An optimized peptide vaccine strategy capable of inducing multivalent CD8+ T cell responses with potent antitumor effects

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Therapeutic cancer vaccines are an attractive alternative to conventional therapies for treating malignant tumors, and successful tumor eradication depends primarily on obtaining high numbers of long-lasting tumor-reactive CD8+ T cells. Dendritic cell (DC)-based vaccines constitute a promising approach for treating cancer, but in most instances low immune responses and suboptimal therapeutic effects are achieved indicating that further optimization is required. We describe here a novel vaccination strategy with peptide-loaded DCs followed by a mixture of synthetic peptides, polyinosine-polycytidylic acid (poly-IC) and anti-CD40 antibodies (TriVax) for improving the immunogenicity and therapeutic efficacy of DC-based vaccines in a melanoma mouse model. TriVax immunization 7–12 d after priming with antigen-loaded DCs generated large numbers of long-lasting multiple antigen-specific CD8+ T cells capable of recognizing tumor cells. These responses were far superior to those generated by homologous immunizations with either TriVax or DCs. CD8+ T cells but not CD4+ T cells or NK cells mediated the therapeutic efficacy of this heterologous prime-boost strategy. Moreover, combinations of this vaccination regimen with programmed cell death-1 (PD-1) blockade or IL2 anti-IL2 antibody complexes led to complete disease eradication and survival enhancement in melanoma-bearing mice. The overall results suggest that similar strategies would be applicable for the design of effective therapeutic vaccination for treating viral diseases and various cancers, which may circumvent current limitations of cell-based cancer vaccines.

Introduction

One goal in cancer immunotherapy is to activate and expand tumor-reactive CD8+ T cells that are capable of recognizing and destroying tumor cells.[1,2] Tumor-reactive CD8+ T cells recognize antigens through the engagement of T cell receptors with a cognate peptide-MHC class I complex on tumor cells. It is evident that tumor cells are poorly immunogenic for initiating CD8+ T cell responses because they express insufficient level of antigens, MHC, and costimulatory molecules.[3,4] On the other hand, professional antigen presenting cells (APCs) such as dendritic cells (DCs), when loaded with appropriate tumor antigens, have been proven to trigger tumor-reactive CD8+ T cell responses both *in vivo* and *in vitro*.[5] In view of this, substantial

efforts have been made to develop effective immunotherapeutic strategies utilizing tumor antigen-loaded DCs as vaccines to induce tumor-specific immunity.[6,7] In spite of their safety and apparent immunogenicity, no clear evidence has been obtained indicating that DC vaccines provide significant protective immunity in cancer patients that is superior to other vaccination strategies. Thus, we believe that there is a clear need for additional strategies to optimize DC-based cancer vaccines to achieve the desired therapeutic benefit.

We previously described a non-infectious, subunit peptide-based vaccine capable of generating robust and long-lasting antigen-specific CD8+ T cell responses effective against established tumors.[8,9] This vaccine called TriVax consists of a mixture of a synthetic peptide corresponding to the minimal CD8+ T cell

epitope, poly-IC adjuvant, and costimulatory anti-CD40 antibodies (Abs), which is administered intravenously. More recently, experiments performed in combination with adoptive cell transfer (ACT) demonstrated that the administration of TriVax after ACT resulted in the expansion of vast number of adoptively transferred CD8$^+$ T cells without the need of lymphodepletion and/or high dose IL2, which are usually required for ACT.[10] These results suggest that TriVax can be very efficient in stimulating and expanding pre-existing antigen-specific T cells. Although these results were highly encouraging for developing therapeutic peptide vaccines, the TriVax immunization strategy by itself is somewhat ineffective in generating primary responses with some MHC binding peptides. In this respect, we hypothesize that CD8$^+$ T cells primed with antigen-loaded DCs may be able to expand to large numbers following TriVax administration. Indeed, we show here that heterologous DCs prime-TriVax booster immunizations in mice achieved very high levels of CD8$^+$ T cell responses capable of persisting long periods of time. This strategy was effective when a single or multiple peptide epitopes were used and were capable of eliciting significant tumor responses in a mouse melanoma model. Moreover, the addition of low dose of IL2 in a form of IL2/anti-IL2 Abs complexes (IL2Cx) or PD-1 blockade further enhanced the efficacy of this heterologous vaccination strategy obtaining complete tumor rejections. The present findings should facilitate the development of more effective immunotherapeutic strategies for cancer that may circumvent current limitations of cell-based cancer vaccines.

Results

TriVax booster immunization after priming with peptide-loaded DCs elicits enhanced and long-lasting CD8$^+$ T cell responses with potent antitumor effects

To assess whether TriVax (peptide mixed with poly-IC and anti-CD40 Abs) would enhance the immune responses generated by DCs vaccination, we compared several combinational prime-boost immunization modes using the minimal heteroclitic epitope Trp1$_{455/9M}$ (TAPDNLGYM), which has a substitution at position 9 M for A to improve MHC-I binding and immunogenicity.[11,12] As shown in **Fig. 1A**, both Trp1$_{455/9M}$-loaded DCs and Trp1$_{455/9M}$TriVax generated 1–2% antigen-specific IFNγ-producing CD8$^+$ T cells in blood 6 d after priming. The numbers of antigen-specific CD8$^+$ T cells in blood after the prime were comparable, but the effectiveness of spleen CD8$^+$ T cells to recognize peptide-pulsed cells and B16 cells was somewhat better in mice vaccinated with antigen-loaded DCs (**Fig. S1**). When homologous or heterologous booster immunizations were administered 7 d after the prime, evident increases in the numbers of antigen specific CD8$^+$ T cells were observed in all cases (**Fig. 1A, B**). However, the magnitude and duration of the secondary T cell response was most dramatic in mice that received Trp1$_{455/9M}$TriVax booster immunization after DC priming (more than 8-fold as compared to the homologous DC vaccination). The reverse heterologous immunization, TriVax prime, DC boost did

not induce the massive immune response observed with DC prime-TriVax boost. The large CD8$^+$ T cell responses induced by DCs prime, TriVax boost (DC_TriVax) were capable of persisting for more than 2 months (**Fig. 1B** and **Fig. S2**). Notably, the mice that received DC_TriVax immunization had higher number of Trp1$_{455}$-specific CD8$^+$ effector T cells with lytic functionality (CD107a/b mobilization) and capable of producing IFNγ observed at day 70 post-vaccination (**Fig. 1C** and **Fig. S3**). The functional activity of freshly isolated CD8$^+$ T cells was evident not only against peptide-pulsed APCs but also toward B16 melanoma cells (**Fig. 1D**). The optimal time interval between prime (Trp1$_{455/9M}$-loaded DCs) and booster (Trp1$_{455/9M}$TriVax) immunizations was observed to be between 7 and 12 d (**Fig. 2**). On the other hand, a significantly lower CD8$_+$ T cell response was observed when the booster immunization was given 5 d post-prime and almost no secondary response was observed when the boost was administered 3 d after the prime. Moreover, we observed that both intravenous and subcutaneous routes of DC vaccination (for priming) were highly effective and comparable for the generation of strong antigen-specific CD8$^+$ T cell responses followed by TriVax booster immunization (**Fig. S4**). So far these results indicate that TriVax immunization 7–12 d after priming with antigen-loaded DCs, generates large numbers of long-lasting antigen-specific CD8$^+$ T cells capable of recognizing tumor cells and that these responses are far superior to those generated by homologous immunization with either TriVax or DCs. The data also imply that although DCs are very effective at priming the CD8$^+$ T cell responses they are quite ineffective at boosting.

Effects of poly-IC and anti-CD40 Abs on booster immunization after priming with peptide-loaded DCs vaccination

Next, we evaluated the role that each of the components of TriVax play in the secondary expansion of antigen-specific CD8$^+$ T cells, which were induced by the Trp1$_{455/9M}$-loaded DC priming vaccination. TriVax booster vaccine containing all three components (Trp1$_{455/9M}$, poly-IC, and anti-CD40 Abs) was significantly superior to the administration of peptide alone, peptide plus poly-IC, or peptide plus anti-CD40 Abs (**Fig. 3A, B**). Moreover, substitution of the anti-CD40 Abs for other agonistic Abs reactive with different costimulatory molecules (OX40 and 4-1BB), known to enhance the magnitude and quality of T cell responses, was quite not as effective as anti-CD40, and induced responses similar to those observed with peptide plus anti-CD40. Freshly isolated splenic CD8$^+$ T cells were effective in recognizing peptide-pulsed EL4 targets and B16 melanoma cells (**Fig. 3C, D**).

Therapeutic effects of DC prime-TriVax boost vaccination against established B16 melanoma

Next, we evaluated whether Trp1$_{455/9M}$DC_TriVax vaccination would offer a therapeutic benefit against 5 d subcutaneously established B16 tumors (3–5 mm diameter). As shown in **Fig. 4A**, homologous prime-boost Trp1$_{455/9M}$TriVax vaccinations had a moderate therapeutic effect, whereas the heterologous
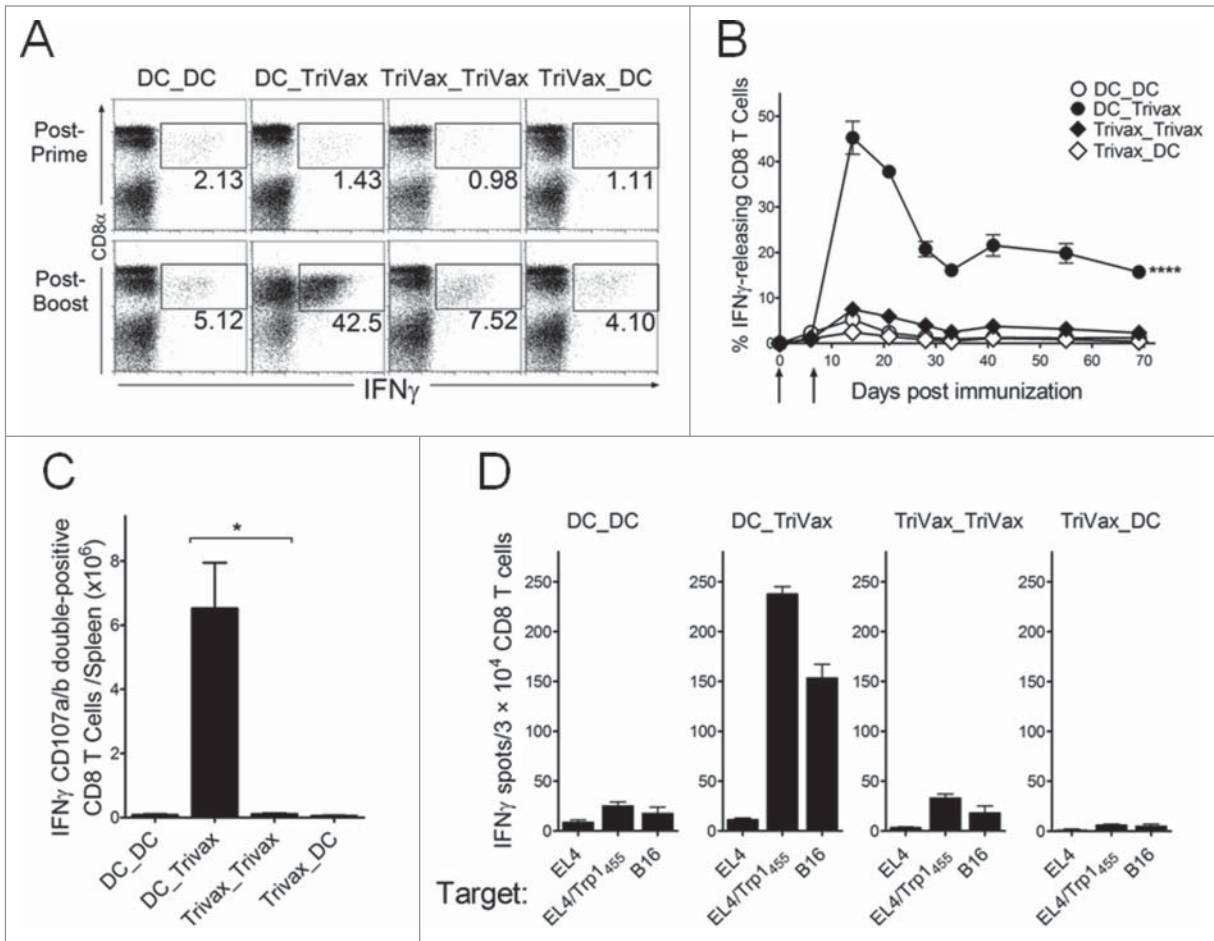
Figure 1. TriVax booster immunization after priming with peptide-loaded DCs elicits enhanced CD8$^+$ T cell responses. B6 mice (three per group) were immunized intravenously with either 2×10^6 DCs loaded with Trp1$_{455/9M}$ peptide or TriVax composed of 100 μg of Trp1$_{455/9M}$, 50 μg of poly-IC, and 100 μg of anti-CD40 (prime); 7 d later, the mice received a booster immunization with DCs or TriVax. (**A**) On day 6 (post-prime) and 14 (post-boost), blood samples were evaluated by intracellular IFNγ staining after coculturing with Trp1$_{455}$ peptide. Dot plot analysis for one mouse of each group is presented. *Numbers* in each rectangular gate represent the percentage IFNγ positive cells of all CD8$^+$ T cells. (**B**) Frequency of Trp1$_{455}$-specific CD8$^+$ T cells in peripheral blood was followed in individual mice throughout various time points. *Points*, mean for each group; *bars*, SD. *Arrows*, times of vaccination. *P* value was calculated using two-way ANOVA test comparing with the homologous prime-boost TriVax-vaccinated group (****, $P < 0.0001$). (**C**) Total numbers of intracellular IFNγ and cell surface CD107a/b double-positive CD8$^+$ T cells was calculated from the experiment in (**B**). On day 70, splenocytes from each individual mouse were stimulated for cell surface mobilization of CD107a/b and intracellular IFNγ staining. *Columns*, mean for each group; *bars*, SD. *P* value was calculated using unpaired Student *t* test (*, $P < 0.05$). (**D**) CD8$^+$ T cells were purified from pooled splenocytes, and antigen-induced IFNγ secretion was evaluated for their capacity to recognize tumor cells using EliSpot assay. APCs used: Trp1$_{455}$-pulsed EL4 (EL4/Trp1$_{455}$), B16 melanoma, and un-pulsed EL4 cells (negative control). Results represent the average number of spots from triplicate wells with SD (*bars*) of the means.

Trp1$_{455/9M}$DC_TriVax immunization exhibited a substantially better antitumor effect. In contrast, the other vaccination protocols tested had negligible therapeutic effects, which were comparable to the no vaccine and the two control groups that received an irrelevant peptide (Ova$_{55}$DC_ Ova$_{55}$TriVax and a Trp1$_{455/9M}$TriVax immunization priming with DCs not pulsed with peptide (DC$_{only}$_Trp1$_{455/9M}$TriVax). The therapeutic antitumor effects induced by these vaccines correlated with the levels of antigen-specific T cells observed in blood (**Fig. 4B**).

Next, we assessed the roles that various immune cell subsets play in the induction of the therapeutic antitumor effects induced by DC_TriVax vaccination. Specifically we studied the participation of CD8$^+$, CD4$^+$ T cells, T regulatory cells and NK cells by *in vivo*

depletion of these cell subsets using specific Abs. Elimination of CD8$^+$ T cells completely abolished the therapeutic benefit of DC_TriVax vaccination, demonstrating that these cells are crucial for the controlling of established tumors (**Fig. 4C**). Interestingly, CD4$^+$ T cell elimination substantially enhanced the therapeutic efficacy of DC_TriVax vaccination with increased frequency of antigen-specific CD8$^+$ T cells (**Fig. 4D**). The combination of anti-CD4$^+$ treatment and DC_TriVax vaccination potentiated the expansion of tumor-reactive CD8$^+$ T cells, by ~4-fold increase compared with non-combined mice (data not shown). On the other hand, anti-CD25 and anti-NK1.1 antibody treatment had no significant effect on either the antitumor effectiveness of DC_TriVax, or the magnitude of antigen-specific CD8$^+$ T cells.
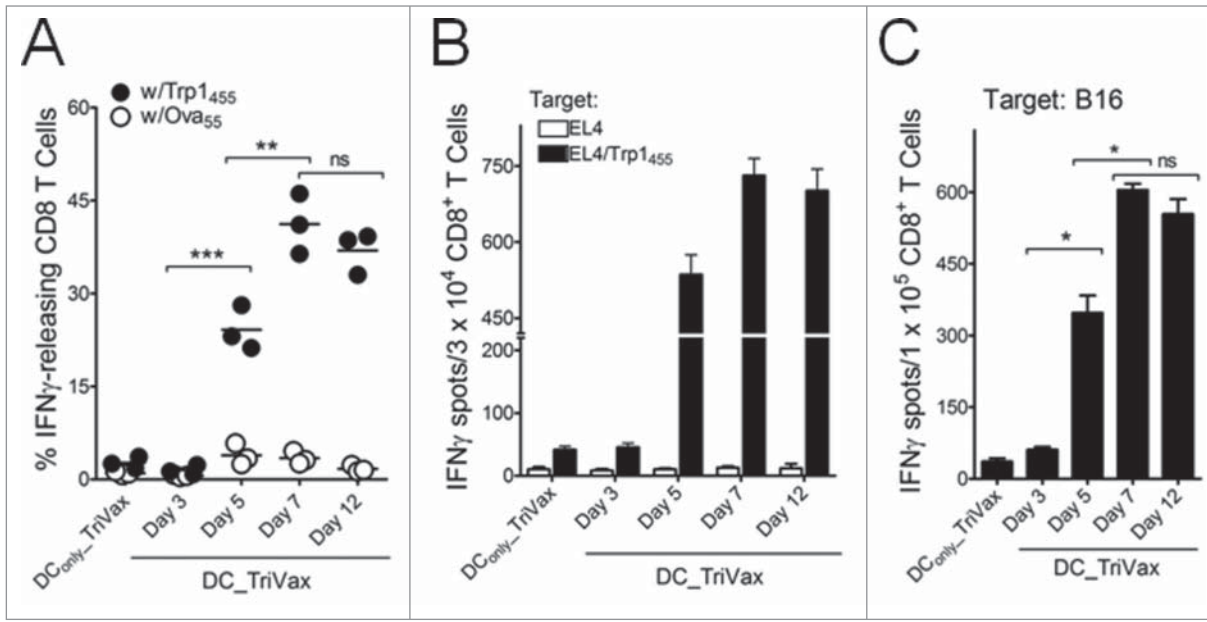
Figure 2. Optimal interval of vaccination in DCs prime and TriVax booster immunization strategies. B6 mice (three per group) were immunized intravenously with $Trp1_{455/9M}$-loaded DCs (prime); 3, 5, 7 and 12 d later, the mice received booster immunization with $Trp1_{455/9M}$TriVax. An experimental group primed with peptide-free DCs alone and boosted with $Trp1_{455/9M}$TriVax on day 7 ($DC_{only\_}$TriVax) was included. (**A**) Eight days after the boost, the presence of $Trp1_{455}$-specific $CD8^+$ T cell responses in spleen was evaluated by intracellular $IFN\gamma$ staining as described in **Fig. 1B**. *Points*, the value for each individual mouse; *horizontal line*, the average value of the group. (**B and C**) Antigen-induced $IFN\gamma$-EliSpot assay was performed with $Trp1_{455}$-pulsed EL4 (**B**) and B16 melanoma (**C**). Results represent the average number of spots from triplicate wells with SD (*bars*) of the means. *P* values were calculated using unpaired Student *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant). These experiments were repeated twice with similar results.

We previously observed that PD-1 blockade (with anti-PD-L1 Abs) and the use of IL2 in the form of IL2Cx resulted in a remarkably enhanced therapeutic antitumor effect in mice treated with adoptive T cell transfers followed by TriVax.[10] In view of this, we evaluated here the addition of anti-PD-L1 Abs and IL2Cx to the $Trp1_{455/9M}$DC_TriVax vaccination strategy. The results shown in **Fig. 4E** indicate that PD-1 blockade or IL2Cx potentiated the therapeutic efficacy of DC_TriVax. The improved antitumor effects observed in the IL2Cx combination were accompanied by a substantial increase of $Trp1_{455}$-specific $CD8^+$ T cells (**Fig. 4F**). On the other hand, although enhanced therapeutic effects were observed with use of PD-1 blockade, no significant increase of antigen-specific $CD8^+$ T cells was observed in comparison to DC_TriVax immunization alone.

Application of DCs prime-TriVax boost vaccination strategy to different epitopes derived from melanosomal antigens

Next, we inquired whether the DC_TriVax strategy could be extended to other MHC-I binding peptides known to function as strong $CD8^+$ T cell epitopes for mouse melanoma. For these experiments, we examined two T cell epitopes derived from the melanosomal antigens, $Trp2_{180}$ (SVYDFFVWL) and human $gp100_{25}$ ($hgp100_{25}$; KVPRNDQWL), which functions as a heteroclitic $CD8^+$ T cell epitope for mouse $gp100_{25}$ ($mgp100_{25}$; EGSRNDQWL).[13] The results showed that $hgp100_{25}$DC_TriVax immunization induced a substantially higher $mgp100_{25}$-specific $CD8^+$ T cell response as compared to homologous $hgp100_{25}$TriVax prime-boost (**Fig. 5A, B**). Although a homologous prime-boost $Trp2_{180}$TriVax vaccination generated high levels of antigen-specific $CD8^+$ T cells, the $Trp2_{180}$DC_$Trp2_{180}$TriVax strategy resulted in approximately 2-fold higher number of antigen-specific melanoma reactive $CD8^+$ T cells. Both peptides generated $CD8^+$ T cells capable of recognizing peptide-pulsed targets and B16 melanoma cells (**Fig. 5C**). A peptide titration curve comparison between these $CD8^+$ T cells revealed that the $Trp2_{180}$-specific T cells induced by DC_TriVax exhibited an approximately 10-fold higher avidity as compared with T cells generated by homologous prime-boost TriVax immunization (**Fig. 5D**).

Multi-epitope-loaded DCs priming followed by TriVax booster immunization induces concurrent effective antitumor $CD8^+$ T cell responses

One of the potential hurdles of tumor immunotherapy using single T cell epitope vaccines is the possibility of the selection of tumor escape variants. In view of this, we explored whether priming using DCs loaded with several peptides followed by multipeptide TriVax would generate a concurrent, multivalent $CD8^+$ T cell response. As shown in **Fig. 6A**, DC_TriVax using a combination of three peptide epitopes ($Trp1_{455/9M}$, $Trp2_{180}$, and $hgp100_{25}$) triggered a substantial simultaneous $CD8^+$ T cell response to all three epitopes, whereas homologous prime-boost TriVax vaccination mostly generated a $Trp2_{180}$-specific $CD8^+$
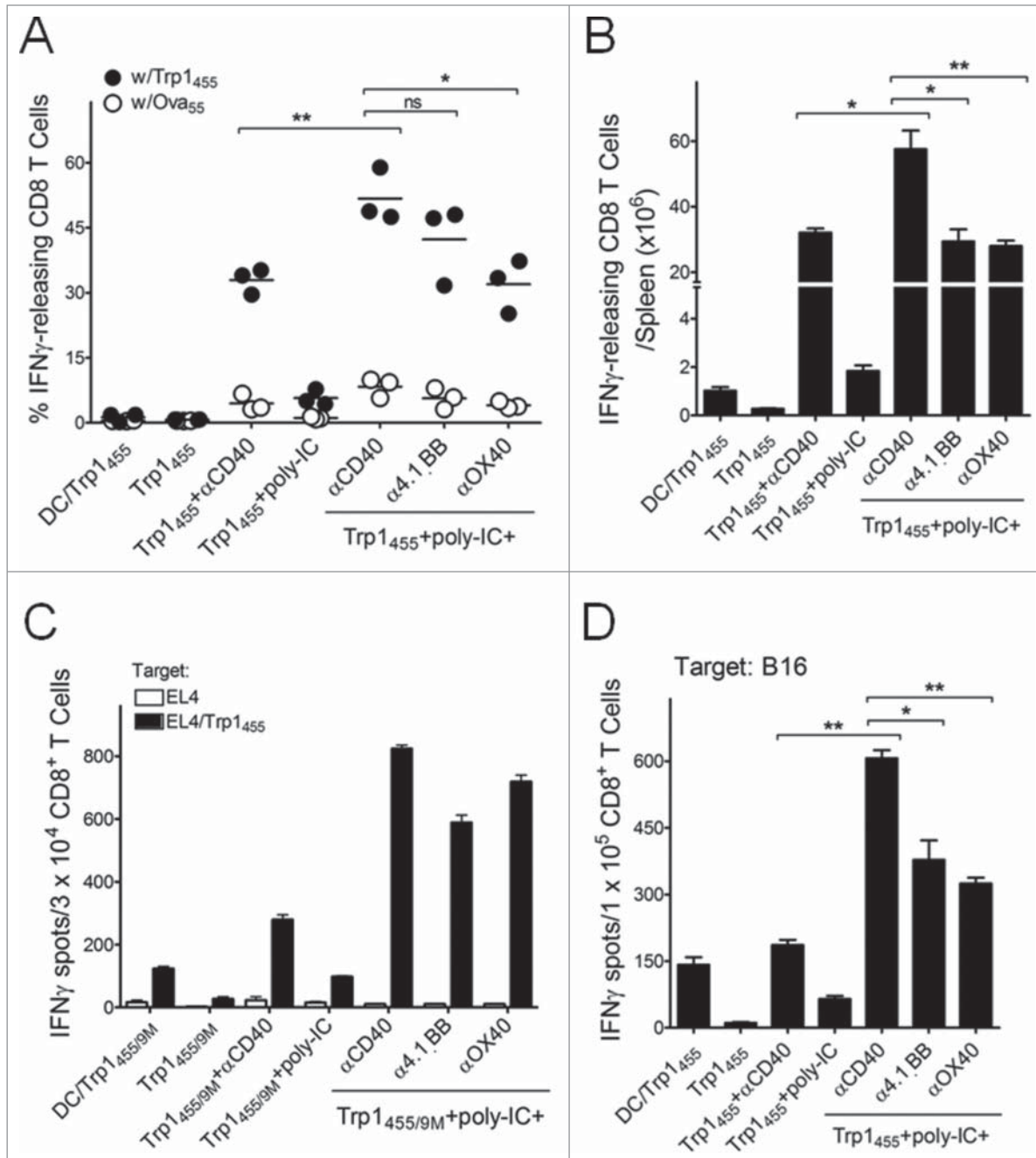
Figure 3. Synergic effects of poly-IC and anti-CD40 Abs for booster immunization after priming with peptide-loaded DCs. B6 mice (three per group) were immunized intravenously with $Trp1_{455/9M}$-loaded DCs (prime); 7 d later, the mice received booster immunization with various combinations of 100 μg of $Trp1_{455/9M}$ peptide, 50 μg of poly-IC, 100 μg of anti-CD40, anti-4.1BB, and anti-OX40 Abs as indicated. (**A**) Eight days after the boost, numbers of $Trp1_{455}$-specific $CD8^+$ T cells in spleen were evaluated by intracellular IFNγ staining after coculturing with $Trp1_{455}$ (w/$Trp1_{455}$) and Ova_{55} (w/Ova_{55}) peptides. *Points*, the value for each individual mouse; *horizontal line*, the average value of the group. (**B**) Total numbers of IFNγ-positive $CD8^+$ T cells were calculated from the experiment in **A**. *Columns*, mean for each group; *bars*, SD. (**C** and **D**) Freshly isolated $CD8^+$ T cells from pooled splenocytes were evaluated for antigen-induced IFNγ secretion using EliSpot assay against $Trp1_{455}$-pulsed (EL4/$Trp1_{455}$) and un-pulsed EL4 cells (**C**), and B16 melanoma (**D**). Results represent the average number of spots from triplicate wells with SD (*bars*) of the means. *P* values were calculated using unpaired Student *t* test (*, $P < 0.05$; **, $P < 0.01$; ns, not significant).

T cell response. The functional activity of purified $CD8^+$ T cells was studied for their ability to recognize various target cells. Notably, $CD8^+$ T cells from DC_TriVax-immunized mice recognized B16 melanoma cells significantly better as compared with $CD8^+$ T cells from homologous prime-boost TriVax-immunized mice (**Fig. 6B**). Administration of either DCs loaded with three peptides at the same time (3pep_MIX) or a mixture of DCs that were pre-loaded with individual peptides did not make
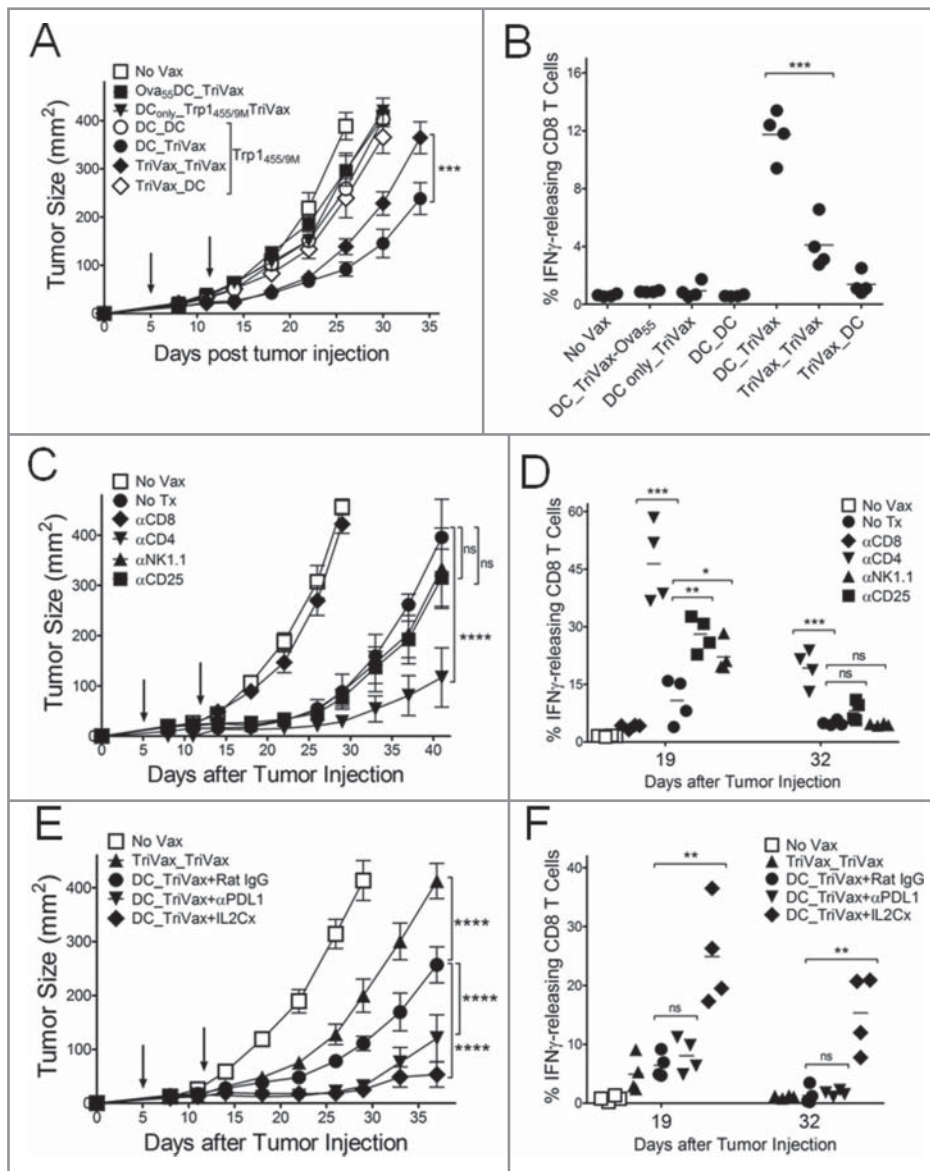
Figure 4. Therapeutic antitumor effect of DCs prime-TriVax boost vaccination strategy against established B16 melanoma. B6 mice (four per group) were inoculated subcutaneously on day 0 with 2×10^5 B16 cells and vaccinated intravenously on day 5, and 12 (vertical arrow) as indicated. (**A**) Effects of combinatorial vaccination on the therapeutic efficacy of antigen-loaded DCs and TriVax immunization. Non-vaccinated mice (No Vax), Ova_{55}-loaded DCs prime/Ova_{55}TriVax booster (DC_TriVax- Ova_{55}) vaccinated, and peptide-free DCs alone prime/$Trp1_{455}$TriVax booster (DC_only_TriVax) vaccinated mice were included as controls. (**B**) On day 19, blood samples were evaluated by intracellular IFNγ staining after coculturing with $Trp1_{455}$ peptide. *Points*, the value for each individual mouse; *horizontal line*, the average value of the group. (**C**) Effector mechanism involved in antitumor effects. B16-bearing mice were administered with indicated Abs -1 and -3 d before immunization. No Vax and no antibody-treated (No Tx) mice were included as controls. (**E**) PD-1 blockade and IL2Cx enhance the therapeutic efficacy of DCs prime-TriVax boost vaccination regimen. No Vax and homologous prime-boost TriVax-vaccinated mice (TriVax_TriVax) were included as controls. Anti-PD-L1, normal rat IgG, or IL2Cx were administered as described in Materials and Methods. (**D** and **F**) Frequency of antigen-specific CD8$^+$ T cells was evaluated at various time points (on day 19 and 32) by intracellular IFNγ staining using blood samples from mice in **C** and **E**, respectively. Tumor sizes were determined in individual mice by measurements of two opposing diameters and are presented as tumor areas in mm^2. *Points*, mean for each group of mice; *bars*, SD. Statistical significance of tumor sizes and numbers of antigen-specific CD8$^+$ T cells was calculated using two-way ANOVA test and unpaired Student t tests, respectively, comparing with the normal DCs prime-TriVax boost group (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant).

a difference for inducing an effective CD8$^+$ T cell responses after a TriVax boost (data not shown).

Next, we examined the therapeutic efficacy of $3pep_{MIX}$-loaded DC_TriVax against established B16 melanoma in comparison with the individual $Trp1_{455/9M}$-, $Trp2_{180}$-, or $hgp100_{25}$DC_TriVax immunizations. As shown in **Fig. 6C**, administration of homologous prime-boost $3pep_{MIX}$-TriVax had a moderate therapeutic effect that was comparable with $hgp100_{25}$DC_ $hgp100_{25}$TriVax. On the other hand, $3pep_{MIX}$DC_$3pep_{MIX}$ TriVax resulted in a remarkably superior therapeutic antitumor effect. Administration of $Trp1_{455/9M}$DC_TriVax and $Trp2_{180}$DC_TriVax resulted in an equally intermediate antitumor effect. The presence of antitumor CD8$^+$ T cells was evaluated in spleens of tumor-bearing mice that received $Trp1_{455/9M}$-, $Trp2_{180}$-, and $3pep_{MIX}$DC_TriVax on day 36. The results shown in **Fig. 6D** indicate that multivalent CD8$^+$ T cells capable of effectively recognizing B16 tumor cells were present in $3pep_{MIX}$DC_TriVax-vaccinated mice, whereas CD8$^+$ T cells from the mice that received $Trp2_{180}$DC_TriVax recognized dominantly $Trp2_{180}$-pulsed EL4 target cells. In addition, CD8$^+$ T cells from the mice that received $Trp1_{455/9M}$DC_TriVax were effective in recognizing the $Trp1_{455}$-pulsed target cells and B16 tumor cells. Interestingly, the CD8$^+$ T cells from these mice were also effective in recognizing $Trp2_{180}$- or $mgp100_{25}$-pulsed target cells, indicating that $Trp1_{455/9M}$DC_TriVax immunization in tumor-bearing mice may be effective in generating epitope spreading. Lastly, we assessed the additional therapeutic benefit of combining PD-1 blockade or IL2Cx with $3pep_{MIX}$DC_TriVax vaccination against established B16 tumors. The results presented in **Fig. 6E** indicate that the addition of either IL2Cx or PD-1 blockade increased dramatically the efficacy of $3pep_{MIX}$DC_TriVax vaccination, where several complete tumor eradications were now attained. Furthermore, we validated the presence of antigen-

specific CD8^{+} T cells in tumor-infiltrating lymphocytes (TIL) and splenocytes. Since the tumor size may impact the effectiveness of T cell immunity, $3pep_{MIX}DC\_TriVax$ vaccination regimen was used to treat 7 d established B16 tumors ($\sim$0.5 cm diameter). Under these circumstances, the addition of PD1 blockade and IL2Cx reduced significantly the median tumor growth rate, but no tumor rejections were observed (**Fig. S5A**). When the frequency of antigen-specific CD8^{+} T cells was measured at day 32, $Trp2_{180}$-specific CD8^{+} T cells were dominantly present in TIL and splenocytes and the combination using IL2Cx helped to maintain higher numbers of the $Trp2_{180}$-specific CD8^{+} T cells as compared with DC_TriVax alone or DC_TriVax/ PD1 blockade (**Fig. S5B**). Notably, less than 5% of $Trp2_{180}$-specific CD8^{+} T cells in TIL ($\sim$0.5% in splenocytes) express PD-1, (receptor for PD-L1), indicating that the addition of IL2Cx and PD-1 blockade had no significant effect on the levels of PD-1 expression on antigen-specific T cells (**Figs. S5C, D**).

Discussion

It is evident that the effectiveness of a therapeutic vaccine will depend in great part on its ability to induce high numbers of long-lasting antigen-specific CD8^{+} T cells with sufficient high avidity to recognize tumor cells. Our findings provide a promising strategy in which one could achieve high levels of tumor-reactive CD8^{+} T cell responses by combined administration of antigen-loaded DCs followed by a TriVax booster immunization. When compared with two homologous prime-boost vaccinations, the DCs prime-TriVax boost vaccination generated >6-fold higher numbers of functional and long-lasting cytotoxic CD8^{+} T cells, which were capable of recognizing tumor cells translating into significant therapeutic benefit.
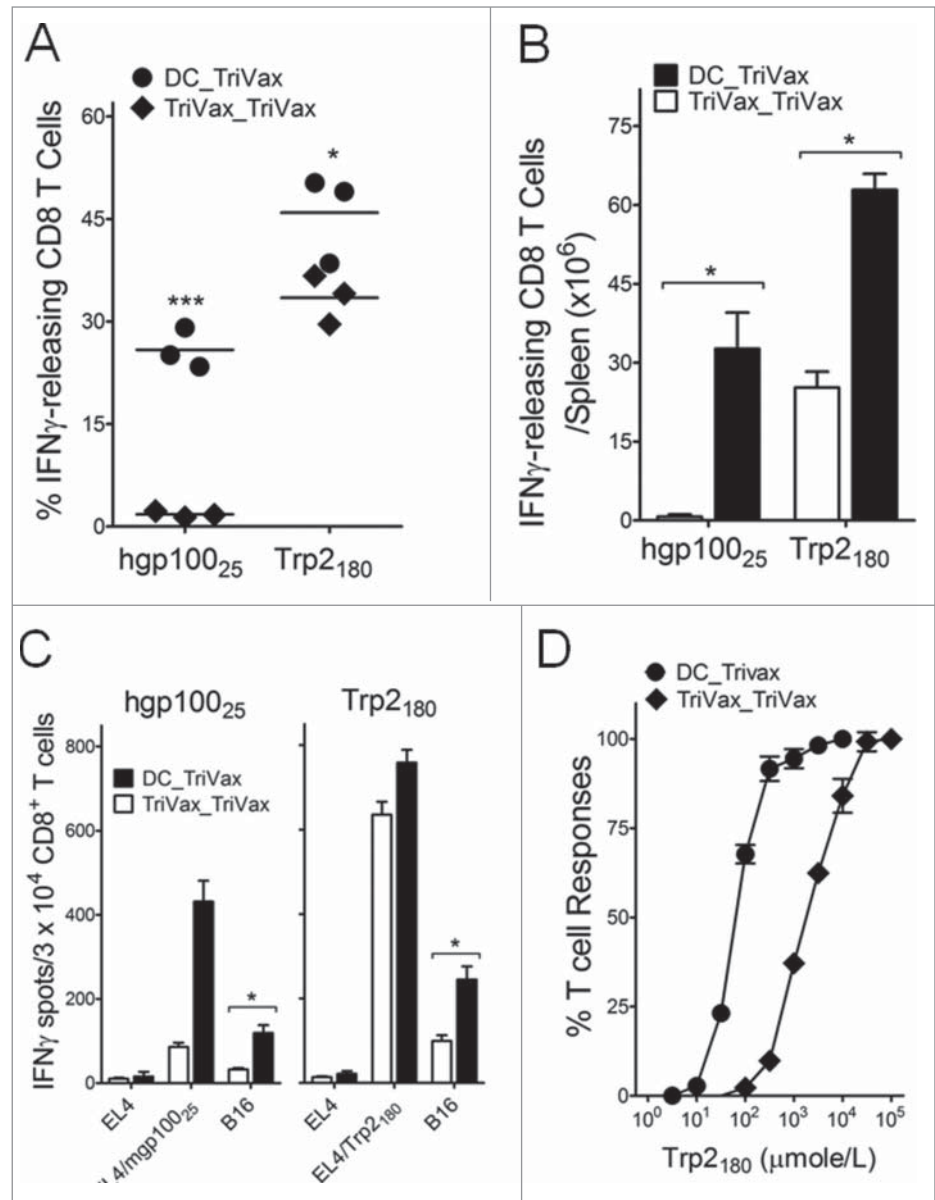

Figure 5. Application of DC prime-TriVax boost regimen to various melanosomal antigen epitopes. B6 mice (three per group) were vaccinated intravenously with either peptide-loaded DCs or TriVax (prime); 7 d later, the mice received TriVax booster immunization in the same manner as described in Fig. 1, except $Trp2_{180}$ and human $gp100_{25}$ ($hgp100_{25}$) peptides were used instead of $Trp1_{455/9M}$ (DC_TriVax and TriVax_TriVax, respectively). (**A**) Eight days after the boost, the presence of antigen-specific CD8^{+} T cell responses in spleen were evaluated by intracellular IFNγ staining after coculturing with $Trp2_{180}$ and mouse $gp100_{25}$ ($mgp100_{25}$), respectively. *Points*, the value for each individual mouse; *horizontal line*, the average value of the group. (**B**) Total numbers of intracellular IFNγ-positive CD8^{+} T cells were calculated from the experiment in **A**. *Columns*, mean for each group; *bars*, SD. (**C**) Freshly isolated CD8^{+} T cells from pooled splenocytes were evaluated for antigen-induced IFNγ secretion with peptide-pulsed EL4 (EL4/ $Trp2_{180}$ and $EL4/mgp100_{25}$, respectively), B16 melanoma, and un-pulsed EL4 cells (negative control). Results represent the average number of spots from triplicate wells with SD (*bars*) of the means. (**D**) Antigen-dose responses of purified CD8^{+} T cells isolated from mice immunized with $Trp2_{180}$ peptide. Results represent the percent T cell response compared to the maximal response (100%) for each T cell line with SD (*bars*) from triplicate cultures. *P* values were calculated using unpaired Student *t* test comparing with homologous prime-boost TriVax-vaccinated group (*, $P < 0.05$; ***, $P < 0.001$).
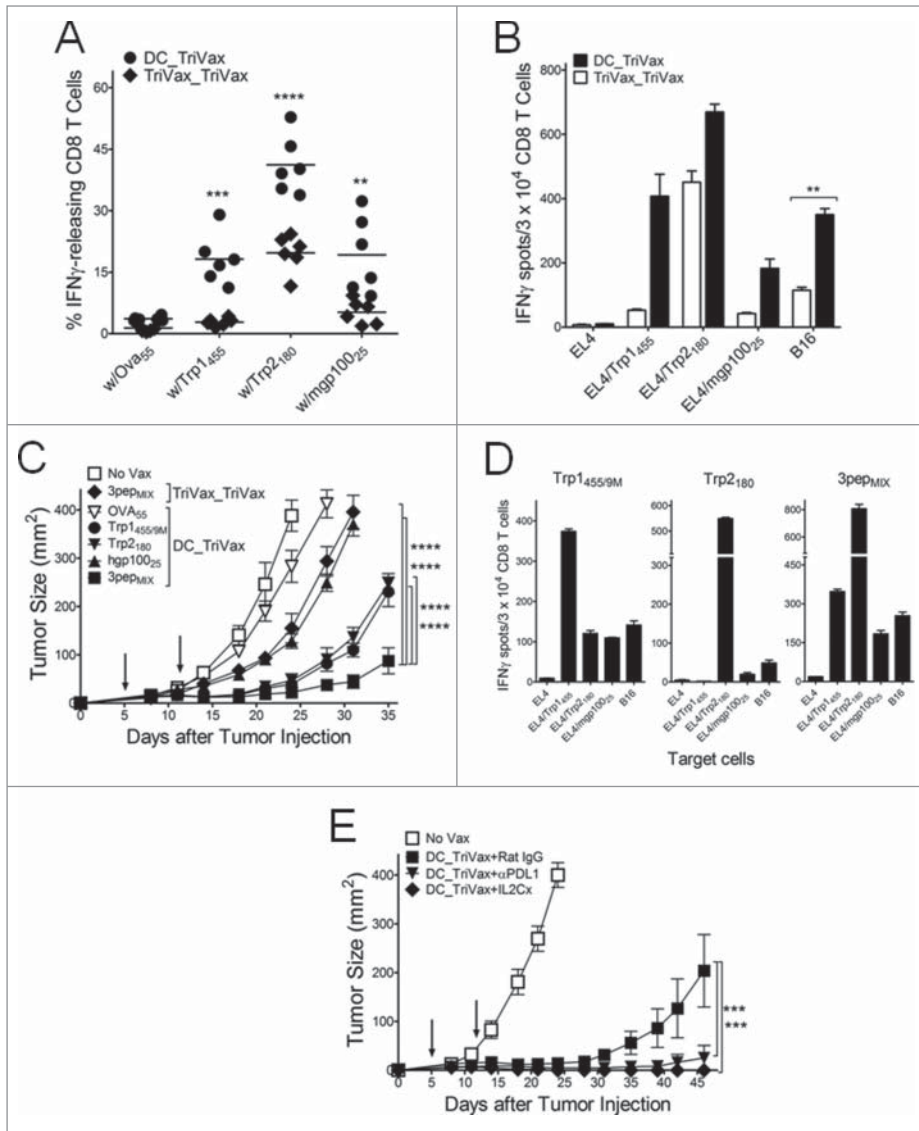
Figure 6. For figure legend, see next page.

vaccine (TriVax). By using antigen-pulsed DCs, naïve $CD8^+$ T cells are first exposed to antigen, costimulation, and appropriate cytokines for activation and formation of primary memory T cells. There is some evidence that pro-inflammatory cytokines during priming can have negative effect on the rate of memory $CD8^+$ T cell formation.[15-17] In view of this, the reason why TriVax prime-DCs boost and Trivax prime-TriVax boost immunizations were not as effective as DCs prime-TriVax boost regimen could be lack of early memory $CD8^+$ T cells formulation following initial TriVax priming. Nevertheless, it should be mentioned that efficient priming could be obtained for some peptides using TriVax or peptide plus poly-IC.[8-11] The ability of a peptide to prime immune responses effectively using TriVax appears to be related to intrinsic properties of the peptide such as size, amphipathicity and whether the peptide represents a self-antigen such as the case of melanosomal proteins. Indeed, we observed that TriVax prime TriVax boost using $Trp2_{180}$ was quite effective in generating preprimary T cell responses (**Fig. 5A**). Nevertheless, our results show that an advantage of using peptide-pulsed DCs for priming with the $Trp2_{180}$ epitope was the generation of higher avidity T cells capable of better recognizing B16 tumor cells and ~10-fold lower amounts of peptide MHC complexes (**Fig. 5C, D**). Although DC-based cellular vaccines have been widely used in multiple clinical studies in cancer patients, in our view the overall therapeutic efficacy remains suboptimal, suggesting that the immune responses these vaccines generate are not sufficiently robust to limit tumor growth.[6,18] Regarding route of delivery, many options have been tested where each route has positive and negative aspects to consider.[19] In a report by Mullins et al., the subcutaneous injection of antigen-loaded DCs may confer superior immunogenicity than intravenous delivery.[20] Our results indicated that TriVax booster immunization could efficiently expand the primed $CD8^+$ T cells, which were generated by DC delivery regardless administration routes. Nonetheless, route of ex vivo-generated DC delivery still remains to be defined for human clinical trials because it would be extremely difficult to achieve intranodal or intralymphatic administration of DCs in mice. Overall, we believe that a vaccination strategy with antigen-loaded DCs in combination with TriVax immunization boost would be a more effective immunotherapy approach

On the other hand, the converse heterologous TriVax prime-DCs boost immunization was inefficient in generating these types of T cell responses, suggesting that DCs are better at priming and Tri-Vax is superior at boosting immune responses. A similar heterologous prime-boost strategy capable of inducing strong $CD8^+$ T cell responses to non-tumor epitopes was described where DC priming was followed by a recombinant *Listeria monocytogenes* boost.[14] Moreover, our results showed that DC-primed antigen-specific $CD8^+$ T cells could be expanded to huge numbers with a TriVax boost administered as early as 5–7 d after the prime (**Fig. 2**). These results are in line with the DCs prime, *L. monocytogenes* boost approach, indicating that peptide-loaded DCs could rapidly generate early memory $CD8^+$ T cells capable of undergoing vigorous secondary expansions followed by a highly inflammatory booster immunization generated either by an infectious agent (e.g., *L. monocytogenes*) or a non-infections synthetic

as compared to DCs vaccines on their own for treating cancer patients.

Recently, numerous reports have shown the efficacy of heterologous prime-boost regimes for generating an increased number of antigen-specific T cells in inducing potent immune response to pathogens, and various cancers.[21-24] Although these strategies have emerged as a more effective vaccination approach in selective enrichment of high avidity T cells,[25,26] manufacturing of recombinant agents expressing foreign antigens can be time consuming and technically challenging and the use of infectious recombinant agents for booster immunizations generate safety issues. Although the combination of all three components of TriVax was most effective in secondary expansion of primed $CD8^+$ T cells, immunization with peptide plus anti-CD40 Abs was also efficient in eliciting secondary $CD8^+$ T cell responses (**Fig. 3**). Moreover, instead of anti-CD40 Abs, the use of costimulatory Abs to 4-1BB and OX40 (together with poly-IC) was also effective (but slightly less than anti-CD40 Abs) in generating potent secondary T cell responses. These results imply that the anti-CD40 Abs play an important part in the effectiveness of TriVax. The CD40/CD40L costimulatory interaction has been proposed to be critical during the prime for the generation of memory $CD8^+$ T cells capable of persisting for long time periods.[27-29] The rationale for the use of anti-CD40 Abs in the generation of $CD8^+$ T cell responses is to provide the strong costimulatory signal to DCs, which is usually supplied by CD40 ligand (CD40L) expressing $CD4^+$ T helper cells.[27] Our results indicate that CD40 ligation during the boost results in the vast expansion of memory T cells. There are some indications that virus-reactive memory $CD8^+$ T cells expand efficiently upon antigen reencounter when DCs receive CD40 ligation by T helper cells or anti-CD40 Abs.[30] Our results suggest that direct costimulation to the $CD8^+$ T cells via 4-1BB or OX40 can substitute for CD40 crosslinking on DCs to allow efficient $CD8^+$ T cell memory expansion (**Fig. 3**). $CD4^+$ helper T cells play an important role when non-inflammatory vaccines such as antigen-loaded DCs, are used during the prime.[27] However, we observed that the combination of anti-$CD4^+$ Ab treatment and DC_TriVax vaccination in tumor-bearing mice resulted in the highest percentages of tumor-reactive $CD8^+$ T cells (**Fig. 4D**), and a 4-fold overall increase in total antigen-specific $CD8^+$ T cells in spleens compared with non-treated mice (data not shown). Moreover, the elimination of $CD4^+$ T cells resulted in significantly increased therapeutic benefits (**Fig. 4C**). In agreement, earlier studies[9,31,32] have also shown that depletion of $CD4^+$ T cells enhanced antitumor immunity with strong $CD8^+$ T cell responses. One possible mechanism to explain the strong $CD8^+$ T cell responses in $CD4^+$ T cell-depleted mice could be reduction of $CD4^+$/CD25Foxp3 T regulatory (Treg) cells, which constitutes one of the major suppressive mechanisms for antitumor immunity.[33,34] However, when anti-CD25 Abs PC-61 were administered to deplete Treg cells,[35] no increase in therapeutic benefit of DCs prime-TriVax boost was observed. These results suggest that $CD4^+$ T cell depletion may enhance $CD8^+$ responses in DCs prime-TriVax boost not only by Treg depletion but also by inducing partial lymphodepletion allowing better access of the antigen-stimulated T cells to homeostatic lymphokines (IL2, IL7 and IL15).

Although DC prime-TriVax boost regimes enhanced the antitumor efficacy, no complete tumor regressions were observed (**Fig. 4A, C**). We observed that administration of IL2 (as IL2Cx) or PD-1 blockade to DC_TriVax vaccination enhanced the antitumor therapeutic effect (**Fig. 4E**) to a similar degree as $CD4^+$ T cell depletion, but no complete tumor rejections were observed. It has been reported that the type of IL2Cx used in the present studies enhances the proliferation of memory $CD8^+$ T cells without expanding $CD4^+$ T regulatory cells.[36,37] Our results also showed that IL2Cx increased not only the frequency of antigen-specific $CD8^+$ T cells but long-term maintenance of the $CD8^+$ T cells, achieving higher therapeutic benefits as compared with

Figure 6 (See previous page). DCs priming followed by TriVax boost with multi-peptides induces concurrent $CD8^+$ T cell responses capable of recognizing numerous antigens. B6 mice were vaccinated intravenously with either DCs loaded with $Trp1_{455/9M}$, $Trp2_{180}$, and $hgp100_{25}$ or TriVax composed of 100 μg of each peptide, 50 μg of poly-IC, and 100 μg of anti-CD40 (prime); 7 d later, the mice received booster immunization with the identical TriVax. (**A**) Eight days after the boost, the presence of antigen-specific $CD8^+$ T cell responses in spleen were evaluated by intracellular IFNγ staining after coculturing with individual peptides (w/Ova$_{55}$, w/Trp1$_{455}$, w/Trp2$_{180}$, and w/mgp100$_{25}$). *Points*, the value for each individual mouse; *horizontal line*, the average value of the group. The results are the sum of two independent experiments with three per group. P values were calculated using unpaired Student t test comparing with homologous prime-boost TriVax-vaccinated group (**, P < 0.01; ***, P < 0.001; ****, P < 0.0001). (**B**) Freshly isolated $CD8^+$ T cells from pooled splenocytes were evaluated for antigen-induced IFNγ secretions with peptide-pulsed EL4 (EL4/Trp1$_{455}$, EL4/Trp2$_{180}$, and EL4/mgp100$_{25}$, respectively), B16 melanoma, and un-pulsed EL4 cells (negative control). Results represent the average number of spots from triplicate wells with SD (*bars*) of the means. P value was calculated using unpaired Student t test (**, P < 0.01). (**C**) Therapeutic effects induced by multi-peptide-loaded DCs prime-TriVax booster vaccination against established B16 melanoma. B6 mice (five per group) were inoculated subcutaneously on day 0 with 2 × 10⁵ B16 cells and vaccinated intravenously on day 5, and 12 (vertical arrow) in combination with DCs or TriVax using either individual peptide or mixture of Trp1$_{455}$, Trp2$_{180}$, and hgp100$_{25}$ (3pep$_{MIX}$) as indicated. Non-vaccinated mice (No Vax) and Ova$_{55}$-loaded DCs prime-Ova$_{55}$TriVax booster-vaccinated mice were included as controls. (**D**) At the termination of the experiment presented in panel **C** (on day 36), splenocytes from mice from the three vaccinated groups were evaluated for antigen-specific $CD8^+$ T cells by antigen-induced IFNγ production against peptide-pulsed EL4 (EL4/Trp1$_{455}$, EL4/Trp2$_{180}$, and EL4/mgp100$_{25}$, respectively), B16 melanoma, and un-pulsed EL4 cells (negative control). Results represent the average number of spots from triplicate wells with SD (*bars*) of the means. (**E**) PD-1 blockade and IL2Cx enhance the therapeutic efficacy of DCs prime-TriVax boost vaccination using multi-peptides. No Vax were included as controls. Anti-PD-L1, normal rat IgG, or IL2Cx were administered as described in Materials and Methods. Tumor sizes were determined in individual mice by measurements of two opposing diameters and are presented as tumor areas in mm². *Points*, mean for each group of mice; *bars*, SD. Statistical significance of tumor sizes was calculated using two-way ANOVA test comparing with the normal DCs prime-TriVax boost group (***, P < 0.001; ****, P < 0.0001). Two of the five mice in 3pep$_{MIX}$DC_TriVax+anti-PD-L1 Abs, and four of the five mice in 3pep$_{MIX}$DC_TriVax+IL2Cx rejected their tumors. These experiments were repeated twice with similar results.

DC_TriVax alone. Interestingly, while additions of anti-PD-L1 Abs resulted in enhancing antitumor effects as compared with DC_TriVax alone, the frequency of antigen-specific CD8$^+$ T cells was similar to DC_TriVax alone. PD-1 blockade prevents the generation of exhausted T cells leading to enhanced activation and persistence of antigen-specific T cells.[38,39] Because PD-L1 expression can be upregulated at the tumor site,[10,40] it is likely that PD-1 blockade prevents exhaustion of tumor infiltrating T cells, enhancing of the effector function without the need of the increase in overall T cell numbers observed with IL2Cx.

Lastly, our results showed that DCs prime-TriVax boost strategies allow the simultaneous induction of CD8$^+$ T cell responses to multiple epitopes derived from different melanosomal antigens and resulted in evidently enhanced therapeutic benefits as compared to DC_TriVax using single antigenic peptide (**Fig. 6C**). Likewise, the combinations of IL2Cx or PD-1 blockade to these regimes resulted in remarkable antitumor effects where the majority of the mice rejected their tumors (**Fig. 6E**). It recently became clear that multiple immune inhibitory mechanisms are present in tumor site and it appears to be involved to facilitate tumor heterogeneity, which could cause a proportion of tumor cells to decrease the expression of target antigens.[4,41] Thus, DCs prime-TriVax boost regimes capable of inducing concurrent multivalent CD8$^+$ T cell responses specific to more than one antigen could overcome the potential limited applicability of single peptide-based vaccines. Collectively, we present data indicating that heterologous multi-epitope DCs prime-TriVax boost vaccines are able to induce large numbers of multivalent CD8$^+$ T cells resulting in remarkable therapeutic antitumor benefits, suggesting that this strategy may achieve desired clinical results in cancer patients.

Materials and Methods

Mice and cell lines

C57BL/6 (B6) mice were purchased from Orient Bio and maintained in our animal facilities under pathogen-free conditions. Animal care and experiments were conducted according to our institutional animal care and use committee guidelines. Murine melanoma B16F10, EL4 thymoma, and RMA-S cells were from the American Type Culture Collection. All of the cell lines were cultured as recommended by the provider.

Peptides, antibodies and reagents

Synthetic peptides representing the CD8$^+$ T cell epitopes Trp2$_{180}$ (SVYDFFVWL), hgp100$_{25}$ (KVPRNQDWL), mgp100$_{25}$ (EGSRNQDWL), Trp1$_{455}$ (TAPDNLGYA), the heteroclitic analog Trp1$_{455/9M}$ (TAPDNLGYM), and Ova$_{55}$ (KVVRFDKL) were purchased >80% pure from A&A Labs. Monoclonal anti-mouse CD40 (FGK45.5) and anti-4.1BB/CD137 (2A) were prepared from hybridoma culture supernatants. Other Abs for *in vivo* use in mice, anti-PD-L1 (10F.9G2), anti-IL2 (JES6-5H4), anti-OX40/CD134 (OX86), anti-CD8 (2.43), anti-CD4 (GK1.5), anti-NK1.1 (PK-136), and anti-CD25 (PC-61.5.3) were purchased from BioXCell. High molecular weight Poly-IC was purchased from Invivogen, and recombinant cytokines were from PeproTech. Fluorescence-conjugated Abs for flow cytometry were from eBioscience.

Immunization

DCs were generated from bone marrow cells cultured for 7 d with 10 ng/mL granulocyte macrophage colony-stimulating factor and 10 ng/mL IL4. For vaccination, mice were immunized intravenously with 2×10^6 DCs pulsed with 10 μg/mL peptide (or in some instance Trp1$_{455/9M}$, Trp2$_{180}$, and hgp100$_{25}$ simultaneously) for 18 h. DCs were mixed with 20 μg poly-IC prior to injection. TriVax consists of 150 μg synthetic peptide, 100 μg anti-CD40 Abs, and 50 μg poly-IC, administered as a mixture via the intravenous (tail vein) route. For PD-1 blockade, anti-PD-L1 Abs were administered intraperitoneally (200 μg/dose) on days 0, 2, and 4 after each immunization (prime and boost). IL2Cx were prepared by incubating 2 μg recombinant mouse IL2 with 10 μg anti-mouse IL2 (JES6-5H4) per dose for 18 h at 4°C. IL2Cx were administered intraperitoneally on days 1 and 3 after prime and boost immunizations.

Evaluation of immune responses

For measuring antigen-specific CD8$^+$ T cell responses, peripheral blood samples or splenocytes were incubated with 1 μg/mL peptide and 1 μl/mL GolgiPlug (BD Bioscience) at 37°C. After 6 h, cells were stained for intracellular IFNγ following the directions provided by vendor (BD Bioscience) using fluorescence-conjugated Abs against MHC class II, CD8a, and IFNγ. Fluorescence was measured using a FACS Calibur flow cytometer (BD Biosciences) and analyzed using FlowJo software. For CD107a/b mobilization shift assay, 2.5 μg/mL of fluorescence-conjugated anti-CD107a and CD107b Abs were added at the beginning of the stimulation period. To evaluate the *in vitro* T cell tumor recognition, IFNγ EliSpot assays were performed as described previously,[10] using freshly isolated spleen CD8$^+$ T cells (Miltenyi Biotec). Peptide-pulsed or unpulsed EL4 cells and B16 melanoma cells were used as target cells. For peptide dose curve responses, serial peptide dilutions were incubated with RMA-S cells overnight and freshly purified CD8$^+$ T cells were added for an additional 40 h before harvesting supernatants to measure IFNγ production by ELISA (eBioscience).

Antitumor effects

B6 mice were inoculated subcutaneously with 2×10^5 B16 melanoma cells, and 5 d later when tumors measured ~3–5 mm in diameter the first immunization was administered. Seven days later, the mice were given a heterologous or identical booster. In some experiments, mice received IL2Cx and PD-1 blockade as described above. For *in vivo* depletion of lymphocyte subsets, each mouse received intraperitoneal administrations of 300 μg Abs against mouse NK1.1, CD4, CD8, and CD25 on days -3 and -1 before the first immunization. Depletions were confirmed with blood samples using flow cytometry (data not shown). Tumor growth was monitored every 3–4 d in individual tagged mice by measuring two opposing diameters with a set of calipers.

Mice were euthanized when the tumor area reached >400 mm^2. Results are presented as the mean tumor size (area in mm^2) $\pm$ SD for every treatment group at various time points until the termination of the experiment.

Statistical analyses

Statistical significance to assess the numbers of antigen-specific CD8$^+$ T cells was determined by unpaired Student t tests. Tumor sizes between two populations throughout time were analyzed for significance using two-way ANOVA. All analyses and graphs were done using Prism 5.01 software (GraphPad). The results are representative of data obtained from at least two independent experiments.

Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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