Tumor immunology

Basic

Research Article

Transcutaneous immunization with CD40 ligation boosts cytotoxic T lymphocyte mediated antitumor immunity independent of CD4 helper cells in mice

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Transcutaneous immunization (TCI) is a novel vaccination strategy that utilizes skin-associated lymphatic tissue to induce immune responses. Employing T-cell epitopes and the TLR7 agonist imiquimod onto intact skin mounts strong primary, but limited memory CTL responses. To overcome this limitation, we developed a novel imiquimod-containing vaccination platform (IMI-Sol) rendering superior primary CD8$^+$ and CD4$^+$ T-cell responses. However, it has been unclear whether IMI-Sol per se is restricted in terms of memory formation and tumor protection. In our present work, we demonstrate that the combined administration of IMI-Sol and CD40 ligation unleashes fullblown specific T-cell responses in the priming and memory phase, strongly enhancing antitumor protection in mice. Interestingly, these effects were entirely CD4$^+$ T cell independent, bypassing the necessity of helper T cells. Moreover, blockade of CD70 in vivo abrogated the boosting effect of CD40 ligation, indicating that the adjuvant effect of CD40 in TCI is mediated via CD70 on professional APCs. Furthermore, this work highlights the so far underappreciated importance of the CD70/CD27 interaction as a promising adjuvant target in TCI. Summing up, we demonstrate that the novel formulation IMI-Sol represents a powerful vaccination platform when applied in combination with sufficient adjuvant thereby overcoming current limitations of TCI.

Keywords: imiquimod · T cells · TLR7 · transcutaneous immunization · vaccination

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

The development of novel vaccination strategies is urgently needed, as standard vaccines are mostly effective for the prophylaxis, but not in therapeutic settings. Moreover, while immunotherapies are an emerging field, there are currently no synthetic vaccines available for the treatment of cancers [1]. In this context, transcutaneous immunization (TCI) approaches are of great interest since they incorporate all desirable properties of an ideal therapeutic vaccine in terms of defined antigen specificities, targeting of specific APC populations and well-defined adjuvants [2]. Deductive to the easy access and the presence of a complex, but functionally rich network of immune cells in the skin, the interest on TCI has successively accumulated, since the proof-of-concept has been made by Glenn and coworkers 20 years ago [3]. Furthermore, the transcutaneous route for vaccination implies a high potential for safe immune stimulation, considering that the direct contact of potent (or sometimes toxic) adjuvants with the systemic blood circulation is avoided [4].

In our previous work, we have demonstrated that the simultaneous application of a synthetic peptide together with the TLR7 agonist imiquimod onto the intact skin elicits potent primary CTL responses [5, 6]. This is achieved by direct access to skin-resident APCs that present ingested peptides on MHC molecules and thus initiate adaptive immunity in a TLR7-dependent fashion [7]. Both, the proof-of-concept of imiquimod-based TCI demonstrated in experimental rodent models by us and others [6, 8, 9] and the effective topical application of imiquimod in patients with HPV infections, skin cancers, or precancerous lesions allow the assumption that such novel vaccination concepts may be also safe and effective in humans [10]. In further support of this notion, pilot studies in humans, including melanoma patients immunized against NY-ESO-1 protein [11] as well as influenza vaccination [12], demonstrate efficacy of imiquimod as an adjuvant in terms of enhancing T cell responses.

CTL responses induced by TCI with imiquimod based on the commercial formulation in Aldara rapidly fade away, resulting in poor memory formation and only partial tumor protection, as previously demonstrated [9]. Beyond this, we have recently developed novel imiquimod formulations with enhanced potency to induce CTL responses in TCI [6, 13, 14]. While the novel imiquimod formulation IMI-Sol induces enhanced primary $CD8^+$ as well as $CD4^+$ T cell responses, memory formation remains a problem in this vaccination approach. However, recently, we were able to prove that T cell responses and tumor protection can be rescued by additional stimuli, i. e. by CD40 ligation, CTLA-4 blockade [7, 15], or low-dose UV-B irradiation [7], highlighting the importance of "high-quality" T cell responses that confer effective memory responses.

Therefore, we were interested to define whether our novel vaccination platform IMI-Sol is limited per se due to suboptimal antigen presentation via skin APCs or by inefficient co-stimulation provided by imiquimod. To address this, we chose to use ligation of CD40 using an agonistic mAb, which has been used by us and others in the past to provide optimal co-stimulatory signals for CTL priming [9, 16]. CD40 is located on APCs and plays an important role as co-stimulatory molecule during T cell activation, interacting with CD40L (CD154) on $CD4^+$ T cells.

In our present work, we demonstrate that concurrent CD40 ligation induced superior CTL responses upon treatment with our novel vaccination platform IMI-Sol and led to both robust memory formation and greatly enhanced tumor protection, thereby illustrating the protruding potential of our novel imiquimod formulation IMI-Sol. Importantly, CTL responses evoked in the primary and memory phase were maintained after $CD4^+$ T cell depletion, indicating that CD40 ligation bypasses the requirement of $CD4^+$ Th cell responses for the generation of CTL memory. Beyond that we deciphered a central role for CD70 in CD40-mediated boosting of TCI, thereby revealing CD70/CD27 interaction as an auspicious new adjuvant target for imiquimod-based TCI.

Results

CD40 ligation augments CTL responses induced by transcutaneous immunization

We recently demonstrated that TCI with a novel imiquimod formulation IMI-Sol and a synthetic CTL epitope elicits superior primary $CD8^+$ as well as $CD4^+$ T cell responses [6]. Nevertheless, memory formation still remains a problem, as CTL responses decay over time resulting in moderate protection against LCMV in a virus challenge model. To resolve whether TCI induced T cell responses are limited by inefficient co-stimulation provided by the TLR7 agonist imiquimod, we chose to investigate the effects of maximized co-stimulation via additional CD40 ligation on CTL responses mediated by TCI using an agonistic anti-CD40. We therefore performed TCI on the shaved dorsum of C57BL6/J WT mice as depicted in Fig. 1A. Using TCI alone, we were able to detect about 2% peptide-specific CTLs on day 7 post treatment (Fig. 1B) and subsequently a modest cytolytic activity (Fig. 1C). In contrast, additional treatment with an anti-CD40 antibody strongly enhanced this frequency as well as the cytolytic activity in vivo (Fig. 1BC).

Next, we evaluated whether this augmented primary CTL response also converts into an improved memory formation. Therefore, mice were treated as before, but elsewise the frequency of specific CTLs and their cytolytic activity was estimated 35 days after vaccination and without prior restimulation (summarized in Fig. 2A). As depicted in Fig. 2, TCI alone was neither able to provoke a significant number of peptide-specific CTLs nor a specific cytolytic activity, whereas the single additional administration of an anti-CD40 antibody during priming phase led to high frequencies of peptide-specific $CD8^+$ T cells (Fig. 2B) with a formidable in vivo cytolysis of target cells (Fig. 2C).

Regarding the route of antibody application, i.p. and s.c. treatments proved to be euqally effective, especially in terms of memory responses (not shown). Dose titration studies of anti-CD40 together with TCI showed a clearly superior CTL response at 30 μg compared to doses of 10 and 3 μg in terms of primary and memory
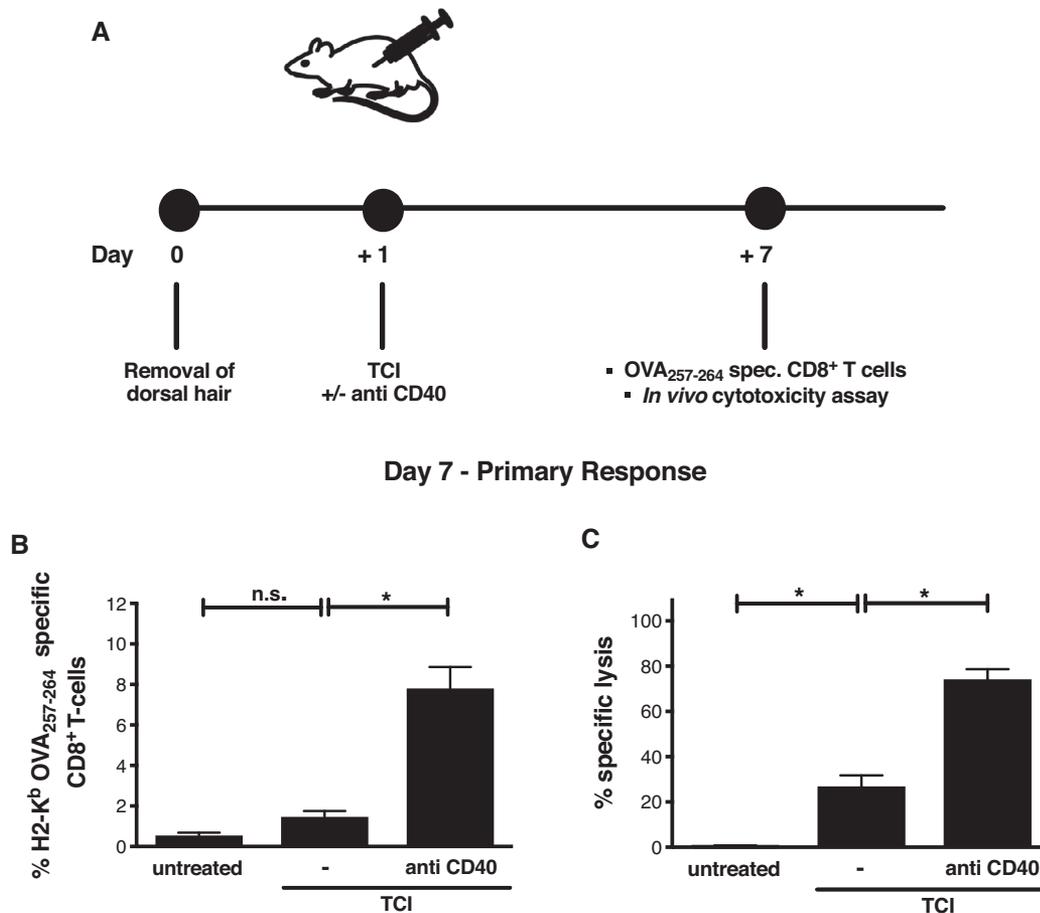
Figure 1. CD40 ligation enhances TCI mediated primary CTL responses. WT mice were immunized by TCI along with OVA$_{257-264}$ on day 1. Thirty micrograms of anti-CD40 were subsequently applied i.p. (A). The frequency of peptide-specific CD8$^+$ T cells was assessed on day 7 via flow cytometry (B) and the in vivo cytolytic activity was analyzed on day 7, 6 h after transfer of peptide-loaded splenocytes (C). One representative experiment of three independent experiments is depicted. In (B) $n = 6$ mice per group, in (C) $n = 6$ mice per group are shown. The data are given as mean with SD. *Significance by one-way ANOVA with Bonferoni Posttest analysis, $p < 0.05$.

CTL responses. Further, we could not observe any compromising adverse effects (not shown).

Taken together, these results clearly demonstrate that the CD40 pathway plays a key role in initiating primary and memory CTL responses upon TCI overcoming current limitations of sole TCI. Moreover, these results allow the conclusion that the efficiency of TCI induced CTL responses is not limited by the amount of antigenic peptide passing the skin, but lack of sufficient co-stimulatory signals that can be overcome by additional CD40 ligation.

CD40 ligation bypasses the need for CD4$^+$ T cell help for the induction of CTL responses

Our previous results have demonstrated that additional co-stimulation through CD40 ligation strongly enhances TCI mediated CTL responses and further promotes to memory formation. Since the CD40 pathway is physiologically mediated by CD4$^+$ T

cells [17], we were now interested to clarify the importance of CD4$^+$ T cells in TCI after additional CD40 ligation. To address this, we depleted the CD4$^+$ T cell population in vivo by i.p. injection of a monoclonal CD4 antibody (clone GK1.5) 1 day prior to immunization and performed vaccination experiments as described before (Fig. 3A). The efficacy of CD4$^+$ T cell depletion was confirmed by flow cytometry prior to TCI demonstrating complete absence of CD4$^+$ T cells in the peripheral blood (data not shown). The frequency of peptide-specific CTLs and their cytotoxic activity in vivo were analyzed on day 7 and on day 35 for evaluation of memory responses. Notably, CD4$^+$ T cell depletion led to an increase of peptide-specific CTLs (Fig. 3B) and cytolytic activity (Fig. 3C) upon TCI alone in the primary response, most likely due to the concurrent depletion of regulatory T (Treg) cells that suppress TCI induced CTL reponses as previously demonstrated by us [15, 18]. However, the groups that received the combined treatment with anti-CD40 showed no significant difference in the primary CTL response in the absence or presence of CD4$^+$ T cells (Fig. 3B and C).
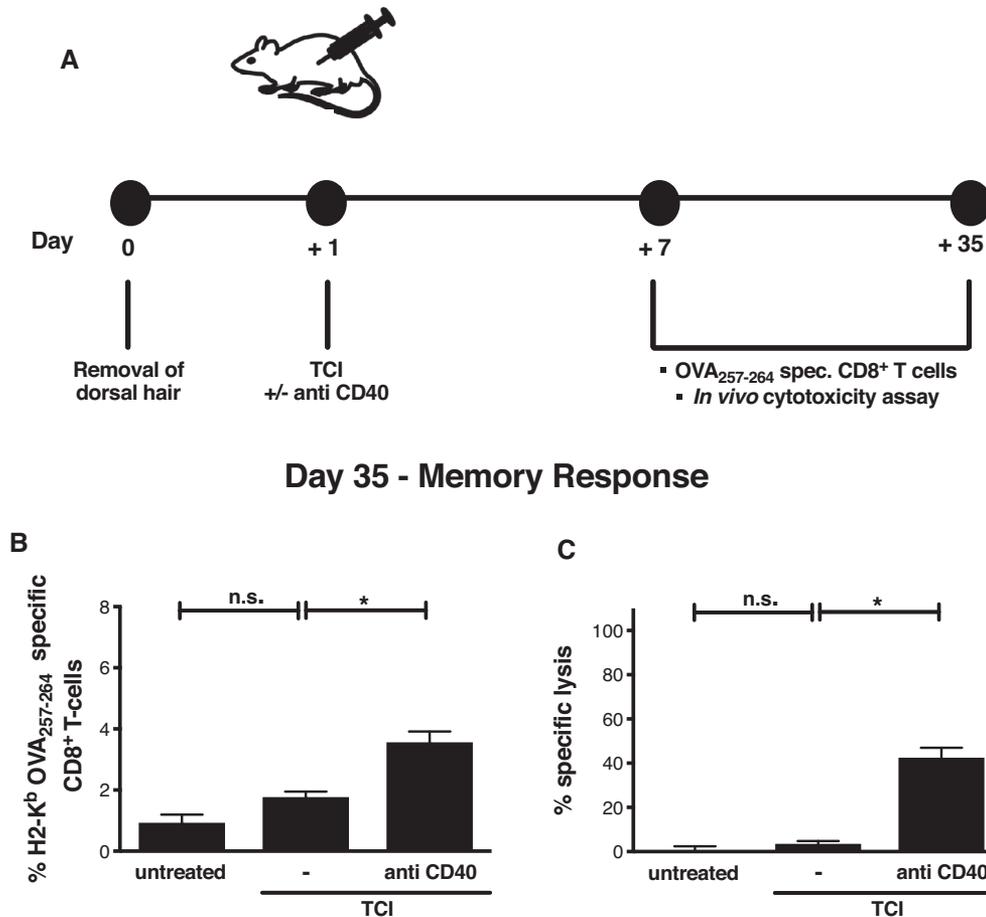
Figure 2. CD40 ligation boosts TCI-mediated CTL responses leading to memory formation. WT mice were immunized with 50 mg IMI-Sol along with 100 μg $OVA_{257-264}$ on day 1. Thirty micrograms of the anti-CD40 were subsequently applied i.p. in the neck (A). For evaluation of memory responses, the amount of $OVA_{257-264}$ specific $CD8^+$ T cells was measured on day 35 (B) and a second cytotoxicity assay was performed on day 35 and evaluated 20 h after transfer of peptide-loaded splenocytes (C). One representative experiment of three independent experiments is depicted. In (B), $n = 3$ mice per group; in (C), $n = 5$ mice per group are shown. The data are given as mean with SD. *Significance by one-way ANOVA with Bonferoni posttest analysis, $p < 0.05$.

In the memory phase, we were able to detect low frequencies of peptide-specific CTLs without any significant cytolytic activities upon TCI alone after $CD4^+$ T cell depletion in the priming phase (Fig. 3D and E). In contrast to this, the high frequency of specific CTLs was retained upon additional CD40 ligation in the absence as well as the presence of $CD4^+$ T cells (Fig. 3D and E), indicating that CD40 ligation in conjunction with TCI is sufficient to bypass the requirement of $CD4^+$ T cell responses for the generation of primary and memory CTL responses. Moreover, CD40 ligation apparently also overrides the suppressive effects of Treg cells on TCI-induced CTL responses.

CD40 ligation boosts CTL responses upon TCI in a CD70 dependent manner

Based on these results, CD40 ligation has shown to be a powerful tool to overcome current limitations of our IMI-Sol based vaccination platform in terms of memory formation. Toward the

underlying mechanisms, others have shown a prominent role for CD70 in CD40 mediated T cell priming by professional APCs [19]. Therefore, we chose to address the role of CD70 in CD40 mediated boosting of TCI. To do so, we treated the mice as before with TCI adding either anti-CD40 or anti-CD70 alone or in combination [20] (Fig. 4A). While TCI upon CD40 ligation was able to evoke high frequencies of specific CTLs, additional administration of FR-70 diminished the frequency of specific CTLs to the level of sole TCI (Fig. 4B). Consistently, the cytolytic capability of CTLs was significantly reduced when blocking CD70 in the priming phase of our vaccination approach (Fig. 4C). Moreover, even sole TCI was largely dependent on CD70 signaling (Fig. 4C). To further elucidate the role of CD70 in our immunization, we were particularly interested in its role in memory formation and functional maintenance. While TCI alone is not sufficient to induce memory formation, the combination of TCI and anti CD40 ligation lead to a significant increase of antigen-specific CTLs and a powerful rejection of antigen-loaded splenocytes. Further, the administration of FR-70 in the priming phase abolished the
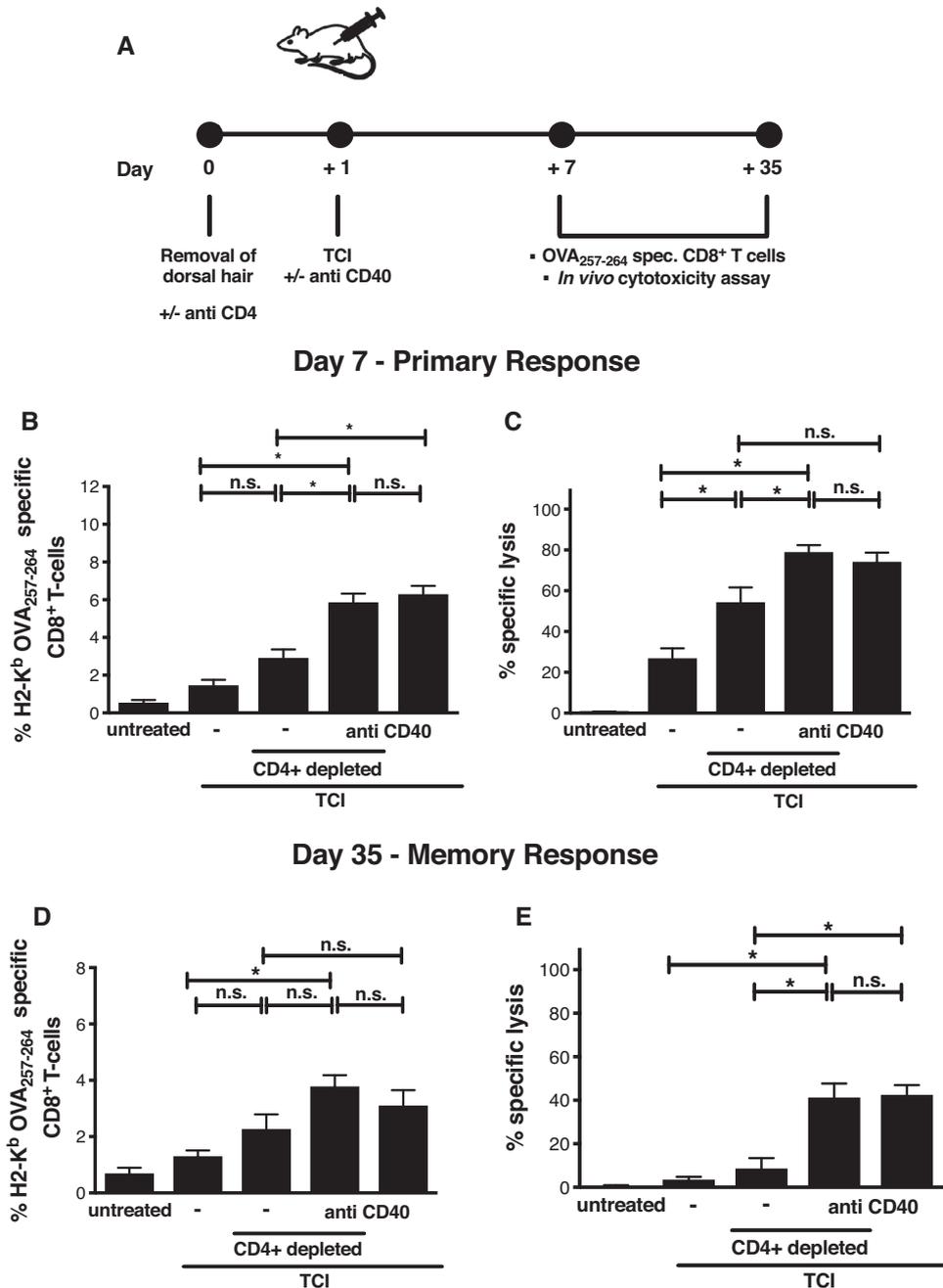
Figure 3. CD40 ligation bypasses CD4^{+} T cell activity in generating primary and memory CTL responses. WT mice were immunized with 50 mg IMI-Sol along with 100 µg OVA$_{257-264}$ on day 1. Thirty micrograms of the anti-CD40 were subsequently applied i.p. Designated animals received 100 µg anti CD4 (clone GK1.5) i.p. on day 0 to deplete CD4^{+} T cells (A). The frequency of peptide-specific CD8^{+} T cells was assessed on day 7 via flow cytometry (B) and the in vivo cytolytic activity was analyzed on day 7, 6 h after transfer of peptide-loaded splenocytes (C). For evaluation of memory responses, the amount of OVA$_{257-264}$ specific CD8^{+} T cells was measured again on day 35 (D) and a second cytotoxicity assay was performed on day 35 and evaluated 20 h after transfer of peptide-loaded splenocytes (E). In (B) and (C), $n = 6$ mice per group; in (D), $n = 3$ mice per group; and (E), $n = 5$ mice per group are shown. The data are given as mean with SD. The depicted data are from one representative out of three independent experiments. *Significance by one-way ANOVA with Bonferoni posttest analysis, $p < 0.05$.

generation of memory CTLs and cytolytic activity, thus underlining the importance of CD70 in T cell priming. Moreover, we aimed to clarify whether the prominent function of CD70 in vivo is restricted to the priming phase of this TCI approach or if there is also a function in memory maintenance. To address this, we immunized mice with TCI and anti CD40. Subsequently and in contrast to previous studies, we applied the FR-70 antibody to these mice in the memory phase of the immune response (Fig. 4A). Whereas the frequency of specific CTLs was not impaired by the delayed FR-70 administration (Fig. 4D), the cytoloytic ability in these mice was
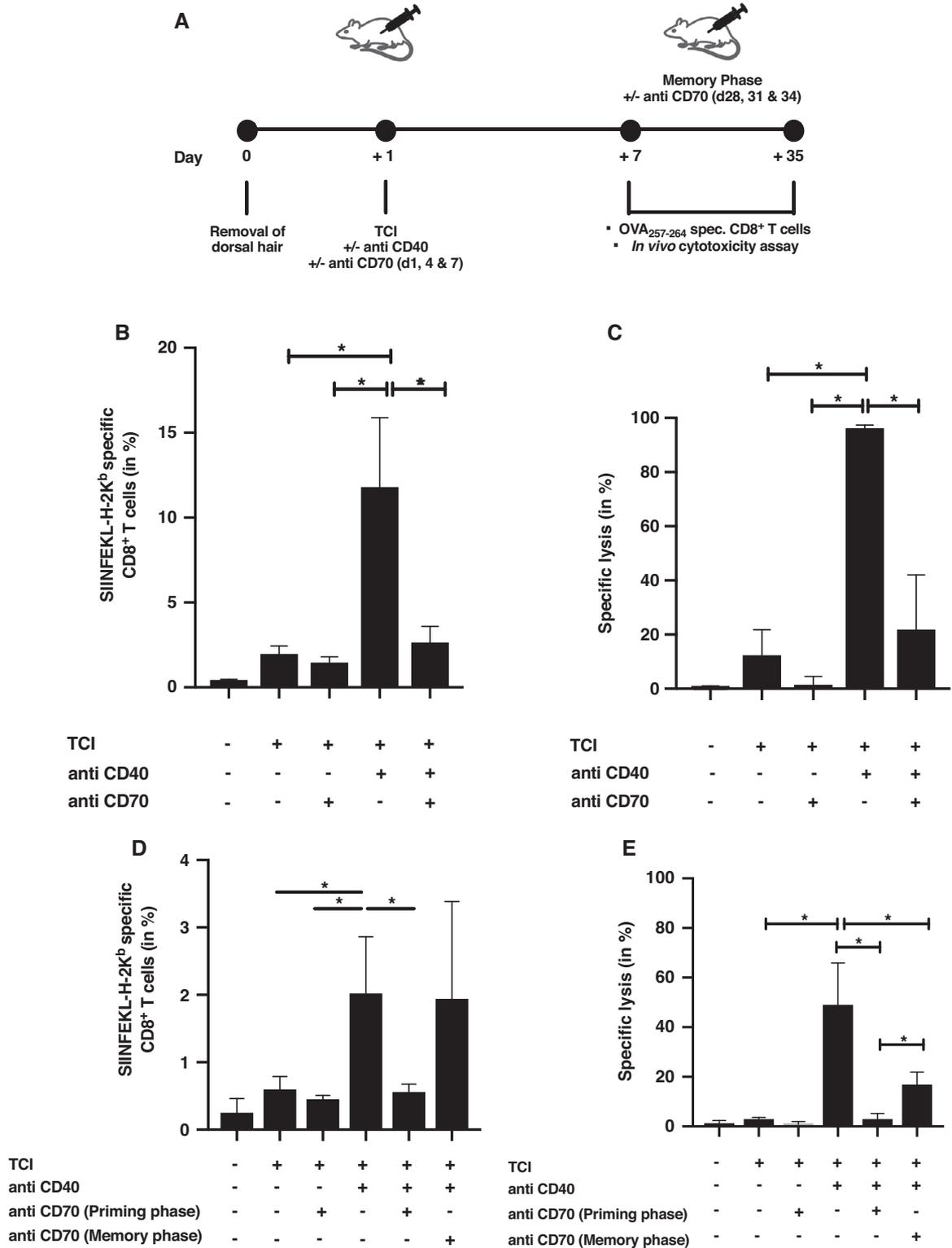
Figure 4. CD40-mediated boosting of TCI depends on CD70 signaling. WT mice were immunized with 50 mg IMI-Sol along with 100 μg OVA$_{257-264}$ on day 1. A total of 250 μg of the anti-CD70 (clone FR-70) were applied on days 1, 4, and 7 via i.p. injection (n = 3–5 per group) (A). The frequency of peptide-specific CD8$^+$ T cells was assessed on day 7 via flow cytometry (B) and the in vivo cytolytic activity was analyzed on day 8, 20 h after transfer of peptide-loaded splenocytes (C). For evaluation of memory responses, the amount of OVA$_{257-264}$ specific CD8$^+$ T cells was measured on day 35 (D) and a second cytotoxicity assay was performed on day 35 and evaluated 20 h after transfer of peptide-loaded splenocytes (E). One representative experiment of two independent experiments is depicted. The data are given as mean with SD. *Significant difference with $p < 0.05$ by one-way ANOVA with Bonferroni's posttest. n.s. indicates "not significant."

significantly reduced in comparison to TCI + anti CD40 treated mice (Fig. 4E). In addition, the diminished immune responses resulting from a delayed FR-70 administration still were significantly stronger compared to those impaired by a CD70 blockade in the priming phase (Fig. 4D and E). Concluding, this findigs clearly illustrate an outstanding importance for CD70 in the priming phase of CD40-boosted TCI. Beyond that, these results also indicate a functional role of CD70 in vivo illustrated by the restricted cytolytic efficiency. Summing up, these results reveal CD70 as a promising target for adjuvant research in imiquimod-based TCI.

CD40 ligation augments antitumor effects of TCI in B16OVA melanoma

Following our preceding findings, suggesting the CD40 pathway as a powerful mechanism to strongly enhance TCI mediated adaptive immune responses, we finally aimed to investigate the efficacy of this new vaccination approach in a therapeutic model of B16OVA melanoma.

Since CD40 may be also expressed on melanoma cell lines [21], we investigated whether CD40 ligation has direct effects on B16OVA melanoma cells. Flow cytometric analysis of B16OVA cells with a fluorescence-labeled anti-CD40 antibody showed no detectable expression of CD40 on the cell surface (not shown). Moreover, we incubated the melanoma cells in vitro with various amounts of the anti-CD40 and performed a proliferation assay to rule out any direct effects on tumor cell proliferation demonstrating that the proliferation of B16OVA cells was neither compromised nor promoted by anti-CD40 (Supporting Information Fig. 1).

For the tumor challenge, we inoculated C57BL6/J WT mice with B16OVA cells s.c. in the left flank and allowed growth until tumor size reached approximately 25 mm^2. Subsequently, the mice were treated with TCI and/or anti-CD40 as indicated in the scheme (Fig. 5A). Untreated (Fig. 5A) and anti-CD40 (Fig. 5C) treated mice showed only minor differences in tumor growth leading to no differences in survival and indicating that CD40 ligation alone has no relevant direct or indirect effects on B16OVA cells in vivo. While TCI treated mice showed a moderate delay in tumor growth (Fig. 5D), this did not result in a significantly prolonged survival. Indicative of the insufficient generation of tumor protective effector memory CTLs, these results were consistent with our previous observation of moderate protection in an LCMV virus challenge model [6]. In contrast, mice that had received TCI in combination with CD40 ligation displayed a significantly enhanced delay in tumor growth (Fig. 5E), leading to a superior survival of about 80% after 40 days (Fig. 5F).

Importantly, even though both, TCI as well as TCI with CD40 ligation, induced high frequencies of peptide-specific CTLs after two treatment cycles (Fig. 6), only mice treated with the combination of TCI and CD40 ligation displayed a significant tumor protection. This observation underlines the importance of high quality CTL responses that cannot be adequately estimated by frequency only.

In conclusion, combined treatment with TCI and CD40 ligation not only leads to enhanced primary and memory CTL responses, but also significantly improves overall survival and delays tumor progression in a therapeutic setup of B16OVA melanoma.

Discussion

While the clinical efficacies and patient outcomes of tumor vaccination approaches have been mostly disappointing so far [22], immunotherapies against cancer have recently gained enourmous interest. Defying cancer by the utilization of the host defense mechanisms appears to be a reasonable and desirable approach. The clinical use of checkpoint inhibitors such as CTLA-4 or PD-1 clearly proves the clinical relevance of such immunotherapies in melanoma and other tumor entities [23–26] and highlights the importance of overcoming tumor driven immunological barriers. At the same time, this might also be an explanation for the limited efficacy of therapeutic vaccines in this setting. Nevertheless, since there is no doubt about the capability of tumor reactive T cells to reject tumors, vaccination approaches that specifically induce or enhance antitumor responses are promising to augment the efficacy of cancer immunotherapies in combinatorial approaches. Along these lines, we were recently able to demonstrate that TCI combined with CTLA-4 blockade enhances tumor specific T cell responses and tumor protection [27].

In our present work, we aimed to optimize CTL priming upon TCI by enhancing co-stimulation via an agonistic CD40 antibody, hypothesizing that our approach using the TLR7 agonist imiquimod provides suboptimal co-stimulatory signals. While we have previously shown with another imiquimod formulation and a different treatment protocol that CD40 ligation enhances CTL responses and memory formation upon TCI [28], our current work now demonstrates the therapeutic efficacy of TCI induced immune responses in a melanoma model (Fig. 5). Importantly and in line with previous data [29, 30], this CTL response is generated by a fully synthetic vaccine and does not require CD4$^+$ T helper cells to create a durable memory response. This is especially remarkable as it is generally acknowledged that CD4$^+$ T cell responses are especially important for forming sufficient CTL memory responses [31]. Toward the underlying mechanisms, CD40 ligation activates the pro-inflammatory CD40 pathway on APCs, promoting the cross-presentation of antigen and activation of dendritic cells (DC) via the production of inflammatory cytokines such as IL-2, IL-7, IL-8, IL-12, TNF-α, and expression of co-stimulatory molecules such as B7 or CD40 [32]. In the current study, we further demonstrate that additional CD40 ligation is able to completely bypass CD4$^+$ T cell help in inducing primary and memory CTL responses (Fig. 3). These results affirm the major relevance of the CD40 pathway in the generation of CD8$^+$ T cell responses [16] and illustrate that CD40 ligation provides a distinct and complementary stimulus in addition to TLR mediated DC activation as previously shown by us and others [19].

To further enrole the underlying mechanism of CD40-mediated boosting in TCI, our present work highlights the importance of
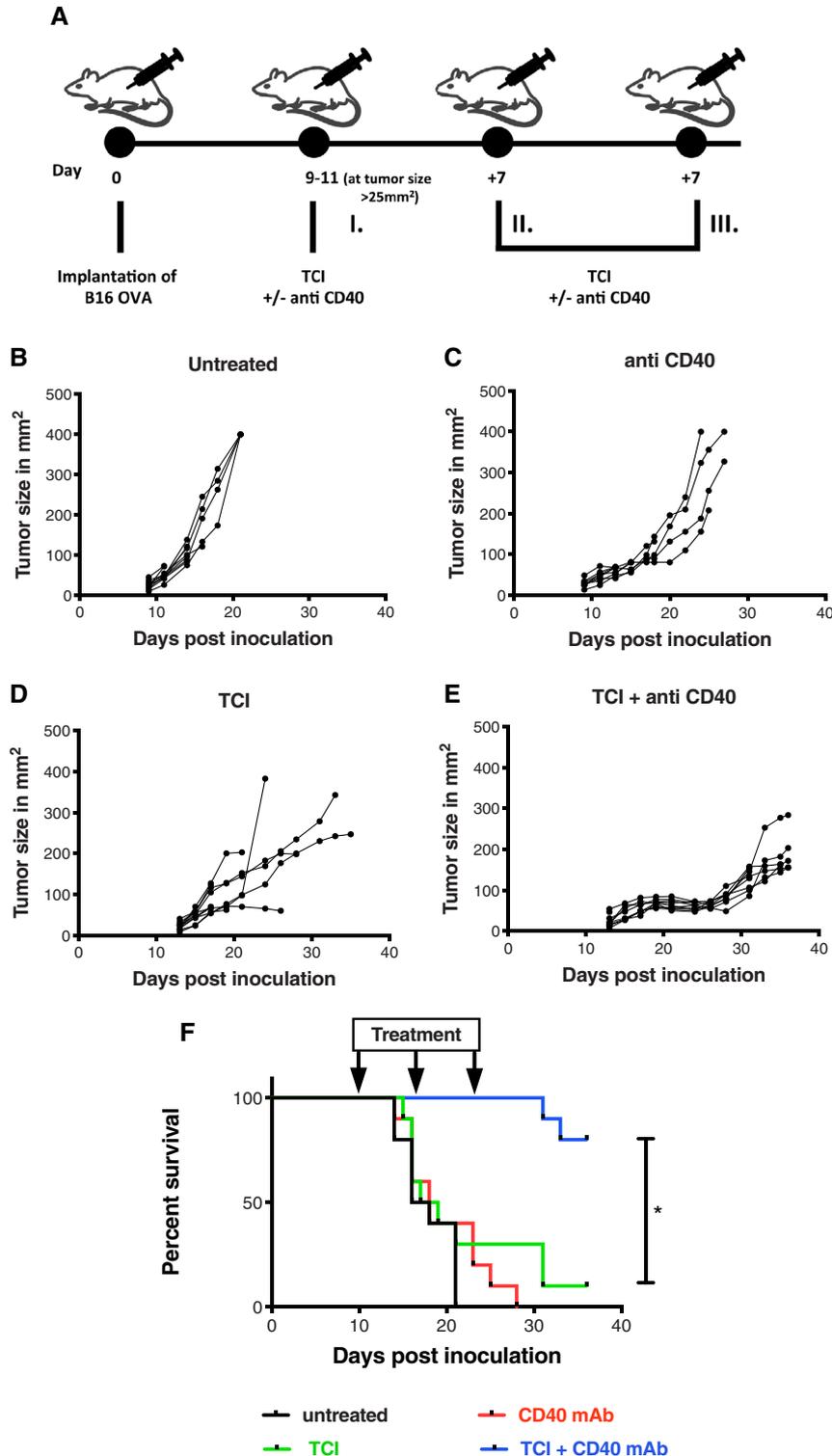
Figure 5. TCI and CD40 ligation delay tumor growth and induce superior survival in B16OVA melanoma. B16OVA melanoma cells (2×10^5 per mouse) were s.c. implanted into the lower left flank of 6–8 weeks old WT mice. Weekly vaccinations were started as indicated at tumor size of >25 mm^2 for 3 weeks along with three s.c. injections of 30 μg anti-CD40 as indicated in (A). Tumor growth and survival were individually assessed for each animal every other day. The depicted data are a cumulative analysis of two independent experiments with $n = 10$ mice for the untreated group (B), $n = 8$ mice for anti-CD40 only (C), $n = 8$ mice for TCI (D), and $n = 10$ mice for TCI + anti-CD40 (E). Each curve represents the tumor growth for one individual animal. Cumulated survival analysis by Kaplan–Meier plot (F). *Significance by Mantel–Cox survival analysis, $p < 0.05$.

CD70/CD27 pathway for the induction of potent CTL responses. While the CD70/CD27 interaction has also been identified to play an essential role in CD8$^+$ T cell priming by CD40-licensed APCs [19], our study fosters the outstanding importance of CD70 molecules in the induction phase of TCI induced T cell responses by an antibody-mediated blockade of CD70 signaling in the priming. Both, the phenotypical appearance and the functionality of antigen-specific CD8$^+$ T cells were impaired when applying a
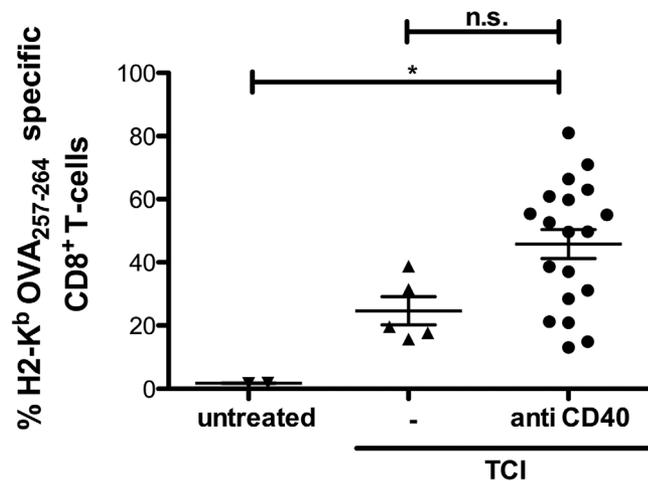
Figure 6. CD40 ligation induces enhanced levels of peptide-specific CD8$^+$ T cells in tumor bearing mice. The frequency of peptide-specific CD8$^+$ T cells was assessed in mice from experiments shown in Fig. 5. Evaluations were performed via flow cytometry on day 18 post tumor inoculation, after two vaccinations ± antibody treatments. The depicted data are the results of the experiment depicted in Fig. 5 (pooled analysis from two independent experiments) with n = 2 mice for untreated (▼), n = 5 mice for TCI (▲), and n = 19 mice for TCI + anti-CD40 (•) treated mice as median with SEM. Each symbol represents an individual mouse (biological replicates). *Significance by one-way ANOVA with Bonferoni posttest analysis; $p < 0.05$.

CD70 blocking antibody (Fig. 4B and C). Further, this study illustrates a significance of CD70 signaling even in sole TCI with our new vaccination platform IMI-Sol (Fig. 4C). In addition, this study highlights that CD70 is indispensable for the induction of immunological memory in the presented TCI model. In addition, our study regarding the role of CD70 in the memory phase led to the assumption that CD70 is unneeded for the maintenance of CD8$^+$ T cells but that blockade via FR-70 affects the cytolytic capability of CD8$^+$ T cells in vivo. This notion is consistent with in vitro studies that indicate an involvement of CD27/CD70 interactions in antigen-specific cytotoxic T-lymphocyte (CTL) activity by perforin-mediated cytotoxicity [33]. Hence, the identification of adjuvantic agents targeting the CD70/CD27 axis through needle-free application seems to be highly desirable.

Moreover, we observed an improved survival of mice and delayed tumor growth using a B16OVA melanoma model (Fig. 5) upon TCI and CD40 ligation. Apparently, this effect is not directly mediated by CD40 ligation on tumor cells or ADCC as B16OVA were negative for CD40 and unimpaired in their expansion in vitro (Supporting Information Fig. 1) or in vivo (Fig. 5C). At present, we are unable to differentiate whether the minor delay in tumor growth in the anti-CD40 treated group is mediated by T cells or by changes in the tumor microenvironment. This needs further investigation. Nevertheless, our results suggest that promoting the synergistic CTL activation through imiquimod and anti-CD40 is a key mechanism for the enhanced antitumor response. Although not directly shown, the most likely mechanism is based on enhanced activation signals for skin resident DC inducing "high-quality" T cells capable of enhanced tumor rejection. Interestingly,

the observed effect was not largely dependent on the quantity of peptide-specific CTL as we did not observe a significant difference in CTL frequency upon TCI with or without CD40 ligation (Fig. 6).

Although the combined treatment led to prolonged tumor protection, it was not able to accomplish complete tumor rejection and tumor growth eventually reaccelerated. Accordingly, we assume that other tumor derived escape mechanisms, such as loss of antigen, MHC I, or other surface receptors such as FasL or TRAIL on the tumor cell surface [34–36] and an immunosuppressive tumor microenvironment caused incomplete tumor rejection. Regulatory T cells, tumor-associated macrophages, or myeloid derived suppressor cells contribute to a protumoral microenvironment by the release of immunosuppressive cytokines (e.g. IL-10, TGF-β) or the inhibition of the checkpoint inhibitors CTLA-4/PD-1 [37, 38]. Interestingly, CD40 can overcome this suppressive milieu by converting M2-like macrophages when combined with CSF1-R inhibition [39–41], indicating a great potential of CD40 ligation in combined immunotherapy approaches, which needs to be further explored.

Towards the potential clinical relevance of these findings, it should be noted that agonistic CD40 antibodies are currently in clinical development and have shown some promising results in various tumors including malignant melanoma and some haematological tumors [41, 42]. While single agent studies with CD40 agonists have confirmed safety they only show limited efficacy [39]. Therefore, the full potential of this treatment will only be revealed when combined with other immunotherapies such as TCI.

In conclusion, CD40 ligation boosts TCI induced primary and memory CTL accomplishing prolonged tumor protection while bypassing CD4$^+$ T cell help. Consequently, combined immunotherapy including advanced vaccination strategies represents a promising approach for the treatment of malignant melanoma and needs to be further explored.

Materials and methods

Mice

Six to 8 weeks old C57BL/6 (WT) mice were used for vaccination experiments and purchased from the animal facility of the University of Mainz (TARC) or the Harlan Laboratories. All animal procedures were conducted according to the institutional guidelines and were reviewed and confirmed by an institutional review board headed by the local animal welfare officer (Prof. Dr. O. Kempski) of the University Medical Center (Mainz, Germany). All assays were approved by the responsible authority (National Investigation Office Rheinland-Pfalz, Koblenz, Germany). The Approval ID assigned by this authority: AZ 23 177-07/ G13-1-012.

Transcutaneous immunizations

Transcutaneous immunizations were performed as described previously [6] using 50 mg IMI-Sol (own production) together with 100 µg of the synthetic peptide $OVA_{257-264}$ (SIINFEKL, peptides&elephants, Potsdam, Germany) dissolved in DMSO and mixed with cremor basalis officinalis. On day 0, dorsal hair of the mice was removed without harming the skin barrier, using an electrical hair clipper. TCI treatments were applied onto the back skin (approximately 10 cm^2) on day 1 if not indicated otherwise. Treatments were scheduled weekly and over a period of 3 weeks in tumor experiments. When indicated, the agonistic CD40 antibody (clone FGK-45, obtained from hybridoma cells of own production) was applied simultaneously with the TCI treatment by an intraperitoneal or subcutaneous injection of 30 µg suspended in 150 µL PBS. In contrast, 250 µg anti-CD70 antibody (clone FR-70, resuspended in 100 µL PBS and kindly provided by Prof. Hideo Yagita, Jutendo University, Tokyo) was applied either on days 1, 4, and 7 (priming phase) or on days 28, 31, and 34 (Memory phase) by intraperitoneal injection.

For depletion of $CD4^+$ cells where indicated, 100 µg anti-CD4 (clone GK1.5, own production) were injected i.p. on day 0.

Mice were anesthetized previous to all procedures using a dilution of Ketamin/Rompun in a weight adjusted dosage.

Flow cytometric analyses and in vivo cytotoxicity assay

Flow cytometric analyses and evaluation of in vivo cytotoxicity were performed as described previously [6] adhering to the current guidelines for the use of flow cytometry in immunological studies [43]. Blood samples were collected via tail vein incision, subjected to hypotonic lysis, and incubated with specific mAbs as indicated. Antibodies used for FACS analysis were APC- or PB-conjugated anti-CD8 (clone 53–6.7; eBioscience, San Diego, USA), PB-conjugated anti-CD3 (clone 17A2; eBioscience, San Diego, USA) and PE-conjugated anti-CD4 (clone GK1.5; BioLegend, San Diego, USA). To measure the frequency of peptide-specific $CD8^+$ T cells, blood samples were stained with PE-conjugated $OVA_{257-264}$-$H2$-K^b (tetrameric labeling, own production).

For detecting in vivo cytolytic activity, splenocytes of syngenic WT mice were labeled with different amounts of 5,6-CFSE (Invitrogen, Carlsbad, USA) resulting in a $CFSE^{low}$ and a $CFSE^{high}$ population. The cells labeled with $CFSE^{low}$ were additionally labeled with 1 µM of $OVA_{257-264}$. Adoptive transfer of 3 × 10^7 cells in a 1:1 ratio ($CFSE^{low}$:$CFSE^{high}$) into immunized and untreated mice was performed via intravenous injection into the tail vein. For assesment of cytolytic activity during the primary response, blood was drawn and subjected to flow cytometry on day 7 post immunization, 6 and 20 h after transfer of target cells. For analysis of the memory response, the assay was performed on day 35 post immunizations. Specific lysis was evaluated as following: Specific lysis (%) = 100 × (1- ($CFSE^{low}$ of immunized mouse/$CFSE^{low}$ of untreated control).

All analyses were performed with a LSRII Flow Cytometer and FACSDiva Software (Becton Dickinson, Franklin Lakes, USA).

Proliferation assay of B16OVA melanoma cells

To evaluate directly mediated effects through CD40 ligation on tumor cell proliferation, B16OVA melanoma cells were incubated in 96-well plates (5000 cells in 100 µL medium per well) with various concentrations of anti-CD40 at 37°C and 5% CO_2. Untreated cells served as negative control, cells incubated with an apoptotic dose (400 µg/mL) of Mitomycin C served as positive control. Cell proliferation was evaluated after 24, 48, and 72 h via photometric measurements at 490 nm using the CellTiter AQuous One Solution-Kit (Promega, Fitchburg, USA). Photometric measurements were performed with a Tecan Microplate Reader.

Tumor rejection assay

For tumor implantation B16OVA melanoma cells (2 × 10^5, ATCC, cultured in Iscove´s medium containing 10% FCS and 1% of each glutamine, Na-pyruvate, penicilline/streptomycine, and G418) were subcutaneously injected into the lower left flank of anesthetized animals. Treatment was initiated at tumor size of approximately 25 mm^2 (9–11 days after inoculation). Tumor growth was traced by measuring the tumor diameter in two dimensions three times a week using a caliper. Mice were sacrificed when tumor size exceeded a diameter of 400 mm^2 or when bleeding ulceration ocurred. The point of death was recorded as the day after sacrifice.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 5.0a for Mac OS X, GraphPad Software). One Way ANOVA with Bonferoni Post-Test was used to compare multiple groups. Survival analysis was accomplished by a Mantel-Cox test. For all analyses, $p < 0.05$ was regarded as statistically significant.

Conflicts of interest: The authors declare no conflict of interest.

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Abbreviation: **TCI**: transcutaneous immunization

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