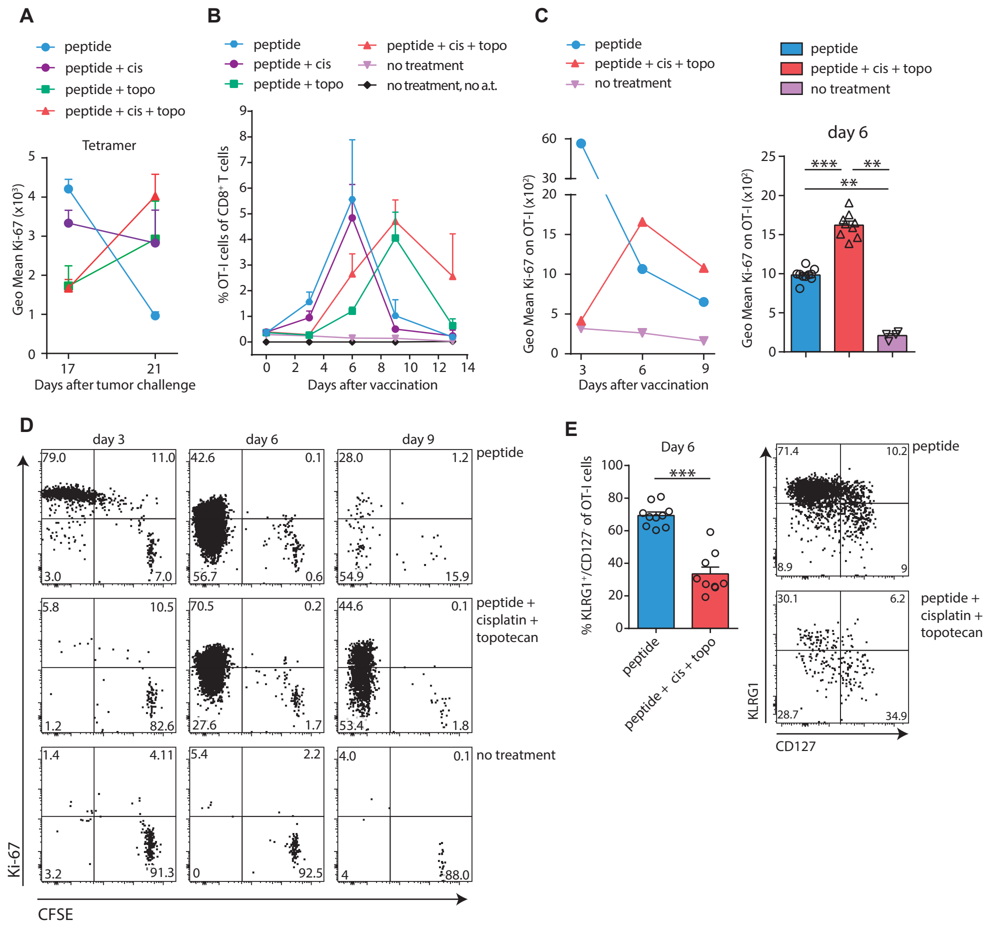

Figure 2. Topotecan delays kinetics of SLP vaccine induced T cell responses.
A) Mice were treated as in figure 1C, tumors were dissected and analyzed by flow cytometry. Shown is the Ki-67 expression on H2-D^b E7$_{49-57}$ Tetramer$^+$ T cells on day 17 and day 19. Data is representative for two individual experiments. B-E) 0.5 × 10^6 Thy1.1$^+$ congenically labeled OT-I T cells were injected i.v. in Thy1.2$^+$ recipient mice on day -1. The next day (day 0), mice were vaccinated with the SLP containing the (SIINFEKL) epitope. Cisplatin was provided on day 0, topotecan on day 0-2. A) The percentage of OT-I T cells in blood was measured on days 3, 6, 9 and 13. B) Samples from one treatment group were pooled and the geometric mean of Ki-67 expression on each time point was plotted. Numbers of cells per animal on day 6 were sufficient to plot data from each individual animal and perform statistics (analyzed by Mann Whitney T test). D) Samples from one treatment group were pooled, dot plots shown indicate Ki-67 and CFSE expression on OT-I cells. E) Percentage of SLECs of OT-I cells on day 6 is plotted in the left section, each symbol represents an individual animal (analyzed by Mann Whitney T test). Representative dot plots are shown on the right. Shown is the mean + SEM (*, $P < 0.05$ and **, $P < 0.01$, ***, $P < 0.001$).

a peak in Ki-67 expression on day 3, while OT-I cell numbers in mice treated with topotecan peaked on day 6. Moreover, we observed that Ki-67 expression in OT-I T cells of mice receiving vaccination in conjunction with cisplatin and topotecan had a remarkably low Ki-67 expression on day 3. The high Ki-67 expression peaked on day 6, corresponding to a peak in the number of

OT-I T cells on day 9. Interestingly, not only were the numbers of OT-I cells in chemotherapy-treated animals on day 9 comparable to the numbers of OT-I cells on day 6 in mice that did not receive topotecan, also the expression of Ki-67 and CFSE on day 9 of triple treated animals was similar as in vaccinated animals on day 6. Together these data show that proliferation of vaccine-activated T cells is severely affected by topotecan, but also that these T cells quickly recover after topotecan treatment, resulting in a delayed peak of the specific T cell response. To further confirm this observation we measured the number of short lived effector cells (SLEC), a group of effector cells that is prone for cell death via apoptosis and is characterized by a KLRG1 expression and an absence of the IL-7 receptor (CD127) (19). Indeed we observed an enhanced number of SLEC T cells in mice that received vaccination in the absence of chemotherapy on day 6 after vaccination, confirming an effect of chemotherapy on T cell kinetics (Figure 2E).

When we further analyzed the kinetics of the T cell response we observed that the contraction of the OT-I response in animals receiving topotecan appeared to be slower: the slope (m) of the % OT-I cells on peak of the response and the next moment measured was -0.537 for the triple treatment group and -1.512 for animals that were vaccinated but received no chemotherapy. The result of this delayed contraction phase is that more T cells are present for a longer period of time. Together these data indicate that topotecan treatment strongly affects proliferation of antigen-specific T cells. When topotecan treatment is stopped, the T cells recover, vigorously expand and display a delayed contraction phase.

Effects of topotecan treatment on the kinetics of vaccine-induced T cells are observed for multiple vaccination protocols.

To examine whether topotecan also affects endogenous responses in a similar way we used 3 models. First, chemotherapy was provided from day 6 after vaccination. The antigen specific response in blood was followed by analyzing the HPV16 E7 tetramer binding of CD8 T cells until the first animals had to be killed due to a large tumor burden. Similar to the OT-I response, the kinetics of the T cell response in vaccinated animals receiving topotecan was delayed compared to that in mice that did not receive topotecan (Figure 3A). Then we repeated this experiment in non-tumor bearing mice where the vaccine-induced response could be followed for a longer period of time. Strikingly, not only was the antigen-specific T cell response in these mice delayed, the maximum number of vaccine-specific T cells at the peak of the response was also twice as high in the mice receiving topotecan and the contraction phase was prolonged (Figure 3B): the slope (m) of the % vaccine-specific cells on peak of the response and the next moment measured was -0.040 for the triple treatment group and -0.063 for animals that were vaccinated but received no chemotherapy. Again the non-vaccine specific $CD8^+$ T cells were not affected by chemotherapy (Supplemental Figure 2D and data not shown).

Next, MC-38 tumor bearing mice were vaccinated with the mutant peptide of *Reps1* (20). Cisplatin was provided on the same day as vaccination (day 6) and this was repeated one week later (day 13), while topotecan was provided on days 6-9 and 13-16. Most mice receiving only vaccination had to be killed for ethical reasons around day 24 and the T cell response could not be analyzed in this group. Mice receiving combination treatment of vaccination with chemotherapy lived longer but no vaccine-induced T cell response could be detected until day

24 (day 18 after vaccination), indicating that also with this neo-epitope vaccine, kinetics of the T cell response were delayed (Fig 3c). Together these data indicate that the effect of topotecan on antigen specific T cells is observed in multiple treatment protocols and with multiple vaccines.

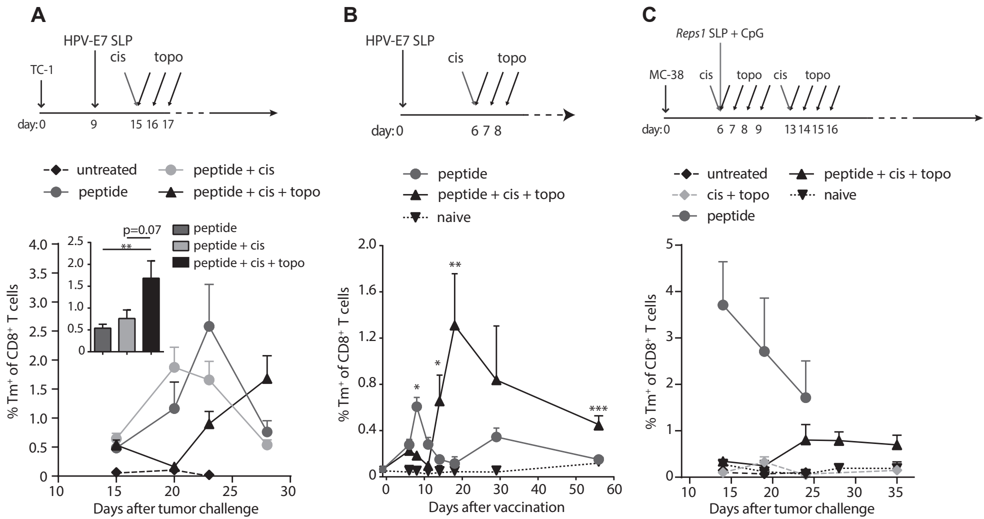

Figure 3. Kinetics of endogenous responses delayed by topotecan.
A) Wild-type C57BL/6 mice were injected s.c. with $1x10^5$ TC-1 tumor cells. Animals were treated with vaccine on day 9 and 6 days later with chemotherapy (cisplatin on day 15, topotecan on day 15-17). *N* = 5-7 mice per group. B) Wild-type C57BL/6 mice were vaccinated. Six days later, animals were treated with systemic chemotherapy (cisplatin on day 6, topotecan on day 6-8). Indicated are significant differences between "peptide" and "peptide + cisplatin + topotecan" treated animals. *N* = 4-8 mice per group, data is representative for 3 individual experiments. C) Wild-type C57BL/6 mice were injected s.c. with $1x10^5$ MC-38 tumor cells. Six days later, when tumors were palpable, mice were treated systemically with chemotherapeutics with or without HPV16 $E7_{43-77}$ peptide in Montanide in the opposing flank. Chemotherapy was repeated one week after initial treatment and vaccination was repeated 2 weeks after initial treatment. *N* = 6 mice per group, experiment is performed once. A-C) For each experiment a schematic diagram of the therapy regimen is shown on top. Presented at the bottom is a quantification of the percentage vaccine-specific cells, as determined by (figure A,B) H2-D^b $E7_{49-57}$ (RAHYNIVTF) tetramer staining, or (figure C) H2-D^b Reps1 (AQLANDVVL) tetramer staining within the $CD8^+$ T-cell population. Small bar graph in figure (A) shows the quantification of the tetramer staining as measured on day 28 after tumor challenge. Shown is the mean + SEM, data in (A) and (B) are analyzed by a Mann Whitney T test (*, $P < 0.05$ and **, $P < 0.01$, ***, $P < 0.001$).

DISCUSSION

Here we have shown that combination treatment of tumor-specific vaccination, cisplatin and topotecan results in a strong and durable cure rate of 95% of TC-1 tumor bearing mice. This effect is significantly stronger than combination of vaccine and single chemotherapeutic compound. While the clinical effect of this triple treatment combination is clearly dependent on CD8 T cells, topotecan affects the kinetics of vaccine-specific CD8+ T cell responses. The effect of topotecan on T cells can be roughly divided into three phases: First topotecan causes a pause in

T cell proliferation. Second, after topotecan treatment is stopped, the T cells enter into a delayed but robust expansion phase and finally a slower contraction phase. Together this indicates that topotecan suppresses vaccine-induced T cell responses, but only for the short time-period that the compound is actively present in the body (Figure 4).

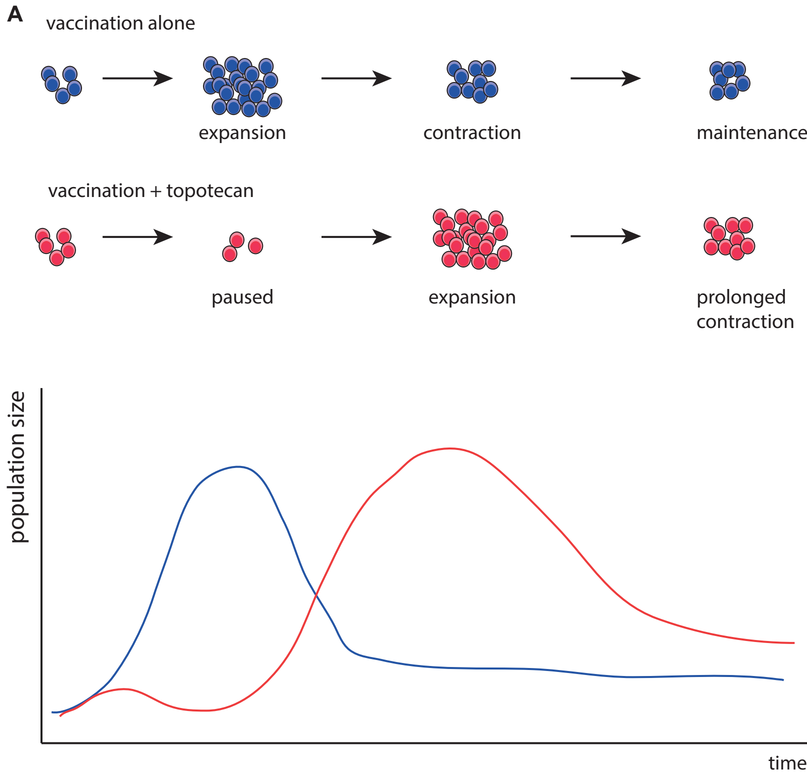

Figure 4. Model for the effect of topotecan on the antigen specific T cells.
A typical CD8 T cell response to vaccination is shown in blue. Upon presentation of antigen by antigen presenting cells to T cells they clonally expand. At the end of the expansion phase vaccine induced T cells undergo an extensive contraction phase, mediated by apoptosis. Some remaining memory CD8 T cells can persist for years. A CD8 T cell response to vaccination in the presence of topotecan is shown in red. Although initially some vaccine-specific CD8 T cells can be characterized, nearly all disappear upon topotecan treatment while some are paused for proliferation. Upon topotecan washout the CTLs rigorously proliferate, resulting in a strong expansion of the number of vaccine specific T cells. The contraction phase is prolonged resulting in more T cells long after vaccination.

We showed that vaccine stimulated OT-I T cells in animals receiving topotecan, unlike those in animals that received no topotecan, had barely proliferated three days after vaccination (Figure 2B). The percentage of OT-I cells in mice treated with vaccine + topotecan was similar to that in non-vaccinated animals, which suggests that the OT-I cells had not died as a result of topotecan. Indeed, the percentage of endogenous T cells in the same mice was not affected

(supplemental figure 2D), indicating that topotecan only hampered the proliferation of activated T cells. This data is corroborated by our *in vitro* observations that an increasing topotecan dose clearly correlates to a decreased T cell proliferation.

We have not formally excluded that vaccine induced T cells redistribute upon topotecan treatment and reappear in the blood a few days later. However, since the Ki-67 and CFSE dilutions display similar kinetics as the number of T cells identified in the blood of the mice, an actual pause in T cell proliferation by topotecan treatment seems the only valid explanation for the unique T cell kinetics observed upon topotecan treatment.

Actually, topotecan is an S-phase specific chemotherapeutic drug (21). Interestingly, it has been shown that sensitivity to apoptosis of lymphocytes by another topoisomerase I inhibitor, etoposide, requires G1 to S-phase transition (22). T cells depend on binding of IL-2 to the high affinity IL-2 receptor to proceed true G1 to S-phase transition (23). The vigorous expansion, although delayed, of vaccine induced CTLs in topotecan-treated animals suggests that some T cells might have escaped the effects of topotecan, possibly via a temporary shutdown of IL-2 production preventing the transition to the S-phase.

Interestingly, when tumor-free mice were vaccinated and treated with cisplatin and topotecan, the peak of the T cell response was 8 days later then in control mice. Also, the percentage of specific CD8 T cells on the peak of the response was higher than in vaccinated mice that received no chemotherapy (Figure 3b). However, in the adoptive transfer setting, the peak of the response in the mice receiving topotecan was only 3 days later than the peak in the T cell response in control mice. Interestingly, the number of OT-I T cells on the peak of the response was comparable between mice that received topotecan and those that did not (Figure 2b). Given that costimulatory signals are important for T cell expansion and temporal differences in expression of various costimulatory signals may affect T cell immunity (24), it will be interesting to dissect the role of costimulatory molecules in the delayed expansion phase induced by topotecan.

The third phase of the T cell response is the (prolonged) contraction phase. The slope (m) of the contraction phase of the OT-I response in animals receiving cisplatin + topotecan is 2.3 times lower than in animals that received no chemotherapy (Figure 2B). The contraction of the T cell response in tumor-free mice in response to HPV16 SLP was also less pronounced (m is 1.6 times lower chemotherapy-treated mice, Figure 3B). Antigen-driven T cell responses contract due to apoptosis and this involves a sophisticated process that depends on the interaction between Bax or Bak with Bim on the mitochondrial surface and possibly decreasing levels of Bcl-2 (25-27). $Bim^{-/-}$ effector T cells give rise to functional and fit memory T cells (27) and this molecule might be affected by topotecan. Currently, we are performing experiments in which T cells are isolated at the peak of the response and levels of various apoptosis-related molecules are compared. Furthermore, SLECs are more prone to cell death as other T cell subsets (19). The high number of OT-I SLECs on day 6 in mice receiving vaccination but not chemotherapy suggests that these T cells are indeed more prone to contraction-phase related cell death as those isolated in topotecan-treated mice.

These effects of topotecan on T cell characteristics and kinetics may not only have implications for the design of chemo-immunotherapy treatment in patients but also for other

types of immunotherapy. Currently used treatment protocols often involve multiple cycles of immunotherapy of which the timing is based on our current knowledge about T cell kinetics. The optimal timing for booster vaccinations is during the late stages of effector to memory transition (28), a moment that is clearly delayed by topotecan. This calls for a careful evaluation of treatment schedules when immunotherapeutics are combined with topotecan and related cell-cycle specific chemotherapeutics. However, the superiority of triple treatment involving cisplatin, topotecan and vaccination over double treatment in anti-cancer treatment makes it worthwhile to further explore the processes involved in this synergy.

REFERENCE LIST

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SUPPLEMENTARY FIGURES

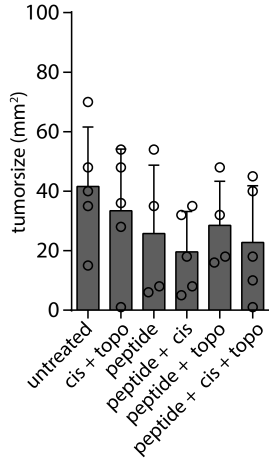

Figure S1. Similar tumor sizes when tumors were dissected.

Wild-type C57BL/6 mice were injected s.c. with $1x10^5$ TC-1 tumor cells. Eight days later, when tumors were palpable, mice were treated with HPV16 $E7_{43-77}$ peptide in Montanide in the opposing flank. Chemotherapy was started on day 14 (cisplatin on day 14, topotecan on day 14-16). Tumor sizes were measured on day 17. Shown is the mean + SEM, each dot represents an individual mouse. Data is analyzed by a one-way ANOVA test followed by a Tukey's multiple comparisons test, no significant differences were observed. Data is representative for 2 individual experiments.

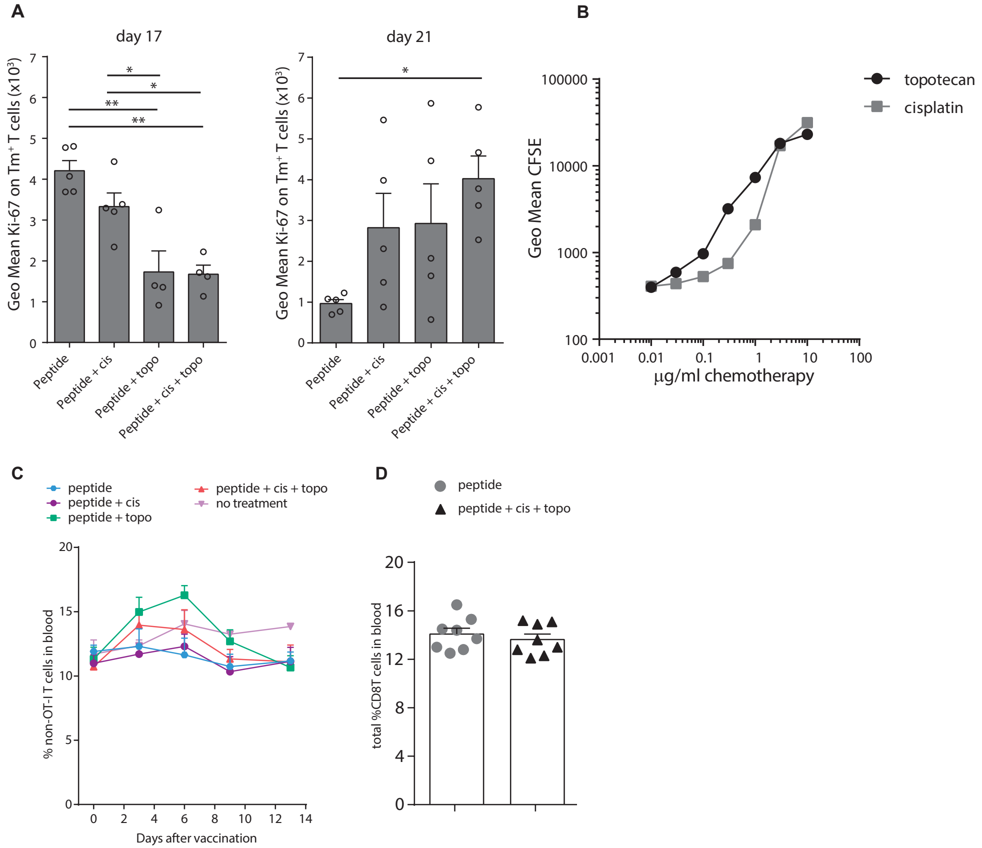

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Figure S2. Topotecan affects proliferation of antigen specific cells but other T cells are not affected.
A) Wild-type C57BL/6 mice were injected s.c. with 1x10^5 TC-1 tumor cells. Eight days later, when tumors were palpable, mice were treated with HPV16 E7$_{43–77}$ peptide in Montanide in the opposing flank. Chemotherapy was started on day 14 (cisplatin on day 14, topotecan on day 14-16). Ki-67 levels were analyzed on H2-D^b E7$_{49–57}$ Tetramer$^+$ T cells on day 17 and day 19. Shown is the mean + SEM, each dot represents an individual mouse. Data is analyzed by a one-way ANOVA test followed by a Tukey's multiple comparisons test (*, $P < 0.05$ and **, $P < 0.01$, ***, $P < 0.001$). B) 50.000 CFSE labeled OT-I cells were incubated with 50.000 OT-I cells and 10 µg/ml OVA$_{241-270}$ and increasing doses of chemotherapy. Three days later cells were washed and CFSE dilution was analyzed by flowcytometry. C) 0.5 × 10^6 Thy1.1$^+$ congenically labeled OT-I T cells were injected i.v. in Thy1.2$^+$ recipient mice on day -1. The next day (day 0), mice were vaccinated with the SLP containing the (SIINFEKL) epitope. Cisplatin was provided on day 0, topotecan on day 0-2. Shown is the percentage of non-OT-I T cells in blood. D) Wild-type C57BL/6 mice were vaccinated. Six days later, animals were treated with systemic chemotherapy (cisplatin on day 6, topotecan on day 6-8). Shown is the total percentage of T cells in blood. *N* = 4-8 mice per group, data is representative for 2 individual experiments.