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Delayed vaccine-induced CD8$^+$ T cell expansion by topoisomerase I inhibition mediates enhanced CD70-dependent tumor eradication

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ABSTRACT

Background The survival of patients with cervical cancer who are treated with cisplatin in conjunction with the topoisomerase I inhibitor topotecan is enhanced when compared with patients treated with only one of these chemotherapeutics. Moreover, cisplatin-based and T cell-based immunotherapy have been shown to synergize, resulting in stronger antitumor responses. Here, we interrogated whether topotecan could further enhance the synergy of cisplatin with T cell-based cancer immunotherapy.

Methods Mice bearing human papilloma virus 16 (HPV16) E6/E7-expressing TC-1 tumors were vaccinated with HPV16 E7 long peptides and additionally received chemotherapy consisting of cisplatin and topotecan. We performed an in-depth study of this combinatorial chemoimmunotherapy on the effector function and expansion/contraction kinetics of vaccine-induced CD8$^+$ T cells in the peripheral blood and tumor microenvironment (TME). In addition, we interrogated the particular role of chemotherapy-induced upregulation of costimulatory ligands by tumor-infiltrated myeloid cells on T cell proliferation and survival.

Results We show that E7 long peptide vaccination combined with cisplatin and topotecan, results in CD8$^+$ T cell-dependent durable rejection of established tumors and 94% long-term survival. Although topotecan initially repressed the expansion of vaccine-induced CD8$^+$ T cells, these cells eventually expanded vigorously, which was followed by delayed contraction. These effects associated with the induction of the proliferation marker Ki-67 and the antiapoptosis molecule Bcl-2 by intratumoral tumor-specific CD8$^+$ T cells, which was regulated by topotecan-mediated upregulation of the costimulatory ligand CD70 on myeloid cells in the TME.

Conclusions Taken together, our data show that although treatment with cisplatin, topotecan and vaccination initially delays T cell expansion, this combinatorial therapy results eventually in a more robust T cell-mediated tumor eradication due to enhancement of costimulatory molecules in the TME.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Topotecan, cisplatin and peptide vaccination work well when used in a dual combination but it remains unclear if the triple combination synergizes.

WHAT THIS STUDY ADDS

⇒ Combining peptide vaccination with cisplatin and topotecan increases antigen-specific CD8$^+$ T cell expansion and prolongs their persistence. In the tumor microenvironment, these CD8$^+$ T cells coexpress Ki-67 and Bcl-2, which is regulated by topotecan-mediated CD70 upregulation on myeloid cells.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study shows that triple therapy with vaccination, cisplatin and topotecan is an effective therapy strategy that can be translated into a clinical treatment protocol to optimize and prolong the effects of immunotherapy.

INTRODUCTION

Immunotherapy has revolutionized cancer treatment, with durable antitumor responses in a subgroup of patients. Since immunotherapy is more effective in patients with earlier stages of cancer than in patients with late-stage cancer, chemotherapy remains a valuable treatment option in patients with advanced, recurrent or metastatic disease. Over the past decade, numerous studies have examined various chemoimmunotherapy combinations. These studies have shown that chemotherapeutic drugs have different immune-potentiating effects and that combining chemotherapeutics could reinforce these properties,[1 2] but the underlying mechanisms behind effective combined chemotherapeutic strategies are often unknown.

Some cancer types are associated with human papilloma viral (HPV) infections. Oncogenic/high-risk HPV infections are

responsible for 4.5% of all cancers worldwide, including virtually all cases of cervical cancer.[3–5] 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 cancer vaccination.[6 7] Previously, we have used mouse models for HPV-induced cervical cancer to test whether different chemotherapeutics could be combined with therapeutic cancer vaccination with synthetic long peptides (SLP).[8 9] Although some chemotherapeutics tested had negligible effects on the vaccine induced-survival, others, including cisplatin and topotecan improved survival.[8]

The synergy between cisplatin and immunotherapy has been studied in detail. The underlying mechanisms include cisplatin-induced upregulation of the T cell costimulatory molecules CD80 and CD86, enhanced T cell infiltration, and T cell-produced TNF mediating increased cisplatin-induced killing of tumor cells.[8 10–16] In contrast to cisplatin, the interaction between topotecan and immunotherapy has been less well studied. However, it is clear that this topoisomerase I inhibitor can synergize with immunotherapy.[8 17 18] For example, it has been shown that exposure to topotecan upregulates Fas/CD95 in glioblastoma cells, enabling T cell-mediated glioma cytotoxicity,[18 19] and that topotecan induces activation of dendritic cells (DCs) with T cell stimulatory effects.[17 20]

The survival of patients with cervical cancer treated with cisplatin in combination with topotecan is improved, compared with single treatment with cisplatin.[21 22] Therefore, we hypothesized that dual treatment with cisplatin and topotecan, combined with SLP vaccination could be an attractive novel treatment modality for patients with cervical cancer. Here, we investigated the therapeutic interaction between cisplatin, topotecan and therapeutic vaccination and determined its impact on the kinetics and characteristics of circulating vaccine-specific $CD8^+$ T cell responses and immune cell infiltration into the tumor.

MATERIALS AND METHODS

Mice

Female C57BL/6J mice aged 6–8 weeks were purchased at Harlan Laboratories (Horst, Netherlands, C57BL/6JRccHsd) and Janvier Laboratories (Le Genest-Saint-Isle, C57BL/6JRj) and housed at the central animal facility of Leiden University Medical Center (LUMC; Leiden, the Netherlands). The ovalbumin (OVA)-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 systems under specific pathogen-free conditions.

Treatment schedules

Tumor cell line TC-1 (a kind gift from T.C. Wu, John Hopkins University, Baltimore, Maryland, USA) was generated by retroviral transduction of lung fibroblasts of C57BL/6 origin with the HPV16 E6 and E7 and c-H-*ras* oncogenes,[23] and maintained as previously described.[24] Tumor cell line C3 was generated by transfection of B6 mouse embryonic cells (MEC) with the complete HPV16 genome and maintained as previously described.[25] Mice were subcutaneously inoculated with 1×10^5 TC-1 tumor cells or 5×10^5 C3 tumor cells in 200 µL PBS and 0.2% BSA in the right flank. When mice developed palpable tumors, they were divided to ensure comparable tumor sizes between different treatment groups. Vaccination was provided subcutaneously (s.c) in the left flank with synthetic long HPV16 $E7_{43–77}$ peptide (GQAEPDRA HYNIVTFCCKCDSTLRLCVQSTHVDIR) (150 µg, $CD8^+$ T cell epitope indicated in bold) dissolved in PBS and 1:1 (v/v%) emulsified in Montanide (ISA-51, Seppic) or Incomplete Freud Adjuvant (Sigma Aldrich). For survival experiments, mice were vaccinated on day 8 in the presence or absence of chemotherapy. Chemotherapy was provided via intraperitoneal injection (i.p.) according to the experimental setup. Cisplatin was used at 4 mg/kg and topotecan at 2 mg/kg.[8] To deplete $CD8^+$ or $CD4^+$ T cells, mice were injected i.p on day 7 with 100 µg anti-CD8 antibody (clone 2.43) or 150 µg anti-CD4 antibody (clone GK1.5) (BioXcell), respectively. Depletions were repeated every 5–6 days with 50 µg antibody. All mice used in the experiments had a >99% depletion efficiency as verified by flow cytometry.

For experiments in which the intratumor immune response was analyzed, tumors were injected on day −8 or −9 for TC-1 or day −14 for C3 tumors and vaccination was provided on day 0. Where indicated, 400 µg CD70 blocking antibodies (Clone FR70, BioXcell) were provided on days 5, 9 and 12 via i.p. injection.[22] In tumor-free settings, the same protocol was followed and mice were boosted on day 100 with 150 µg HPV16 $E7_{43–77}$ peptide+20 µg CpG (ODN1826, InvivoGen) dissolved in PBS. Tumor size is displayed as mm^2 (l×h) or mm^3 (l×w×h×0.52). Of note, concurrent with previous clinical observations,[26] neutropenia was observed in tumor-bearing mice receiving topotecan treatment.

Preparation for flow cytometry

Peripheral blood was collected from the tail vein. Erythrocytes were removed using a hypotonic ammonium chloride buffer. Mice were sacrificed on indicated time points and lymph nodes were removed. To obtain tumor infiltrated leukocytes, mice were transcardially perfused with PBS supplemented with 2 mM EDTA. Isolated tumors were disrupted in small pieces and incubated for 15 min 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. Tumor immune filtrates containing more than 5% B cells after perfusion were excluded from analysis.

Adoptive transfer experiments

Isolation of T cells to be transferred has been described previously.[8] Thy1.1 (CD90.1) TCR transgenic OT-I $CD8^+$ T cells were carboxyfluorescein diacetate succinimidyl

ester (CFSE, Invitrogen) labeled and subsequently intravenously injected in Thy1.2 recipient mice. The next day mice were vaccinated with SLPs containing the SIINFEKL epitope ($OVA_{241-270}$) together with 20 µg CpG emulsified in montanide (ISA-51, Seppic). Cisplatin (4 mg/kg, i.p.) was provided on day 0, topotecan (2 mg/kg, i.p.) on days 0, 1 and 2. The kinetics of the OT-I T cells were measured in blood by flow cytometry. The slope (m) was calculated using the following formula m=(mean % OT-I T cells of $CD8^+$ T cells on peak of response–mean % OT-I T cells of $CD8^+$ T cells on next time point)/(day of peak of response–day next time point).

Flow cytometry

Following 10 min incubation with Fc-block and naïve mouse serum, cells were resuspended in staining buffer (PBS+1% FSC) and incubated with HPV16 $E7_{49–57}$ peptide (RAHYNIVTF) loaded H-$2D^b$ tetramers. 7-aminoactinomycin D (Invitrogen) or Zombie-NIR (Biolegend) was used for dead cell exclusion. Surface stain with antibodies (listed in online supplemental file 1) was performed for 30 min at 4°C. For intracellular Ki-67/Bcl-2 staining, surface-stained cells were fixed and permeabilized with FoxP3 Fix/Perm buffers (Biolegend), and subsequently incubated with Ki-67 and Bcl-2-specific antibodies or corresponding isotype controls. Samples were analyzed with BD-LSRII flow cytometers or 3L-Cytek Aurora flow cytometers. Results were analyzed using FlowJo software (Tree Star, V.10.8.1) and OMIQ (Omiq, California, USA, www.omiq.ai).

Intracellular cytokine staining

Lysed blood samples were incubated with HPV16 $E7_{49-57}$ peptide (5 µg/mL). Single cell suspensions from tumor draining lymph nodes or tumors were incubated with 1 µg/mL ionophore and 100 ng/mL PMA in the presence or absence of 1 µg/mL topotecan. To compare intratumoral $CD8^+$ T cell function between groups, 40,000 D1 cells[27] were preloaded with the SLP HPV16 $E7_{43–77}$ (10 µg/mL) and incubated with single-cell suspensions of tumors. Incubations were performed for 5 hours in the presence of brefeldin A (2 µg/mL, Sigma Aldrich) after which an intracellular cytokine staining was performed.

After cell surface staining, cells were fixed overnight with 0.5% paraformaldehyde and permeabilized with Perm/Wash buffer (BD Biosciences). Subsequently, cells were stained for 0.5 hour at 4°C with antibodies.

In vitro effect of topotecan treatment

TCR transgenic OT-I $CD8^+$ T cells were CFSE labeled and plated on 25,000 adherend SAMBOK (MEC.B7.SigOVA) cells,[28] in the presence of 1 µg/mL topotecan. Following 3 days of incubation, cells were harvested, stained and analyzed by flow cytometry.

Immunokinetic parameter calculations

To determine the availability of $E7_{49–57}$-specific and $KLRG1^+$ $CD8^+$ T cells in the differently treated mice, the area under the curve (AUC, (%specific cells×day)) was calculated in GraphPad Prism (software V.9.3.1).

To calculate the half-life of $E7_{49–57}$-specific and $KLRG1^+$ $CD8^+$ T cells in the circulation, the responses of these $CD8^+$ T cell populations were first normalized to the peak response per mouse (the peak response was set as 100% at time point zero). Subsequently, the responses after the peak were calculated as a percentage of the peak response and were plotted as a function of time after peak response. Finally, data were fitted in GraphPad Prism using a one-phase decay model (constrains: Y0=100%; Plateau>0%, and K>0.02) to estimate the half-life ($t_{1/2}$) of the induced antigen-specific T cells.

Statistical analysis

Survival for differentially treated mice was compared using the Kaplan-Meier method and the log-rank (Mantel-Cox) test. Analysis of variance or Mann-Whitney U tests were used for comparing >2 or 2 groups, respectively. Values of $p<0.05$ were considered significant. For all analyses, we used GraphPad Prism.

RESULTS

Treatment by combined cisplatin, topotecan and SLP vaccination results in $CD8^+$ T cell-dependent durable control of established tumors

The survival of patients with cervical cancer treated with cisplatin in combination with topotecan is enhanced compared with single treatment with cisplatin.[21 22] To test whether the addition of vaccination to this treatment regimen further improves survival, mice were subcutaneously inoculated with HPV16 E6 and E7 expressing TC-1 tumors. On day 8, when a palpable tumor was present, treatment was initiated (figure 1A). In comparison to the single chemotherapy treatments the combined treatment of these chemotherapeutics resulted in delayed tumor growth and improved survival (figure 1A–C), similar to what was observed in the clinic. Single treatment with HPV-E7 SLP induced a strong but temporary decrease in tumor size and the addition of topotecan or cisplatin improved vaccine related survival (29% topotecan; 57% cisplatin; figure 1A–C). Triple treatment with cisplatin, topotecan and SLP vaccination resulted in durable tumor rejection in nearly all mice (94% survival), without significant weight loss (online supplemental figure S1A). $CD8^+$ T cells are crucial for the durable anti-tumor responses observed with this triple therapy (figure 1D). Notably, $CD8^+$ T cell depleted and triple therapy treated animals lived significantly longer than untreated mice, likely due to direct chemotherapy-mediated inhibition of tumor cell growth (figure 1D). In line with these observations, peptide+topotecan as well as peptide+cisplatin mediated survival depends on $CD8^+$ T cells (online supplemental figure S1B), and ref.[8] Additionally, survival induced by cisplatin+topotecan is also largely dependent on $CD8^+$ T cells (online supplemental figure S1C). Previously, we showed that animals treated with peptide and

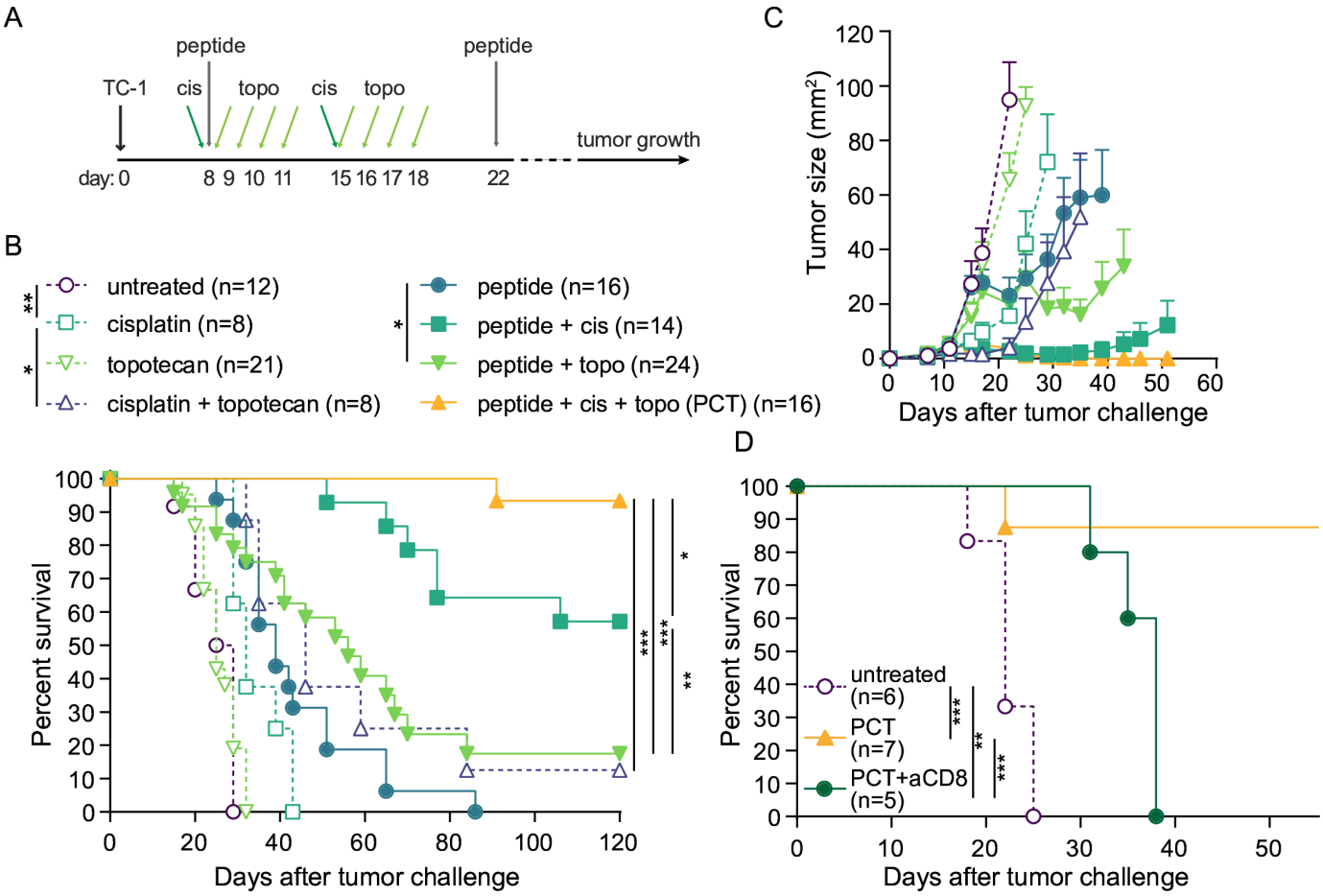

Figure 1 Strong synergy between SLP vaccination, cisplatin and topotecan is CD8⁺ T cell dependent. (A) Schematic diagram of the therapy regimen for TC-1 tumor-bearing mice treated with peptide vaccination, cisplatin and topotecan. (B, C) Kaplan-Meier survival plot (B) and corresponding average tumor growth (mean+SEM) pooled from multiple experiments. n, number of mice per condition. (D) Mice were treated according to the scheme shown in (A). In addition, the peptide vaccine, cisplatin, and topotecan (PCT) treated group received CD8 depleting antibodies starting at day 7 post-tumor challenge. *p<0.05, **p<0.01, ***p<0.001. SLP, synthetic long peptides.

peptide+cisplatin are protected against rechallenge with TC-1,[8] and that this required a vaccine-induced CD8⁺ T cell response of >0.3% of the total circulating CD8⁺ T cell population.[29] All vaccinated animals displayed a percentage of $E7_{49–57}$-specific CD8⁺ T cells above 0.3% on day 130, indicating long-term protection against rechallenge (online supplemental figure S1D). All together, these data show that the combination of cisplatin, topotecan and SLP vaccination results in durable, CD8⁺ T cell dependent, rejections of established tumors.

Decreased abundance of tumor-infiltrating CD8⁺ T cells after topotecan treatment

To prevent a confounding bias effect of tumor size on immune responses,[9 30 31] we adapted our treatment protocol in such a way that tumors were analyzed before objective tumor regressions occurred, which ensured that tumors were similar in size at the time of analysis (figure 2A, online supplemental S2A). This setup, in which the peptide vaccine was provided before chemotherapy, still provided a significant survival benefit of the triple therapy when compared with vaccination alone (figure 2B). Following treatment, increased leukocyte infiltration was observed in nearly all treatment groups as compared with tumors from untreated animals (figure 2C, online supplemental figure S2B). However, while cisplatin enhanced peptide-related leukocyte infiltration, topotecan reduced this (figure 2C). Strikingly, in comparison to the groups receiving peptide and peptide+cisplatin, a decrease in total CD8⁺ T cells and $E7_{49–57}$-specific CD8⁺ T cells infiltrated in the tumor was found in mice treated with peptide+topotecan and in mice treated with this combination in conjunction with cisplatin (figure 2D,E), indicating that topotecan but not cisplatin negatively affected the number of vaccine-induced intratumoral CD8⁺ T cells at this time point. Thus, early after topotecan treatment tumor infiltration by CD8⁺ T cells is strongly reduced.

Topotecan changes the kinetics of antigen-specific CD8⁺ T cells in the circulation and TME

Since the survival after this combinatorial chemoimmunotherapeutic regimen is critically dependent on CD8⁺ T cells, a reduction in abundance of tumor-infiltrated CD8⁺

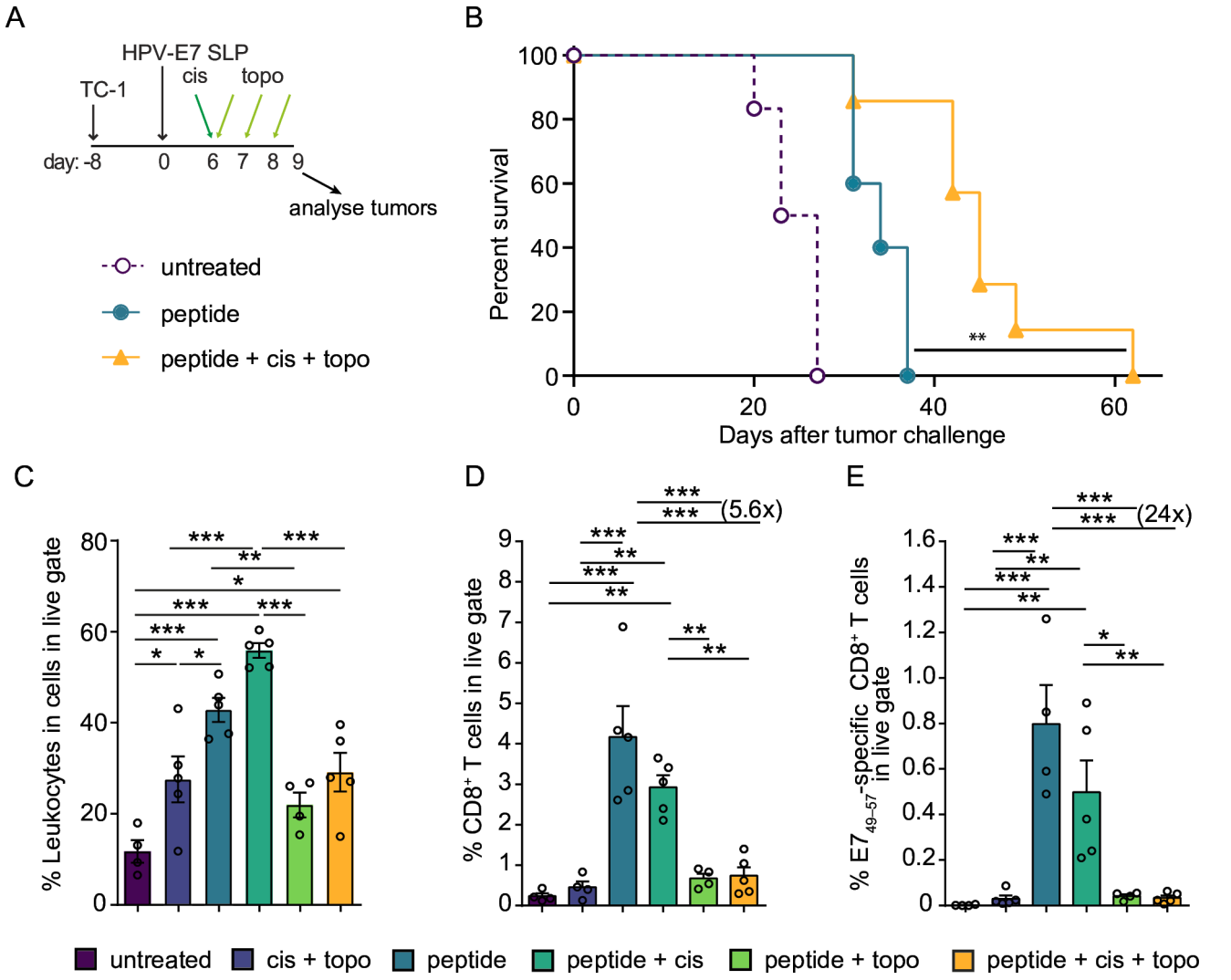

Figure 2 Reduced numbers of tumor-specific CD8$^+$ T cells in tumors of mice receiving topotecan. (A) Scheme of experimental setup: Mice were injected with TC-1 tumor cells and when a palpable tumor was present vaccination was provided with the HPV16 E7$_{43–77}$ peptide. Cisplatin chemotherapy was provided i.p on day 6 while topotecan chemotherapy was given i.p on days 6–8. (B) Corresponding survival plot. (C–E) Within the tumors from untreated, cisplatin+topotecan, peptide, peptide+cisplatin, peptide+topotecan, and peptide+cisplatin+topotecan treated animals the percentage of (C) leukocytes, (D) total CD8$^+$ T cells and (E) E7$_{49–57}$-specific CD8$^+$ T cells out of the total live cells on day 9 postvaccination is shown. Fold decrease between peptide and peptide+cisplatin+topotecan group is indicated. Data shown as mean±SEM, *p<0.05, **p<0.01, ***p<0.001. HPV, human papilloma viral; i.p. intraperitoneal.

T cells was unexpected. Therefore, we studied the kinetics of the CD8$^+$ T cell response by using Thy1.1 (CD90.1) congenically marked OT-I CD8$^+$ T cells, which allows analysis of a monoclonal population of T cells responding to vaccination. To this end, mice received CFSE labeled OT-I T cells followed by SLP vaccination and treatment with cisplatin, topotecan or both (figure 3A). As previously observed, cisplatin treatment had no effect on the number of OT-I T cells and the response peaked on the same day as peptide vaccination alone (figure 3B).[8] In contrast, mice receiving topotecan with or without cisplatin had reduced OT-I T cells early after vaccination but the response peaked at a later time point (figure 3B). The percentage of endogenous circulating CD8$^+$ T cells in mice receiving topotecan was comparable during this experiment (online supplemental figure S3A). Since the data so far indicated that topotecan influences the kinetics of vaccine-specific T cells, independent of the cotreatment with cisplatin and given the remarkable synergy following peptide vaccination in conjunction with cisplatin and topotecan as shown in figure 1, we subsequently focused on the combined effect of cisplatin with topotecan on vaccine-induced cells.

The proliferation marker Ki-67, expected to increase preceding the peak of the T cell response, was used to further study the kinetics of the OT-I response. Indeed, the OT-I T cells of mice receiving vaccination without chemotherapy displayed a peak in Ki-67 expression on day 3, preceding the peak of the T cell response on day 6. Ki-67 expression on OT-I T cells of mice receiving vaccination in conjunction with cisplatin and topotecan was remarkably low on day 3, but peaked on day 6,

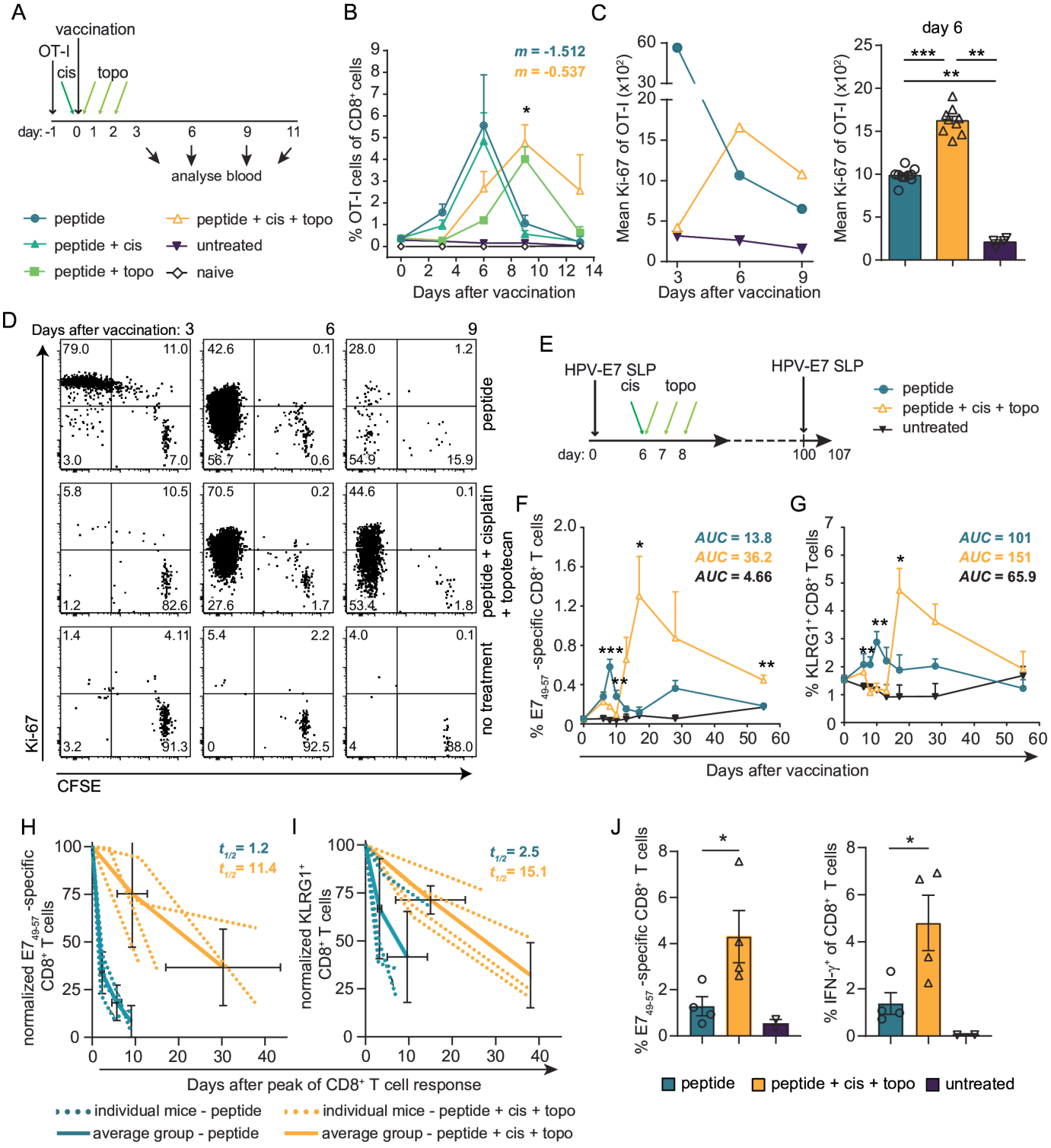

Figure 3 Kinetics of antigen-specific CD8⁺ T cells. (A) Scheme of the experiment shown in (B–D). (B) The percentage of OT-I T cells in the circulation. (C) Left: geometric mean of Ki-67 expression of OT-I T cells plotted in time. Right: geometric mean of Ki-67 expression of OT-I T cells on day 6. (D) Samples from one treatment group were pooled, dot plots indicate Ki-67 expression and CFSE of OT-I T cells on the indicated days postvaccination. (E) Scheme of the experiment shown in (F–J). (F) Kinetics of the percentage of $E7_{49–57}$-specific CD8⁺ T cells and of (G) KLRG1⁺ CD8⁺ T cells in the circulation, area under the curve is indicated as AUC. (H, I) Normalized $E7_{49–57}$-specific CD8⁺ T cell response (H) and normalized KLRG1⁺ CD8⁺ T cell response (I) to the peak of the response is shown per mouse (dotted thin line) and as an average per group (solid thick line), including SD in both the horizontal and vertical directions. Average $t_{1/2}$ per group is indicated. (J) The percentage of $E7_{49–57}$-specific (left) and intracellular IFN-γ⁺ (right) CD8⁺ T cells on day 107, 7 days after the booster. AUC, area under the curve; CFSE, carboxyfluorescein diacetate succinimidyl ester; SLP, synthetic long peptides.

corresponding to a peak in the number of OT-I T cells on day 9 (figure 3B–D). In addition, we observed that the contraction of the OT-I T cell response appeared altered in animals receiving topotecan. The slope (m) of the contraction of the OT-I T cell response was −0.537 for the triple treatment group and −1.512 for animals that were vaccinated but received no chemotherapy (figure 3B), which indicated that more T cells are long-term present due to delayed contraction. In vitro activation assays where $CD8^+$ T cells proliferate after topotecan removal confirmed the potential of $CD8^+$ T cells to display delayed expansion after topotecan removal (online supplemental figure S3B).

Next, we studied the endogenous $CD8^+$ T cell response following HPV-E7 long peptide vaccination in the absence of a tumor, allowing us to study the vaccine-induced response for a longer period of time without tumor outgrowth consequences (figure 3E). Similar to OT-I T cells, the vaccine-induced $E7_{49–57}$-specific $CD8^+$ T cell response was initially reduced in vaccinated animals that also received cisplatin and topotecan as compared with those that only received peptide vaccination. However, the ensuing antigen-specific $CD8^+$ T cell response in these mice was eventually two times higher and the contraction phase was prolonged (figure 3F). This resulted in a 2.6 times increase in area under the curve (AUC) of $E7_{49–57}$-specific $CD8^+$ T cells in the circulation of mice that received cisplatin and topotecan in addition to vaccination, as compared with animals receiving only vaccination. Similarly, the kinetics of the $CD8^+$ T cells expressing KLRG1, a marker for the total number of vaccine-induced $CD8^+$ effector T cells,[24] were similar to the $E7_{49–57}$-specific $CD8^+$ T cell response, as these cells display a delayed but higher peak of the response and a prolonged contraction phase in response to triple therapy (figure 3G).

To better visualize the contraction phase, we plotted the normalized $E7_{49–57}$-specific $CD8^+$ T cell response as a function of the days starting from the peak of the response and indicated this for each mouse (dotted line) and per treatment group (solid line). The half-life ($t_{1/2}$) of the $E7_{49–57}$-specific $CD8^+$ T cells was 9.5 times higher in mice receiving peptide with dual chemotherapy versus animals receiving peptide without chemotherapy. This ranged from 1.2±0.3 days (mean±SEM) in the peptide group to 11.4±4.4 in the triple-therapy group (figure 3H). Despite a much higher default expression of KLRG1 compared with $E7_{49–57}$-tetramer staining, the AUC was still 1.5 times higher (101 vs 151) (figure 3G) and $t_{1/2}$ was 6.1 times higher (ranging from 2.5±1.1 days (mean±SEM) to 15.1±2.7) in animals receiving triple therapy versus animals receiving peptide only (figure 3I). The total percentage of $CD8^+$ T cells were not affected by chemotherapy (online supplemental figure S3C). When mice were rechallenged with the same antigen on day 100 after priming, significantly more vaccine-specific $CD8^+$ T cells, which were also able to produce IFN-γ, were induced in the groups that received chemotherapy in conjunction with vaccination as compared with mice that only received vaccination (figure 3J).

To study if the different dynamics of the circulating $CD8^+$ T cell response was reflected in the TME, tumor-bearing mice were vaccinated with or without chemotherapy and the TME was analyzed (figure 4A). Indeed, while in the peptide only treated group the $E7_{49–57}$-specific $CD8^+$ T cell response peaked on day 12, the percentage of $E7_{49–57}$-specific $CD8^+$ T cells in the TME of animals who had received the vaccination in conjunction with cisplatin and topotecan gradually increased from day 9 to day 15 after peptide vaccination (figure 4B). This gradual increase of $E7_{49–57}$-specific $CD8^+$ T cells in the TME was reflected in the blood circulation. Specifically, the circulating $E7_{49–57}$-specific $CD8^+$ T cell response of peptide+chemotherapy-treated mice was initially low but increased over time until 19 days after vaccination while in peptide only treated mice the response at that time had already contracted (figure 4C). Moreover, Ki-67 expression peaked later in animals receiving topotecan as compared with those that did not receive topotecan (figure 4D,E, online supplemental figure S3D).

To interrogate the T cell functionally, we restimulated the intratumoral cells with $E7_{49–57}$ peptide at day 13, and noticed that the percentage of IFN-γ producing cells among the $CD8^+$ T cells was similar between these groups (figure 4F,G). Remarkably, the ability to produce different cytokines and the amount of cytokines on a per cell basis, was increased in animals that received triple therapy as compared with the mice that only received peptide vaccination (figure 4F–K, online supplemental figure S3E), indicating improved cytokine polyfunctionality in chemo-immunotherapy treated mice.

Together, these data show that topotecan temporally inhibits proliferation of vaccine-activated T cells but that these T cells rapidly recover after treatment, resulting in a delayed contraction of the vaccine-specific $CD8^+$ T cell response with increased effector function.

Topotecan promotes proliferation and survival of tumor-specific $CD8^+$ T cells

Antigen-driven T cell responses contract due to apoptosis involving Bcl-2 family members.[32–36] For example, the canonical prosurvival molecule Bcl-2 plays a role in T cell survival, expansion and antitumor effectivity.[33 36] To further study the T cell kinetics we costained Bcl-2 with the proliferation marker Ki-67. While Bcl-2 was near-absent in the circulating $E7_{49–57}$-specific $CD8^+$ T cells (online supplemental figure S3D), Ki-67 and Bcl-2 coexpression was clearly detected in the $E7_{49–57}$-specific $CD8^+$ T cells in the TME (figure 5A–E, online supplemental figure S3D). On day 9 after peptide vaccination we observed that on average 20% of all $E7_{49–57}$-specific $CD8^+$ T cells expressed both Ki-67 and Bcl-2, irrespective of cisplatin or topotecan treatment (figure 5B–E). However, at day 15 after vaccination, the Ki-67/Bcl-2 double positive $E7_{49-57}$-specific $CD8^+$ T cells in the TME were decreased in groups receiving peptide as a standalone therapy or in conjunction with

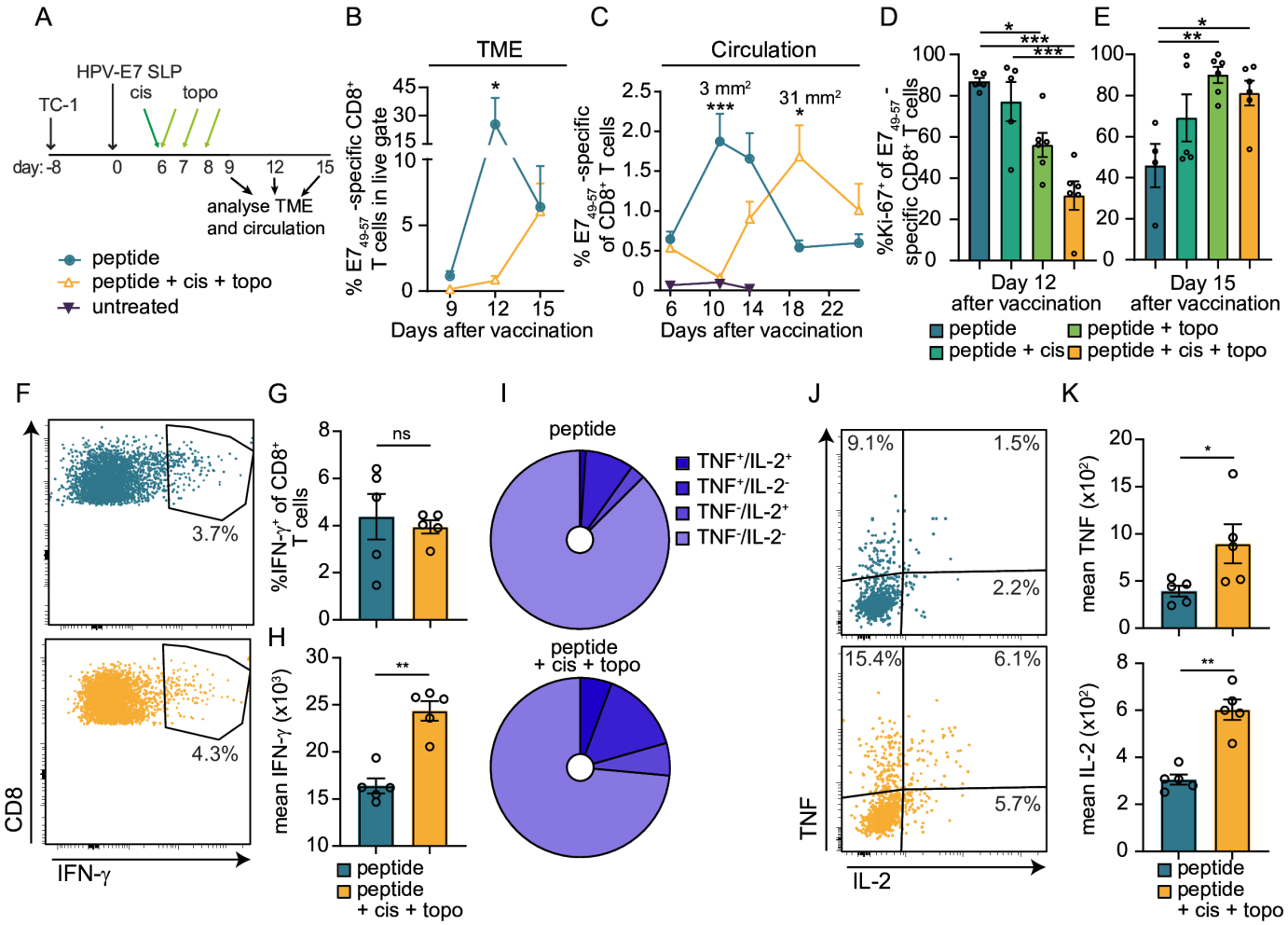

Figure 4 Kinetics and functionality of tumor-infiltrating CD8+ T cells. (A) Scheme of the experiment. (B,C) Percentage of $E7_{49–57}$-specific CD8+ T cells in the TME (B) and circulation (C) in mice receiving peptide or peptide+cisplatin+topotecan. (D, E) Percentage Ki-67+ of $E7_{49–57}$-specific CD8+ T cells in the circulation on day 12 (D) and day 15 (E) after vaccination. Data shown are representative for 2–3 experiments. (F–K) Tumor suspensions isolated on day 13 were restimulated with D1 cells that were preloaded with SLP HPV16 $E7_{43–77}$ and analyzed by flow cytometry. (F) Overlay of equal numbers of CD8+ T cells, showing IFN-γ vs CD8 (n=5 per group). (G) Percentage IFN-γ producing CD8+ T cells. (H) Mean expression of IFN-γ within the IFN-γ-producing cells. (I) Distribution of TNF and/or IL-2 producing cells among IFN-γ producers for peptide treated animals (top) and triple-therapy treated animals (bottom). (J) Overlay of equal numbers of CD8+ T cells (n=5 per group). (K) Mean expression of TNF (top) and IL-2 (bottom) within the IFN-γ producing cells. *p<0.05, **p<0.01, ***p<0.001. HPV, human papilloma viral; SLP, synthetic long peptides; TME, tumor microenvironment.

cisplatin while, these cells increased after topotecan or topotecan/cisplatin cotreatment (figure 5B). This topotecan-induced increase in Ki-67/Bcl-2 double positive cells is mainly attributed to an increase in Bcl-2 expression and only slightly due to an increase in Ki-67 expression (figure 5B–E). The effects were not limited to the vaccine-induced CD8+ T cells since other activated CD8+ T cells (KLRG1+/$E7_{49–57}$-Tm^{neg} cells) also displayed increased Ki-67/Bcl-2 double positive cells (figure 5F). Next, we validated our findings by injecting mice with HPV-transformed C3 tumor cells (online supplemental figure S4A), which is also known as a tumor model to study the effect of therapeutics for HPV-induced malignancies.[25] Analysis of the C3 tumor infiltrate on day 15 after vaccination showed that chemotherapy boosted the infiltration of leukocytes, CD8+ T cells and $E7_{49-57}$ -specific CD8+ T cells (online supplemental figure S4B–D). Similar to the TC-1 model, the Ki-67/Bcl-2 double positive $E7_{49-57}$-specific CD8+ T cells were increased in the TME due to the addition of topotecan (online supplemental figure S4E,F). Together, these data show that vaccine-induced CD8+ T cells in the circulation and tumor of mice treated with peptide in conjunction with cisplatin and topotecan are temporally hampered in their proliferative capacity, and that the subsequent recovery after topotecan treatment relates to increased systemic and intratumoral proliferation and upregulation of the antiapoptotic molecule Bcl-2 in the tumor-infiltrated CD8+ T cells.

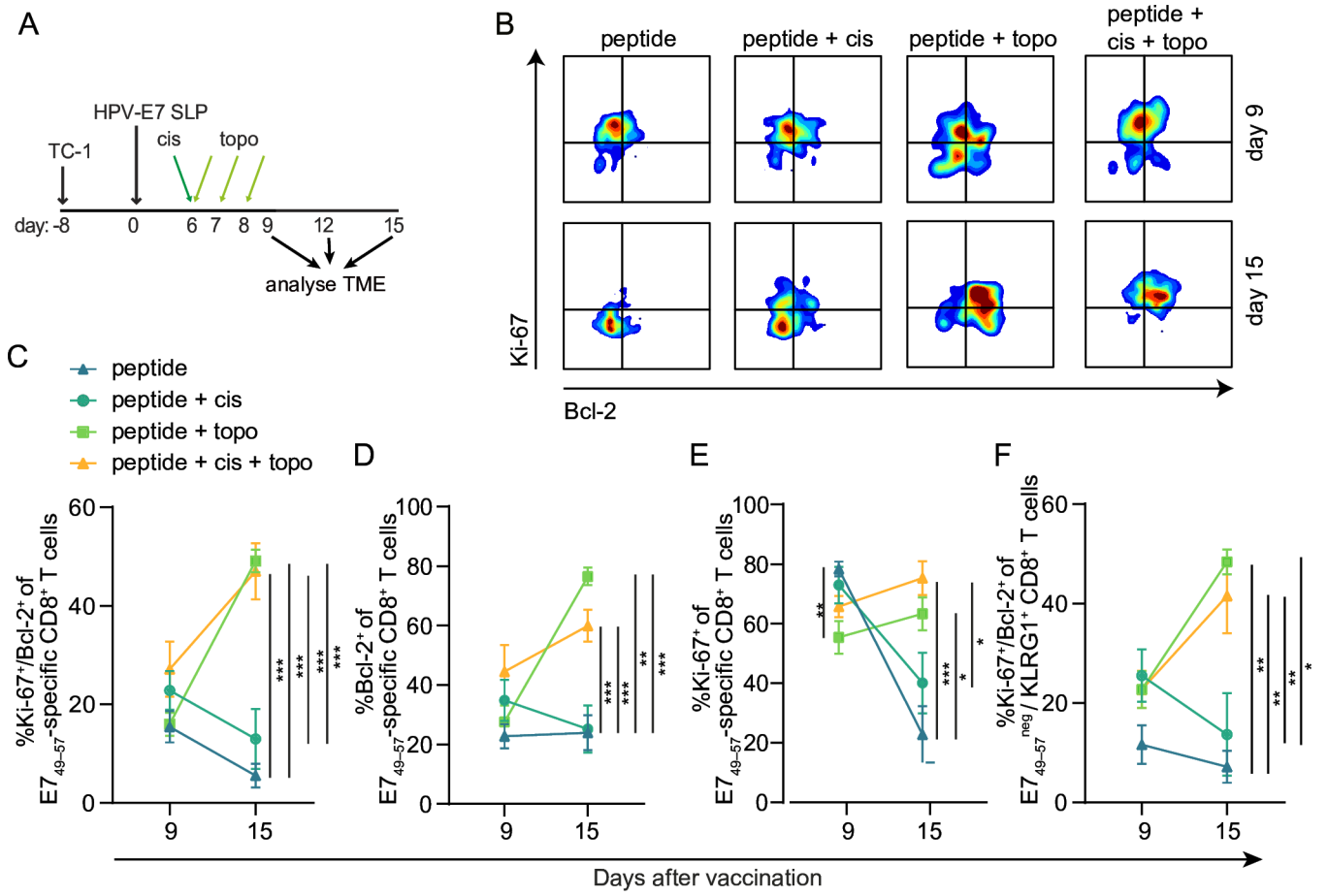

Figure 5 Analysis of tumor-specific CD8$^+$ T cells in the tumor microenvironment (TME). (A) Scheme of the experiment shown in (B–E). (B) Bcl-2 vs Ki-67 expression of E7$_{49–57}$-specific CD8$^+$ T cells, pooled from 4 to 5 animals per group. (C) Percentage of Ki-67$^+$/Bcl-2$^+$ cells of E7$_{49–57}$-specific CD8$^+$ T cells (upper right quadrant in (B)). (D) Bcl-2$^+$ cells of E7$_{49–57}$-specific CD8$^+$ T cells (right quadrants in figure (B, C) Ki-67$^+$ cells of E7$_{49–57}$-specific CD8$^+$ T cells (upper quadrants in (B). (F) Percentage of Ki-67$^+$/Bcl-2$^+$ cells of KLRG1$^+$/E7$_{49–57}^{neg}$ CD8$^+$ T cells. Data shown are pooled data from two experiments, n=6–10 animals per group, and analyzed by on day 9 and day 15. Data are shown as mean±SEM. *p<0.05, **p<0.01, ***p<0.001.

CD70/CD27 mediated costimulation regulates Bcl-2 levels of tumor specific CD8$^+$ T cells in the TME

To gain insight in the upregulation of Bcl-2 in the tumor-infiltrated CD8$^+$ T cells after topotecan and dual chemotherapy, we studied the expression of the costimulatory molecules CD86 and CD70, which are known to enhance T cell survival.[37 38] Previously, we have shown that tumor-specific CD8$^+$ T cell infiltration coincided with a CD8$^+$ T cell-dependent myeloid cell infiltration, more specifically inflammatory macrophages expressing costimulatory molecules and MHC class II.[39] In line with our previous data, we observed that the groups treated with peptide and topotecan having a low CD8$^+$ T cell infiltration at day 9 after vaccination, consequently also displayed a low infiltration with myeloid cells (figure 2E,6A and online supplemental figure S5A). As expected, the tumors of mice treated with peptide plus cisplatin displayed a strong myeloid infiltration in the tumor (figure 6A, online supplemental figure S5A). We noticed that high frequencies of CD86-expressing myeloid cells were present after peptide vaccination and even more after peptide vaccination with low dose cisplatin treatment (figure 6B).[12]

In contrast, topotecan specifically stimulated the upregulation of CD70 and MHC class II on tumor-infiltrating myeloid cells (figure 6C–E). As a result, the total number of tumor-infiltrating CD11b^{high}/CD70^{high} myeloid cells, which have predominantly a DC-like macrophage phenotype (CD11c$^+$/F4/80$^+$), was similar in the groups receiving peptide and topotecan compared with groups receiving vaccination with or without cisplatin (online supplemental figure S5B,C). The relatively higher expression of CD70 on myeloid cells in mice that had received topotecan and cisplatin in conjunction with vaccination was temporal (online supplemental figure S5D), in line with previous data that describe transient expression of CD70 after stimulation.[40]

Next, we analyzed whether CD70 and its receptor, the TNF receptor superfamily member (TNFRSF) CD27, impacted the kinetics of the T cell response. After confirming that CD27 was expressed on E7$_{49–57}$-specific CD8$^+$ T cells (online supplemental figure S5E), tumor-bearing mice were treated with the triple therapy in the presence or absence of CD70 blocking antibodies (figure 6F). At day 15 post vaccination, the percentages

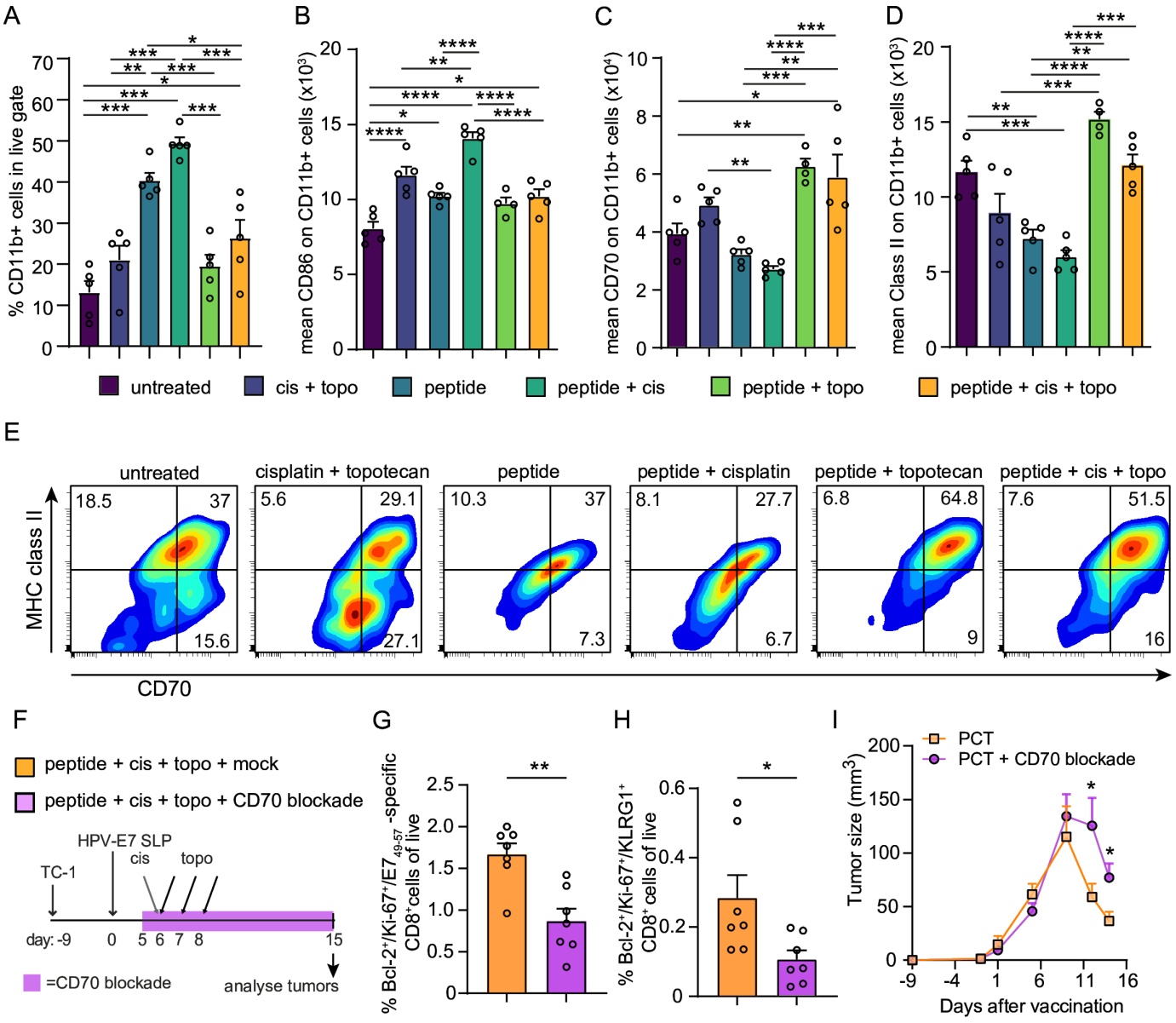

Figure 6 CD70/CD27-mediated costimulation regulates Bcl-2 and Ki-67 levels of tumor-specific CD8$^+$ T cells in the TME. (A–E) Mice were treated according to the experiment scheme shown in figure 4A. Tumors were resected on day 9 after vaccination. (A) Percentage of live/CD45$^+$/CD11b$^+$ cells within the live gate. (B–D) Mean expression of (B) CD86 (C) CD70 and (D) class II on the tumor-infiltrated CD11b$^+$ myeloid population. (E) CD70 expression versus MHC class II expression on tumor-infiltrated CD11b$^+$ myeloid cells. Overlay of 300 cells per mouse, 5 animals per group. (F) Experimental setup and color indication for (G–I). CD70 blockade is provided twice per week. (G, H) Tumors were resected on day 15. Shown is the percentage of (G) Bcl-2$^+$/Ki-67$^+$/E7$_{49–57}$-specific CD8$^+$ T cells and (H) Bcl-2$^+$/Ki-67$^+$/KLRG1$^+$ CD8$^+$ T cells within the live gate. (I) Kinetics of tumor size (mm^3). Shown is the mean+SEM *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are representative of 2–3 experiments. TME, tumor microenvironment.

of both Bcl-2$^+$/Ki-67$^+$ E7$_{49–57}$-specific and Bcl-2$^+$/Ki-67$^+$ KLRG1 expressing CD8$^+$ T cells within the tumor were approximately 50% reduced when the triple chemo-immunotherapy was combined with CD70 blockade (figure 6G,H). Correspondingly, CD70 blockade delayed the triple therapy-mediated tumor regression (figure 6I).

Together these data show that in response to peptide vaccination provided in conjunction with cisplatin and topotecan, myeloid cells express high levels of class II and the costimulatory molecule CD70. When CD70/CD27 mediated costimulation is inhibited, Bcl-2$^+$/Ki-67$^+$ coexpression on intratumoral tumor-specific CD8$^+$ T cells is strongly decreased, indicating a role for topotecan-induced CD70 expression on myeloid cells in the sustained immune response.

DISCUSSION

Here, we have shown that the topoisomerase I inhibitor topotecan affects the kinetics of vaccine-specific CD8$^+$ T cell responses. During topotecan treatment, proliferation of vaccine-induced CD8$^+$ T cells is hindered. However,

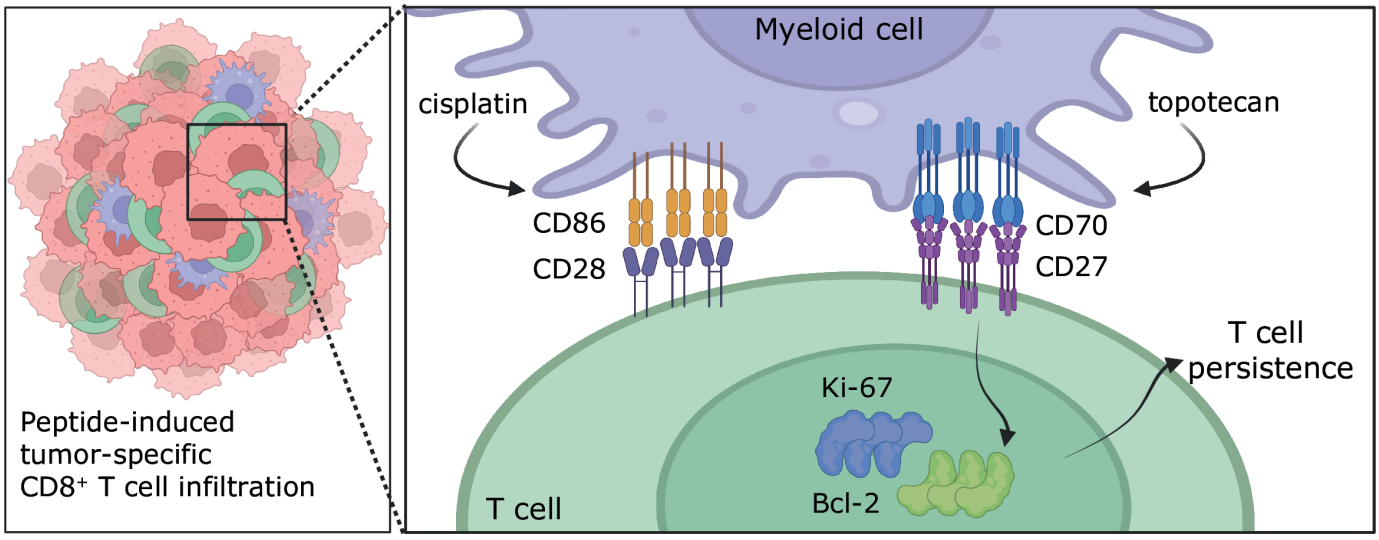

Figure 7 The mechanism of cisplatin and topotecan to improve therapeutic peptide-based vaccination against solid tumors. Cisplatin induces the expression of DAMPs in the TME leading to upregulation of the costimulatory molecule CD86 on tumor-infiltrating myeloid cells; topotecan facilitates expression of the costimulatory molecule CD70 on myeloid cells, thereby enhancing the prosurvival molecule Bcl-2 in proliferating $CD8^+$ T cells. The costimulatory molecule driven enhancement of vaccine-induced T cells improves control of tumor progression. Drawn by Biorender (www.biorender.com). DAMPs, danger-associated molecular patterns; TME, danger-associated molecular patterns.

after treatment termination, these $CD8^+$ T cells enter a delayed but vigorous expansion phase, followed by a prolonged contraction phase, characterized by Bcl-2^+/Ki-67^+ $E7_{49–57}$-specific $CD8^+$ T cells in the TME. While cisplatin enhanced CD86 expression on myeloid cells, topotecan enhanced functional CD70 expression, which mediates the induction of high numbers of Bcl-2^+/Ki-67^+ $E7_{49–57}$-specific $CD8^+$ T cells and tumor regression (figure 7).

Previously, we have shown that treatment with high dose cisplatin results in the release of multiple danger associated molecular patterns (DAMPs) and type I interferons, which triggered functional upregulation of CD80 and CD86.[12] Here, we show that a lower dose of cisplatin still upregulated CD86 but had no effect on CD80 or CD70 expression. On the other hand, topotecan had no effect on CD86 but rather enhanced CD70 expression. This suggests that the regulation of the expression of these costimulatory molecules by chemotherapy is complex and may depend on differences in tumor cell death leading to distinctions of the released DAMPs.

The impact of CD70/CD27-mediated costimulation on T cell immunity was previously studied. Depending on the context, CD70-CD27 interactions can strengthen or impair T cell responses. In the context of acute but also during smoldering infections (eg, with herpesviruses) and malignancies, CD70 expression by APCs contributes to strengthen the T cell response by enhancing the magnitude of antigen-specific $CD8^+$ T cells during expansion, delaying the contraction phase and increasing memory formation.[41–44] However, during chronic infection with highly replicating viruses, the CD70/CD27 pathway results in impaired T cells responses,[45] which relates to settings in which chronic CD70-CD27 interactions eventually result in dysfunctional and waning T cell responses.[46 47] These studies emphasized that expression of CD70 needs to be tightly controlled. Our data indicated that topotecan treatment induced a transient CD70 upregulation on myeloid cells, which normalized within a few days after therapy discontinuation.

In-depth studies showed that CD27 signaling induces the expression of the anti-apoptotic Bcl-2 family member $Bcl\text{-}X_L$, as well as Bcl-2 itself, thereby promoting both survival and expansion of antigen-specific stimulated T cells.[37 48–50] Mechanistically, the level of Bcl-2 expression determines the amount of the pro-apoptotic molecule Bim that a $CD8^+$ T cell can tolerate to survive.[35] Bcl-2 also impacts memory formation as adoptively transferred T cells with a high Bcl-2 expression at the peak of the immune response show better survival and improved capacity to become memory cells than their Bcl-2^{low} counterparts.[34] Furthermore, Bcl-2 expression also reduces activation-induced apoptosis of CARTs and improves their survival, expansion and antitumor efficacy.[33 36] Along these lines it was shown that adoptively transferred pre-activated CD27-silenced OT-I $CD8^+$ T cells display reduced survival compared with CD27 expressing control cells.[51] Together these studies highlight that enhancement of costimulatory molecules, such as CD70 can regulate the persistence of antigen-specific $CD8^+$ T cells via Bcl-2 and Bcl-2 family members. Topotecan-mediated CD70 enhancement in the TME is thus likely instrumental for the enhanced expansion and survival of vaccine-induced $CD8^+$ T cells and related sustained tumor control. However, whether topotecan regulates other molecules than CD70, which influence levels of Bcl-2 and/or Bcl-2-family members remains to be investigated.

The costimulatory molecule CD70 co-localizes with MHC class II molecules within so called "MHC class II compartments" in DCs.[52 53] The here observed increase in both MHC class II and CD70 expression on myeloid cells and their "DC-like" phenotype suggests that the relative abundance of cells with T cell stimulating capacity in the myeloid-immune infiltrate is enhanced in the animals receiving topotecan in conjunction with peptide vaccination as compared with vaccination alone. Indeed, topotecan-conditioned TC-1 tumors contained $Ly6C^{+}$/MHC-II^{+}/$CD11c^{hi}$/$CD64^{hi}$ APC's with high proliferative, and $CD8^{+}$ T cell stimulatory capacity.[17] The enhanced expression of MHC class II may also contribute to additional stimulation of the vaccine-induced $CD8^{+}$ T cells via improved $CD4^{+}$ T help.[54]

In conclusion, we have shown that topotecan initially delays the proliferation of recently primed $CD8^{+}$ T cells. Yet, after termination of the therapy, $CD8^{+}$ T cells are programmed to proliferate strongly while the contraction phase is prolonged. Mechanistically, we showed that topotecan-mediated CD70 upregulation on intratumoral myeloid cells stimulates the coexpression of Ki-67 and Bcl-2 in $E7_{49–57}$-specific $CD8^{+}$ T cells. Further elucidation of the chemo-immunotherapeutic mechanisms should pave the way to develop a treatment regimen that stimulates a high peak of the $CD8^{+}$ T cell response and a slow contraction while at the same time preserving and enhancing antitumor activity.

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Contributors Conception and design: TCvdS, CJMM, SHvdB and RA. Experiments: TCvdS, SvD, INP, JFdG and WV. Analysis and interpretation of data: TCvdS, FJvH, KvdM and RA. Writing, review, and/or revision of the manuscript: all authors. Responsible for the overall content as the guarantor: RA

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Competing interests This study has been conducted by LUMC, which holds a patent on the use of synthetic long peptides as vaccine (US 7.202.034). CJMM and SHvdB are named as inventors on this patent. Additionally, the LUMC holds a patent on the use of T cell cycle synchronization to optimize antitumor responses (No2019548). SHvdB, RA, CJMM and TCvdS are named inventors on this patent. CJMM is employed full time by ISA Pharmaceuticals, which exploits the long-peptide vaccine patent, and has been granted options on ISA Pharmaceuticals stock. SHvdB is a member of the Scientific Advisory Board and receives consultancy fees from ISA Pharmaceuticals. RA is a member of the Scientific Advisory Board of QIAGEN.

Patient consent for publication Not applicable.

Ethics approval Experiments were approved by the Animal Experiments Committee of the LUMC, in line with guidelines of the European Commission, and by local and national committees of animal experiments under permit number AVD1160020186804, and performed according to recommendations and guidelines set by LUMC and by the Dutch Act on Animal Experimentation and EU Directive 2010/63/EU.

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Data availability statement All data relevant to the study are included in the article or uploaded as online supplemental information. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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