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Adjuvant Combination and Antigen Targeting as a Strategy to Induce Polyfunctional and High-Avidity T-Cell Responses against Poorly Immunogenic Tumors

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Abstract

Low antigen expression and an absence of coimmunostimulatory signals may be partly responsible for the low immunogenicity of many tumors. It may be possible to overcome this situation by defining a combination of adjuvants and antigens that can activate a high-avidity antitumor response. Using the poorly immunogenic B16-OVA melanoma cells as tumor model, we tested different combinations of adjuvants and antigens to treat established tumors. In the absence of exogenous antigens, repeated administration of the TLR7 ligand Imiquimod together with anti-CD40 agonistic antibodies activated only innate immunity, which was insufficient to reject intradermal tumors. Administering this adjuvant combination together with OVA as a tumor antigen induced T-cell responses that delayed tumor growth. However, administering a combination of anti-CD40 plus TLR3 and TLR7 ligands, together with antigen targeting to dendritic cells through TLR4, was sufficient to induce tumor rejection in 50% of mice. This response was associated with a greater activation of innate immunity and induction of high-avidity polyfunctional $CD8^+$ T-cell responses, which each contributed to tumor rejection. This therapy activated T-cell responses not only against OVA, which conferred protection against a rechallenge with B16-OVA cells, but also activated T-cell responses against other melanoma-associated antigens. Our findings support the concept that multiple adjuvant combination and antigen targeting may be a useful immunotherapeutic strategy against poorly immunogenic tumors. *Cancer Res; 71(9); 3214–24. ©2011 AACR.*

Introduction

Tumor immunotherapy is aimed at inducing immune responses to destroy tumor cells (1). However, tumors usually behave as poorly immunogenic for T cells, because as opposed to professional antigen presenting cells such as dendritic cells (DC), they express low levels of antigens, MHC, and costimulatory molecules (2). Moreover, unlike danger signals associated with pathogens, such as toll-like receptor (TLR) ligands, or inflammatory signals (cytokines or CD40L expressed on activated CD4 T cells), which induce DC maturation, tumor cells do not release immunogenic danger signals, unless they are submitted to radio- or chemotherapy (3). DC maturation, which involves upregulation of costimulatory, adhesion, and antigen-presenting molecules as well as the production of cytokines and chemokines, is necessary for correct T-cell activation (4). Therefore, to overcome the low tumor immunogenicity, new therapeutic strategies are based on the use of adjuvants (molecules able to induce DC maturation) with or without exogenously added tumor antigens, which will activate DC to properly present tumor antigens to T cells and trigger their effector functions (5). With this aim, a great effort has been made to characterize DC-activating ligands to be used as adjuvant molecules, due to their immunostimulatory and/or targeting properties, and thus avoid the use of microorganisms containing undefined adjuvant mixtures (6). These activating molecules have been shown to increase the immunostimulatory properties of DC by signaling through different activation pathways, and in some cases, synergistic effects have been observed (7–11). Thus, development of therapeutic strategies based on the use of molecularly defined components is a main goal in tumor immunology.

We had previously shown that administration of an adjuvant mixture containing the TLR3 ligand poly(I:C) and agonistic anti-CD40 antibodies, together with ovalbumin (OVA) as tumor antigen, protected mice against the growth of E.G7-OVA thymoma and rejected established tumors in this model (12). B16 melanoma cell lines (13) are poorly immunogenic tumors which have many characteristics of tumors found in patients and consequently, may be a good model to develop immunotherapeutic strategies with potential clinical

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applications. Several approaches have been used to induce immune responses able to reject B16 melanoma. They are based on the use of DC pulsed or expressing different types of antigens (14–16) or complex immunogens such as recombinant viruses, bacteria, or virus-like particles which contain many DC-activating signals (17–19). All these data prompted us to develop an immunotherapeutic strategy to treat B16-OVA established tumors using combinations of adjuvants and antigens. We report below the antitumor efficacy of this strategy, together with the immunological mechanisms responsible of its outcome.

Materials and Methods

Reagents

Peptides OVA(257–264) and TRP-2(180–188) were synthesized in an automatic APEX 396 multiple peptide synthesizer (Aapptec LLC). Peptide identity was confirmed by mass spectrometry and their purity was >95%, as determined by HPLC. OVA protein was purchased from Sigma Aldrich. Proteins containing the extra domain A from fibronectin EDA and EDA-OVA were produced as described (20). Endotoxin levels in these proteins were below 0.2 EU/μg protein as analyzed using the LAL test (Cambrex). Imiquimod was used *in vivo* as Aldara cream (Meda Pharma) and for *in vitro* experiments was obtained from Invivogen. Poly(I:C) was obtained from Amersham and agonistic anti-CD40 antibody was purified from FGK45.5 hybridoma cells as described (12).

Mice

Female C57BL/6 mice (6–8 weeks old; Harlan) were maintained in pathogen-free conditions and treated according to guidelines of our institution, after study approval by the review committee.

Cell lines

Parental EL-4 thymoma cells, OVA-transfected E.G7-OVA cells, and the NK-sensitive cell line YAC-1 were purchased from American Type Culture Collection and were grown as described (21). Cells were thawed and passaged according to ATCC guidelines for less than 6 months from the time they were received or thawed. ATCC authenticates cell lines by DNA fingerprinting by short tandem repeat analysis. B16-OVA (22) and B16.F10 tumor cells (13), obtained from Dr. G. Kroemer, were grown in DMEM containing 10% fetal calf serum and antibiotics. No authentication was done, but the expression of OVA and TRP-2 antigens and MHC-I molecules was tested before the experiments by RT-PCT and flow cytometry, respectively.

Immunization of mice

Naïve mice received combinations of Imiquimod cream (topical application; 2.5 mg/mouse) plus subcutaneous administration of poly(I:C; 50 μg/mouse), anti-CD40 (50 μg/mouse), OVA protein (2–11 nmol/mouse), EDA (2 nmol/mouse), or EDA-OVA protein (2 nmol/mouse). Six days later, animals were killed and splenocytes were obtained for immunological analysis.

Tumor treatment experiments

Mice were injected intradermally with 10^5 tumor cells and when the tumor diameter reached 4 to 5 mm, treatment protocols containing combinations of the following adjuvants and antigens were applied in a 20-day protocol: Imiquimod cream was topically applied daily (2.5 mg/mouse) to shaved skin at the tumor site, whereas poly(I:C; 50 μg/mouse), anti-CD40 (50 μg/mouse), OVA protein (11 nmol/mouse) and EDA-OVA protein (2 nmol/mouse) were administered intratumor (i.t.) 3 times per week. Untreated mice challenged with tumor cells were used as positive controls of tumor growth. In some cases, tumor treatment was accompanied by administration of 200 μg of depleting anti-CD8, anti-NK1.1 or isotype control antibodies on days −1, 0, 2, 4 and 6, being 0 the day when treatment starts. Tumor volume was calculated according to the formula: $V = (\text{length} \times \text{width}^2)/2$. Mice were killed when tumor diameter reached 17 mm.

Ex vivo analysis of DC

Mice bearing 4 to 5 mm tumors ($n = 5$) received a single administration of different adjuvant combinations as described earlier, and 1 day later tumors or tumor-draining lymph nodes were removed, pooled, and DC analyzed directly by flow cytometry or by real-time PCR after purification with anti-CD11c–conjugated magnetic beads (Miltenyi).

Analysis by real-time PCR

Total RNA extraction from tumor cell lines or dendritic cells and real-time PCR were performed as described (23), using primers shown in Supplementary Table S1. Results were normalized according to β-actin. The amount of each transcript was expressed by the formula: $2^{\Delta Ct}$ [ΔCt = Ct (β-actin) − Ct(gene)].

ELISPOT

Cells producing IFN-γ were enumerated by ELISPOT assays using a kit from BD-Biosciences as described (12). For T-cell responses, splenocytes were stimulated with peptides OVA (257–264; 1 μg/mL), TRP-2 (180–188; 10 μg/mL), OVA protein (10 μg/mL) or 4×10^4 irradiated (20,000 rads) tumor cells. When measuring NK-cell–derived production of IFN-γ, splenocytes were incubated with 4×10^4 mitomycin C–treated YAC-1 cells.

Flow cytometry

Expression of MHC K^b class I molecules in tumor cells was analyzed using anti–K^b-FITC labeled antibodies. To analyze NK cells and DC, spleens, tumor-draining lymph nodes or tumors were treated with collagenase and DNAse for 15 minutes and homogenized. Then, cells were first incubated for 10 minutes with Fc Block (BD-Biosciences) and stained with specific antibodies. For NK cell analysis, cells were stained with anti–CD69-FITC, anti–CD3-PE and anti–NK1.1-APC–labeled antibodies. DC were analyzed using anti–CD11c-APC, anti-IA^b (either PE or FITC), anti–CD86-FITC, anti–CD80-FITC, and anti–CCR7-PE antibodies. To measure IL-12 production, DC were incubated for 5 hours with GolgiStop and after fixation and permeabilization were

labeled with anti–CD11c-APC, anti–IA^b-FITC, and anti–IL-12 p70-PE. OVA(257–264)/H-2K^b tetramer-specific T cells were enumerated as described (21), using a 1/1000 tetramer dilution. T-cell activation was analyzed after stimulation of splenocytes with 1 ng/mL of OVA(257–264) in the presence of GolgiStop and GolgiPlug (BD-Biosciences) with or without anti–CD107-FITC antibodies for 4 hours. Then cells were labeled with anti–CD8-APC and fixed, permeabilized, and stained with anti–IFN-γ-PE. For triple cytokine analysis, after surface staining with anti–CD8-FITC, cells were stained with anti–IFN-γ-PE, anti–TNF-α-PE-Cy7, and anti–IL-2-APC labeled antibodies. All antibodies were from BD-Biosciences, except anti-NK1.1 (e-Bioscience). Expression of the different markers was analyzed with a FACSCalibur flow cytometer (Becton Dickinson) and Flowjo software (Tree Star, Inc.).

Measurement of cytokines by ELISA

IL-12 and TNF-α content in the serum obtained from the retroorbital plexus of mice, and IFN-γ from 48 hours culture supernatants of splenocytes stimulated with different concentrations of OVA(257–264) were measured using OptEIA Sets from BD-Biosciences.

Statistical analysis

Survival curves of animals treated with different protocols were plotted according to the Kaplan–Meier method and were compared using the log-rank test. Immune responses were analyzed using nonparametric Kruskal–Wallis and Mann–Whitney *U* tests. $P < 0.05$ was taken to represent statistical significance.

Results

Immunotherapy based only on adjuvant administration has a poor effect on B16-OVA tumor-bearing mice, inducing innate but not adaptive immunity

We have previously reported that a single prophylactic administration of adjuvant molecules poly(I:C) and agonistic anti-CD40 antibodies plus OVA protein induced potent T-cell responses which protected 100% of mice from the growth of subcutaneous E.G7-OVA tumors (12). When this protocol was applied to the B16-OVA melanoma model, no differences in tumor growth and survival were observed between control and vaccinated mice (Supplementary Fig. S1A and B), demonstrating the poor immunogenicity and recognition of B16-OVA cells. Indeed, OVA expression of B16-OVA cells was very low, as compared with E.G7-OVA cells, and the same low expression was obtained for MHC class I K^b molecules (Supplementary Fig. S1C and D). This prompted us to adopt the poorly immunogenic B16-OVA tumor model to develop therapeutic strategies.

Because repeated intratumor administration of poly(I:C) and anti-CD40 rejected 35% of established E.G7-OVA tumors (12), we decided to test the therapeutic efficacy of repeated adjuvant administration on the B16-OVA tumor model. In these experiments, we combined topical application of Aldara cream (containing the TLR7 ligand Imiquimod) and intratumor injection of anti-CD40 antibodies. Mice bearing 7-day intradermal tumors (5 mm) received repeated adjuvant administrations in a 20-day interval. As shown in Figure 1A, treated mice had a slight delay ($P < 0.05$) in tumor growth, but all mice died by day 40.

Rejection of E.G7-OVA tumors after administration of poly (I:C) plus anti-CD40 was associated with the induction of innate and adaptive immunity (12). Therefore, we studied these parameters when using Imiquimod and anti-CD40 as adjuvants. Naïve mice received a single administration of Imiquimod plus anti-CD40 and NK cell activity was studied by measuring CD69 upregulation on CD3$^-$NK1.1$^+$ cells and IFN-γ production against the NK-sensitive YAC-1 cell line. A high activity in both functions was found at 24 hours (Fig. 1B). Regarding spleen DC, a clear upregulation of CD86 was observed (Fig. 1C). Finally, analysis of serum cytokines showed a peak for IL-12 at 6 hours (226 pg/mL), whereas TNF-α levels grew until 48 hours (625 pg/mL).

Analysis of antitumor adaptive immune responses was carried out in tumor-bearing mice, untreated or treated with Imiquimod plus anti-CD40. None of these groups displayed IFN-γ production against the CD8 T-cell epitope OVA(257–264) or OVA protein in ELISPOT assays (Fig. 1D). These results suggest that administration of adjuvants to B16-OVA tumor-bearing mice induces innate but not adaptive immunity.

Combination of adjuvants Imiquimod and anti-CD40 with the tumor antigen OVA induces adaptive immunity with better antitumor effects

The lack of adaptive immunity in B16-OVA-tumor bearing mice treated with adjuvants alone led us to hypothesize that inclusion of an antigen together with adjuvants might be able to induce T-cell responses. Indeed, repeated administration of OVA together with poly(I:C) and anti-CD40 enhanced E.G7-OVA tumor rejection from 35% to 100% (12). Immunization of naïve mice with OVA plus the adjuvant combination Imiquimod and anti-CD40 had a strong effect on the induction of T-cell responses (Fig. 2A), similar to responses observed when combining OVA plus poly(I:C) and anti-CD40 (12). We thus treated tumor-bearing mice with OVA plus Imiquimod and anti-CD40 3 times per week during a 20-day period. In this case, tumor volume was kept below 0.5 cm^3 during the treatment period, as compared to untreated animals, which at day 20 had a mean tumor volume above 1 cm^3 ($P < 0.05$; Fig. 2B). Moreover, only 33% of untreated mice were alive at the end of treatment (day 20), whereas 100% of treated mice survived ($P < 0.05$; Fig. 2C). Also, as opposed to mice treated only with adjuvants (Fig. 1A), once treatment finished, animals treated with OVA plus Imiquimod and anti-CD40 had slow-growing tumors until day 30, when they started to grow and finally all mice died at day 40. We also analyzed the antitumor effect of OVA plus the combination of poly(I:C) and anti-CD40, a similar T-cell activation strategy (12) which induced rejection of E.G7-OVA tumors. Equivalent results were obtained in these experiments, with a significant delay in tumor growth ($P < 0.01$), but without tumor rejection (Supplementary Fig. S2).

The lack of tumor rejection in treated animals could not be attributed to the absence of T-cell responses, because

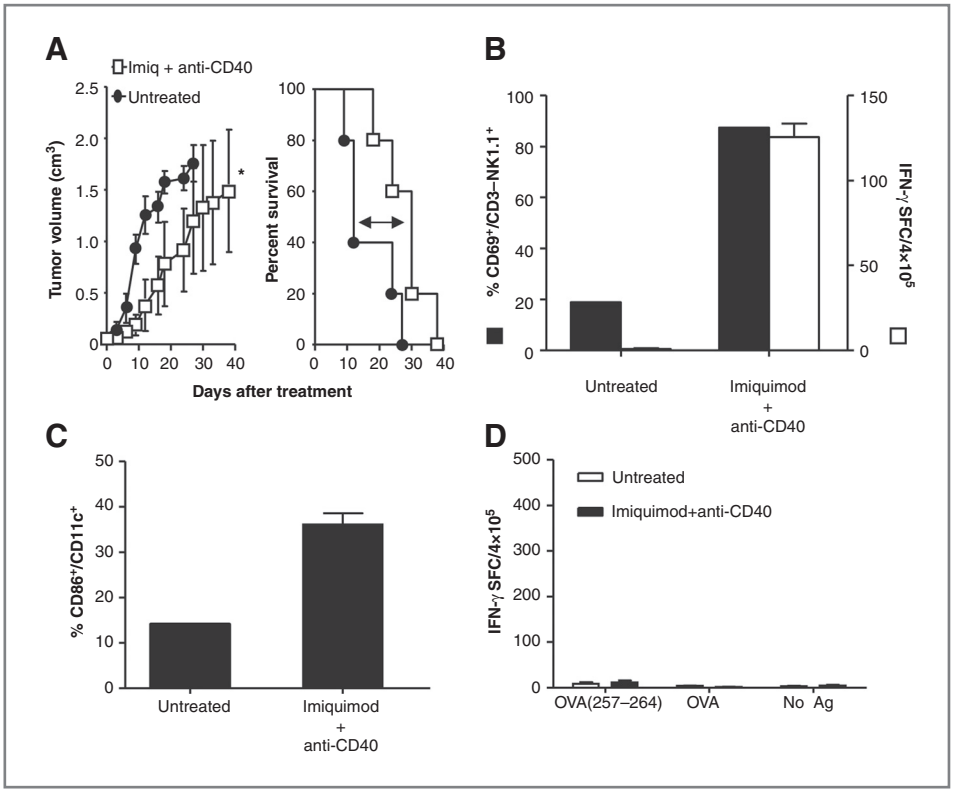

Figure 1. Immunotherapy based only on adjuvant administration has a poor effect of B16-OVA tumor-bearing mice. A, C57BL/6 mice ($n = 6$) were injected s.c. with 10^5 B16-OVA tumor cells and when tumor diameter reached 5 mm they were treated 3 times per week during a 20-day period with Imiquimod + anti-CD40 or left untreated. Graph represents average tumor volume per group of animals studied and survival (*, $P < 0.05$, untreated vs. Imiquimod + anti-CD40). B, naïve mice ($n = 3$) were injected with Imiquimod plus anti-CD40 and NK-cell activation was measured 1 day later in the spleen as CD69 upregulation in $CD3^-NK1.1^+$ cells (filled bars) and as IFN-γ production against the NK-cell–sensitive YAC-1 cell line (open bars). C, activation of splenic DC in animals shown in B was measured as % of $CD86^+$ cells in the $CD11c^+$ population. D, mice ($n = 3$) with 7-day tumors were treated twice 2 days apart with Imiquimod plus anti-CD40 or left untreated and 1 week after first treatment recognition of peptide OVA(257–264) and OVA protein by splenocytes was measured by ELISPOT. Results are representative of 2 independent experiments.

tumor-bearing mice treated with as few as 2 immunizations showed clear responses against tumor antigens (a representative example of mice treated with OVA plus Imiquimod and anti-CD40 is shown in Fig. 2D). Thus, a protocol which combines the tumor antigen with double-adjuvant mixtures induces T-cell responses, delays tumor growth during treatment period and some days beyond, but does not lead to tumor rejection.

Multiple adjuvant combination and antigen targeting enhances innate immunity

The poor but significant effect that antigen plus adjuvant administration had on tumor growth suggested that new strategies inducing enhanced T-cell responses might be more suitable to attain tumor rejection. Thus, looking for a higher synergistic effect between adjuvants, all 3 molecules (anti-CD40, poly(I:C) and Imiquimod) were included in a multiple adjuvant combination (MAC). Moreover, to enhance not only innate immunity, but also to target antigen to antigen presenting cells, OVA was coupled to the extra domain A of fibronectin (EDA), a TLR4 ligand which enhances innate and adaptive immunity, and when coupled to antigens, targets them to TLR4-expressing antigen presenting cells (20). Concerning the activation of innate immunity, in vitro experiments using bone marrow-derived DC from naïve mice stimulated with the different combinations showed that MAC + EDA induced the highest levels of mRNA corresponding to costimulatory molecules associated with T-cell activation and cytokines important for induction of CD8 T-cells (Supplementary Fig. S3). Most importantly, ex vivo analysis of tumor-draining lymph node DC from mice treated with different combinations showed that MAC or EDA+MAC induced the highest CD80 and CD86 expression levels, as occurred with IL-12 and the chemokine receptor CCR7, in accordance with the higher numbers of DC found (Fig. 3A). Equivalent results were obtained analyzing mRNA of costimulatory molecules, IL-15 and IL-15R ($P < 0.05$; MAC or EDA + MAC *vs.* remaining groups; Fig. 3B). Administration of EDA without additional adjuvants had a negligible effect on these parameters. No clear differences were observed when analyzing tumor DC (data not shown), probably due to migration of activated DC to draining lymph nodes.

Ex vivo analysis of NK cell activation, after a single administration of adjuvant combinations to naïve mice showed that, EDA-OVA + MAC induced the strongest NK-cell derived IFN-γ production of the 4 combinations tested (Supplementary Fig. S3;

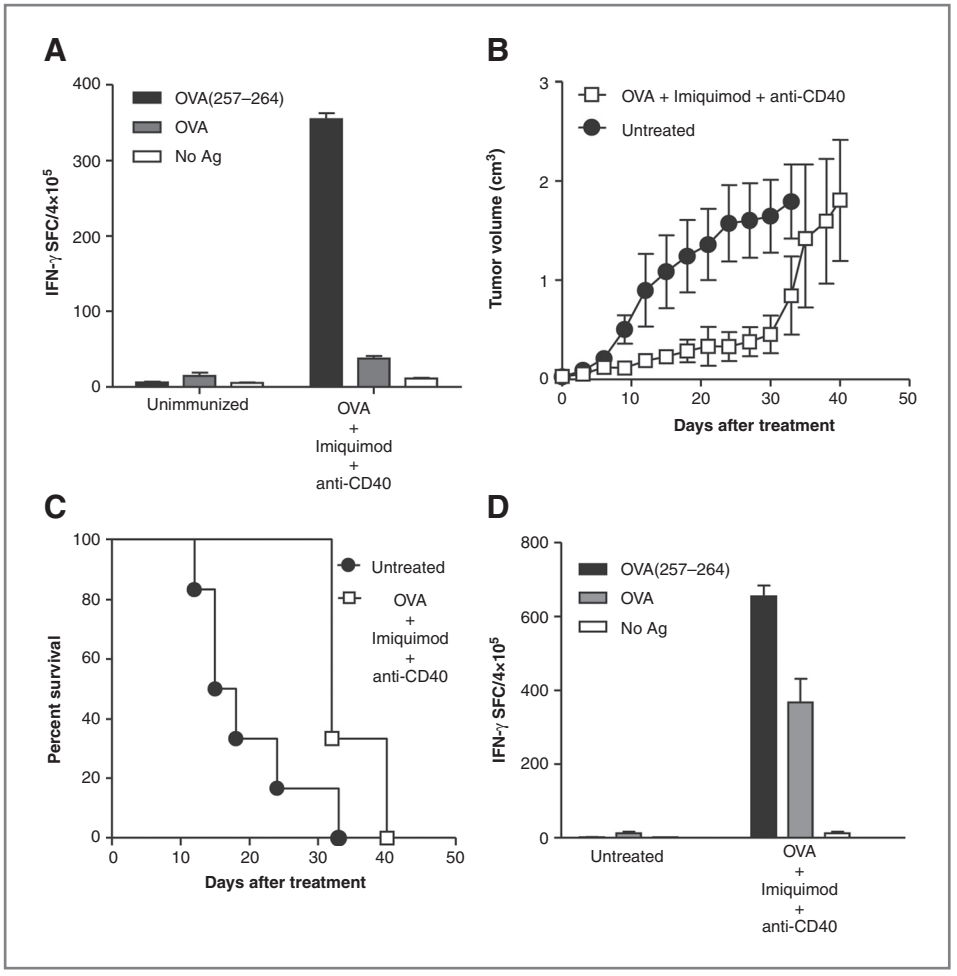

Figure 2. Combination of adjuvants Imiquimod + anti-CD40 with the tumor antigen OVA induces adaptive immunity with better antitumor effects. A, C57BL/6 mice ($n = 3$) received a single immunization with OVA + Imiquimod + anti-CD40 or were left untreated. Six days later, they were killed and the number of IFN-γ–producing cells after stimulation with OVA antigens was measured by ELISPOT. C57BL/6 mice ($n = 6$) were injected s.c. with 10^5 B16-OVA tumor cells and when tumor diameter reached 5 mm they were treated 3 times per week during a 20-day period with OVA + Imiquimod + anti-CD40. Average tumor volume per group of animals studied (B) and survival (C) are represented. Mice ($n = 2$) bearing 5 mm tumors received 2 administrations of OVA + Imiquimod + anti-CD40 and 6 days after the first injection their splenocytes were stimulated with OVA antigens and IFN-γ–producing cells were measured by ELISPOT (D). Results are representative of 2 independent experiments.

$P < 0.01$; EDA-OVA + MAC *vs.* EDA-OVA + Imiquimod + anti-CD40, or OVA + MAC *vs.* OVA + Imiquimod + anti-CD40). When adjuvants were administered to tumor-bearing mice, MAC and EDA + MAC were the most potent combinations ($P < 0.05$; MAC or EDA + MAC *vs.* remaining groups; Fig. 3C).

Multiple adjuvant combination and antigen targeting induces polyfunctional high avidity T-cell responses

Analysis of adaptive immunity in naïve mice immunized with the different combinations was done by ELISPOT assays which measured the number of CD8 and CD4 T-cells producing IFN-γ against OVA(257–264) peptide and OVA protein, respectively. EDA-OVA + MAC showed a synergistic effect, as compared with immunization with OVA plus triple or double adjuvant combinations (Fig. 4A; $P < 0.01$; EDA-OVA + MAC vs. remaining groups). Antigen coupling to EDA was necessary to obtain these high responses, because immunization with MAC plus EDA and free OVA induced lower responses, mainly for those mediated by CD8 T cells (Supplementary Fig. S4).

Besides quantitative parameters of T-cell activation, the quality of the CD8 T-cell responses was studied by stimulating splenocytes with decreasing OVA(257–264) concentrations. Interestingly, splenocytes from mice immunized with EDA-OVA + MAC and stimulated with concentrations as low as 0.1 to 1 ng/mL of OVA(257–264) still produced high amounts of IFN-γ (Fig. 4B), as compared to the other groups. These higher IFN-γ levels were due not only to a expansion of high-avidity OVA(257–264)-specific T-cells, as found by staining with low OVA(257–264)/K^b tetramer concentrations, but also to enhanced activation of IFN-γ–producing cells, as demonstrated in ELISPOT assays using 0.1 ng/mL of peptide (Fig. 4C). Finally, other qualitative aspects were analyzed at the single cell level by studying polyfunctional CD8 T-cells (cells producing several cytokines and displaying different effector functions). These analyses were carried out on high-avidity T cells, by stimulating splenocytes with 1 ng/mL of OVA(257–264). First, a higher number of CD8 T cells with lytic activity against OVA(257–264), determined by the expression of CD107, was found in splenocytes from mice immunized with EDA-OVA + MAC; and second, these animals had the highest percentage of CD8 T cells simultaneously producing IFN-γ, TNF-α, and IL-2 (Fig. 4D). These results suggest that immunization with EDA-OVA + MAC induces stronger responses with a higher avidity for the antigen, which are able to display several effector functions.

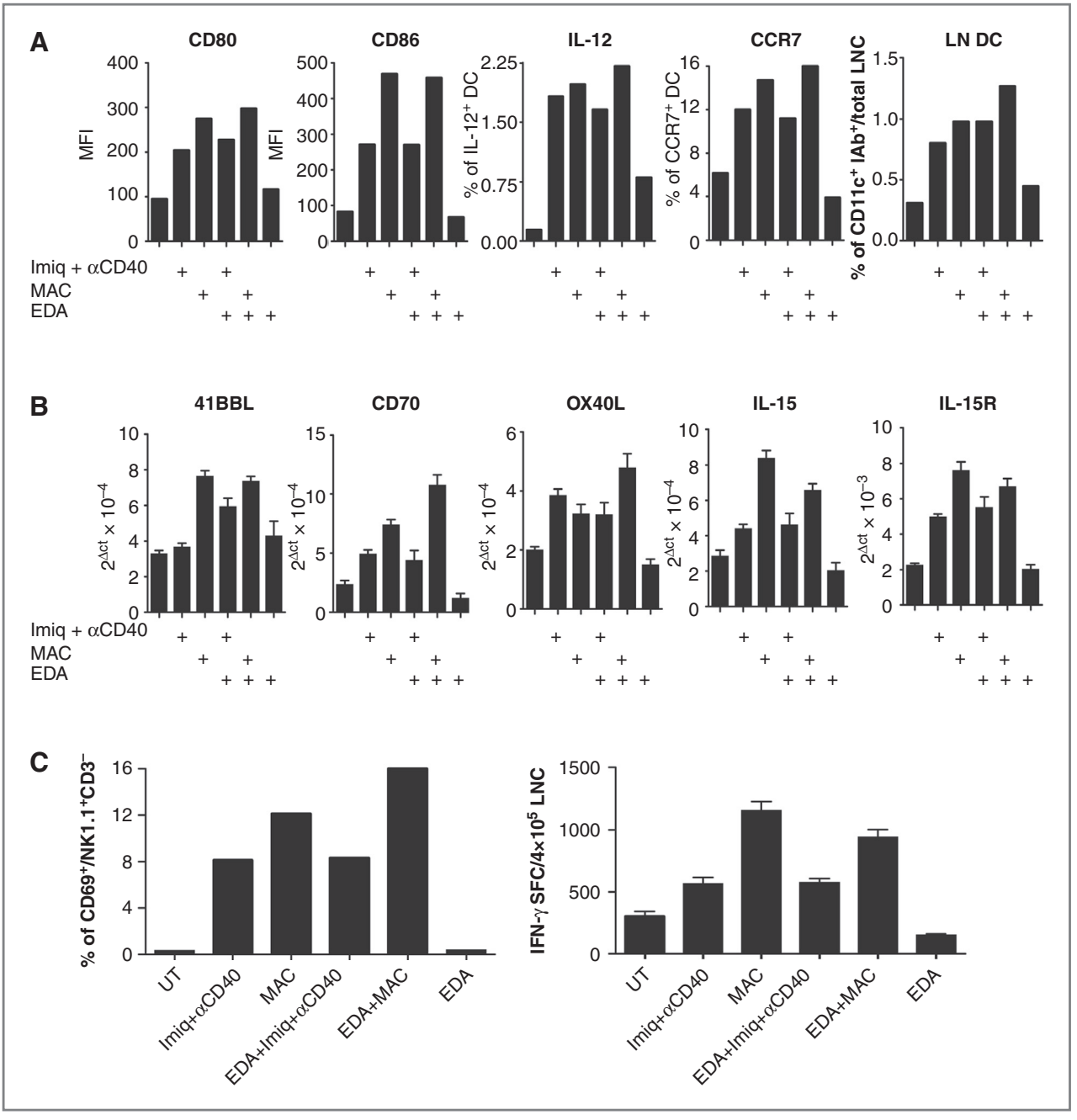

Figure 3. *In vivo* activation of innate immunity by a multiple adjuvant combination. C57BL/6 mice ($n = 5$/group) were injected s.c. with 10^5 B16-OVA tumor cells and when tumor diameter reached 5 mm they were treated with different adjuvant combinations. One day later, tumor-draining lymph nodes were removed and pooled. A, expression of CD80, CD86, CCR7, IL-12 p70 molecules in the DC population, defined as $CD11c^+IAb^+$, as well as the number of DC, were measured by flow cytometry. B, expression of mRNA of costimulatory molecules, IL-15 and IL-15R were measured by real-time PCR in DC ($n = 5$ wells/group) purified with magnetic beads. C, NK cells were analyzed as the percentage of activated NK cells ($CD69^+$ in $NK1.1^+CD3^-$ cells) and by IFN-γ production against the NK-sensitive cell line YAC-1 in ELISPOT ($n = 5$ wells/group). Results correspond to 1 of 2 independent experiments.

With these results, we measured immune responses against tumor cells in tumor-bearing mice treated during 10 days with the different combinations. Mice treated with EDA-OVA + MAC had the lowest tumor size at day 10 and the highest proportion of tumor-free mice (Supplementary Fig. S5), in agreement with the ability of their splenocytes to recognize *in vitro* B16-OVA tumor cells (Fig. 4D), as well as B16.F10 cells. By contrast, a lower or nil recognition by splenocytes from remaining groups was observed. No differences in OVA expression were observed in tumors belonging to groups treated with different adjuvant combinations (Supplementary Fig. S5).

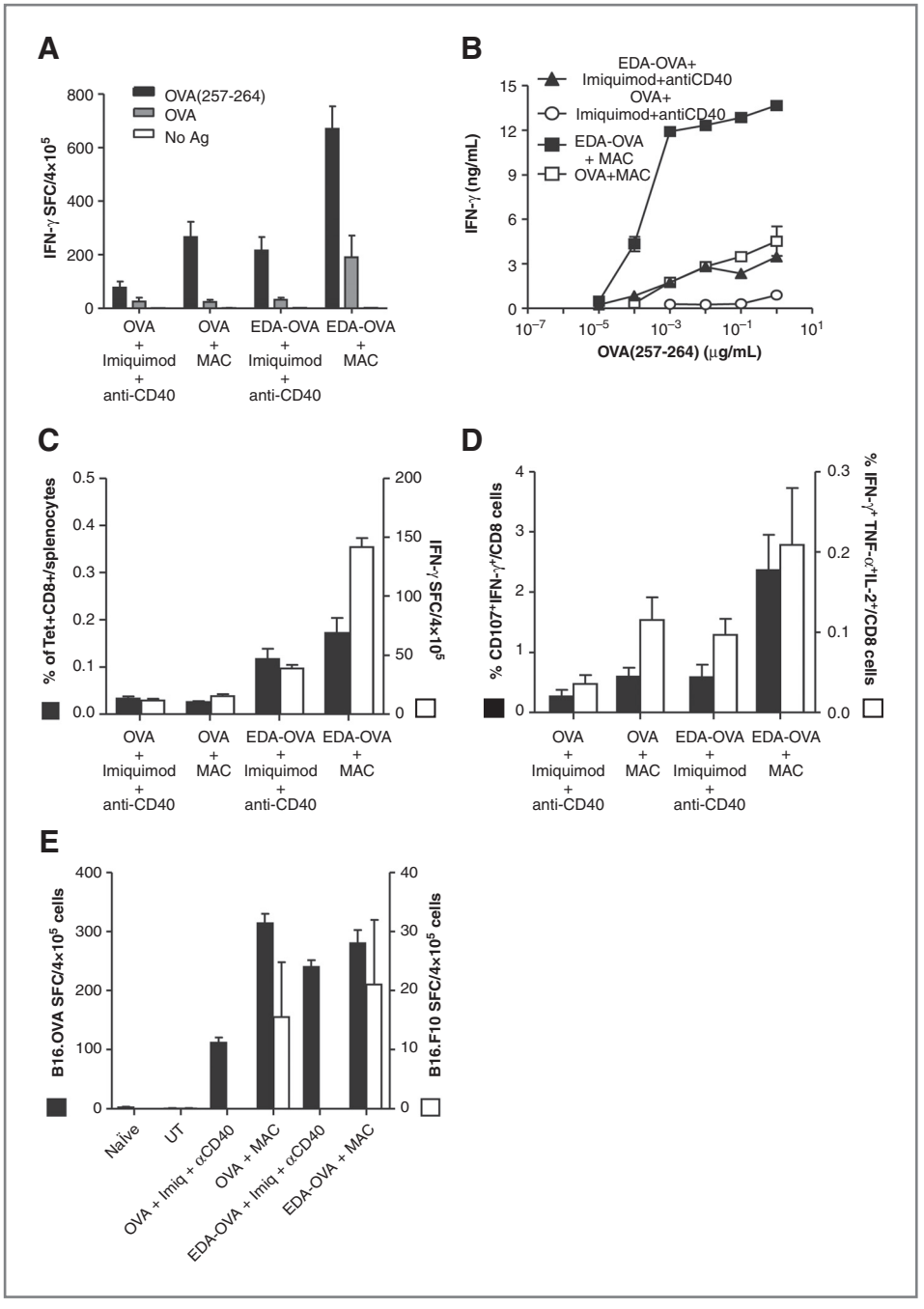

Figure 4. A multiple adjuvant combination plus antigen-targeting strategy induces polyfunctional high-avidity T-cell responses. A, C57BL/6 mice ($n = 3$–4) received a single immunization with OVA plus different adjuvant combinations and 6 days later the number of IFN-γ–producing cells was measured by ELISPOT after stimulation with OVA antigens. B, IFN-γ content, as determined by ELISA, of cell culture supernatants of splenocytes obtained from groups shown in A and stimulated with different concentrations of OVA(257–264). C, percentage of high-avidity OVA(257–264)/K^b tetramer$^+$ CD8$^+$ cells/total splenocytes (1/1000 tetramer dilution) and number of IFN-γ SFC (stimulation with 0.1 ng/mL) in splenocytes from mice immunized as earlier. D, percentage of CD107$^+$IFN-γ$^+$ and IFN-γ$^+$TNF-α$^+$IL-2$^+$ CD8 cells in splenocytes from mice immunized as earlier after stimulation with 1 ng/mL of OVA(257–264). E, splenocytes obtained from naïve mice, B16-OVA-tumor–bearing mice untreated (UT) or treated for 2 weeks with different adjuvant combinations ($n = 6$/group) were stimulated *in vitro* with irradiated B16-OVA or B16.F10 cells and the number of IFN-γ–producing cells was measured by ELISPOT. Results are representative of 2 to 3 independent experiments.

EDA-OVA + MAC administration induces B16-OVA tumor rejection mediated by CD8$^+$ cells

EDA-OVA + MAC, characterized as the best combination to induce innate and adaptive immune responses, was used to treat B16-OVA tumor-bearing mice in long-term experiments. In mice treated with this strategy, tumor growth was completely blocked until day 30, whereas all untreated mice had already died by day 28. Moreover, beyond this day, tumor growth rate was slower in the treated group, and half of mice rejected their tumor and still survived at day 80 ($P < 0.001$; EDA-OVA + MAC vs. UT; Fig. 5A and B).

Administration of EDA-OVA + MAC s.c. at a distal site almost blocked tumor growth for 2 weeks, but as opposed to intratumor administration, all tumors grew after treatment, suggesting that local administration was required to maintain the beneficial effect (Supplementary Fig. S6).

The relevance of effector populations on tumor rejection induced by EDA-OVA + MAC administration was studied by depleting CD8 and NK cells in tumor-bearing mice before treatment. These experiments (Fig. 5D) showed that CD8 depletion abrogated most of the beneficial effect provided by EDA-OVA + MAC ($P < 0.01$; CD8-depleted vs. Isotype, in

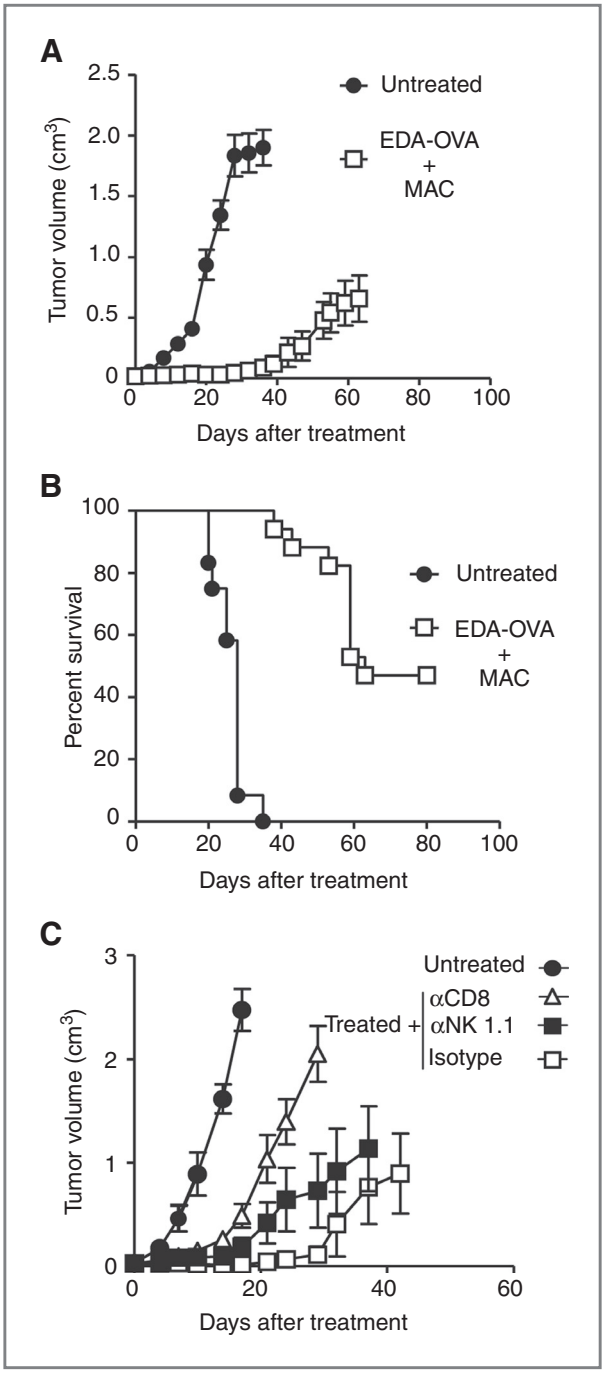

Figure 5. Therapeutic administration of EDA-OVA+MAC induces tumor rejection mediated by $CD8^+$ cells. C57BL/6 mice (14–17 per group) were injected s.c. with 10^5 B16-OVA tumor cells and when tumor diameter reached 5 mm they were treated during a 20-day period with EDA-OVA+MAC or left untreated. Tumor volume (A) and mice survival (B) was monitored twice a week. Results correspond to the sum of 2 independent experiments. C, C57BL/6 mice (7–9 per group) were injected s.c. with 10^5 B16-OVA tumor cells and when tumor diameter reached 5 mm they were depleted of CD8 cells, NK cells, or administered control antibodies previously to treatment with EDA-OVA+MAC as earlier. Undepleted untreated mice were used as control, and tumor growth was measured in all groups.

survival). Depletion of NK cells had only an initial minor effect on tumor growth, in agreement with a decrease of 15% to 20% in the induction of CD8 responses when using mice depleted of NK cells (Supplementary Fig. S7), but no differences were finally observed in survival ($P = 0.77$; NK-depleted vs. Isotype). These results suggest that CD8 cells are the main effector population responsible for tumor rejection.

Administration of EDA-OVA+MAC to tumor-bearing mice induces T-cell responses against different tumor antigens

To analyze the role of the different tumor antigens recognized by T-cells, at day 80 (60 days after finishing treatment), re-challenge experiments were carried out in surviving animals. None of cured animals after treatment with EDA-OVA + MAC developed tumors when re-challenged with B16-OVA cells, whereas a quick tumor growth was observed in control untreated mice. Moreover, when equivalent mice cured after treatment with EDA-OVA + MAC were re-challenged with B16.F10 tumor cells, which do not express OVA, 80% of mice remained tumor free (Fig. 6A). Study of responses against tumor antigens expressed by B16-OVA tumor cells in cured mice showed that they not only recognized OVA, but also the melanoma antigen TRP-2 (Fig. 6B), suggesting that this treatment not only induces responses against the administered antigen, but also against other antigens expressed by tumor cells. Indeed, in agreement with results shown in Figure 5, as early as 10 days after starting treatment, responses against TRP-2(180–188) peptide were observed in mice receiving EDA-OVA + MAC or OVA + MAC (Fig. 6C). This prompted us to see the efficacy of a new therapy based on administration of MAC plus EDA fused to fragment 59–257 of TRP-2 (EDA-TRP2), a protein that activates innate and adaptive immunity (Supplementary Fig. S8). As shown in Figure 6D, all untreated animals died by day 13, whereas 30% of mice treated with EDA-TRP2 + MAC still survived at day 40 ($P < 0.001$; EDA-TRP2 + MAC vs. UT).

Discussion

The failure of many preclinical immunotherapeutic strategies when applied to patients is partially related to the poor immunogenicity of human tumors, as opposed to malignant cells used in animal models that are usually more immunogenic. Thus, protection or tumor rejection could not be achieved in the B16-OVA tumor model using a mixture containing OVA plus poly(I:C) and anti-CD40, a successful protocol used in the E.G7-OVA model. Indeed, B16-OVA cells express much lower levels of OVA antigen and MHC molecules, as compared to E.G7-OVA cells, which would impair tumor recognition and rejection by antigen-specific T cells. Thus, using this poorly immunogenic model, we have analyzed the efficacy of different immunotherapeutic strategies based on the use of different adjuvant combinations, with or without tumor antigens. As opposed to the E.G7-OVA, B16-OVA tumors are not rejected after repeated administration of different double-adjuvant combinations. Adjuvant administration in the former model induces both innate and adaptive

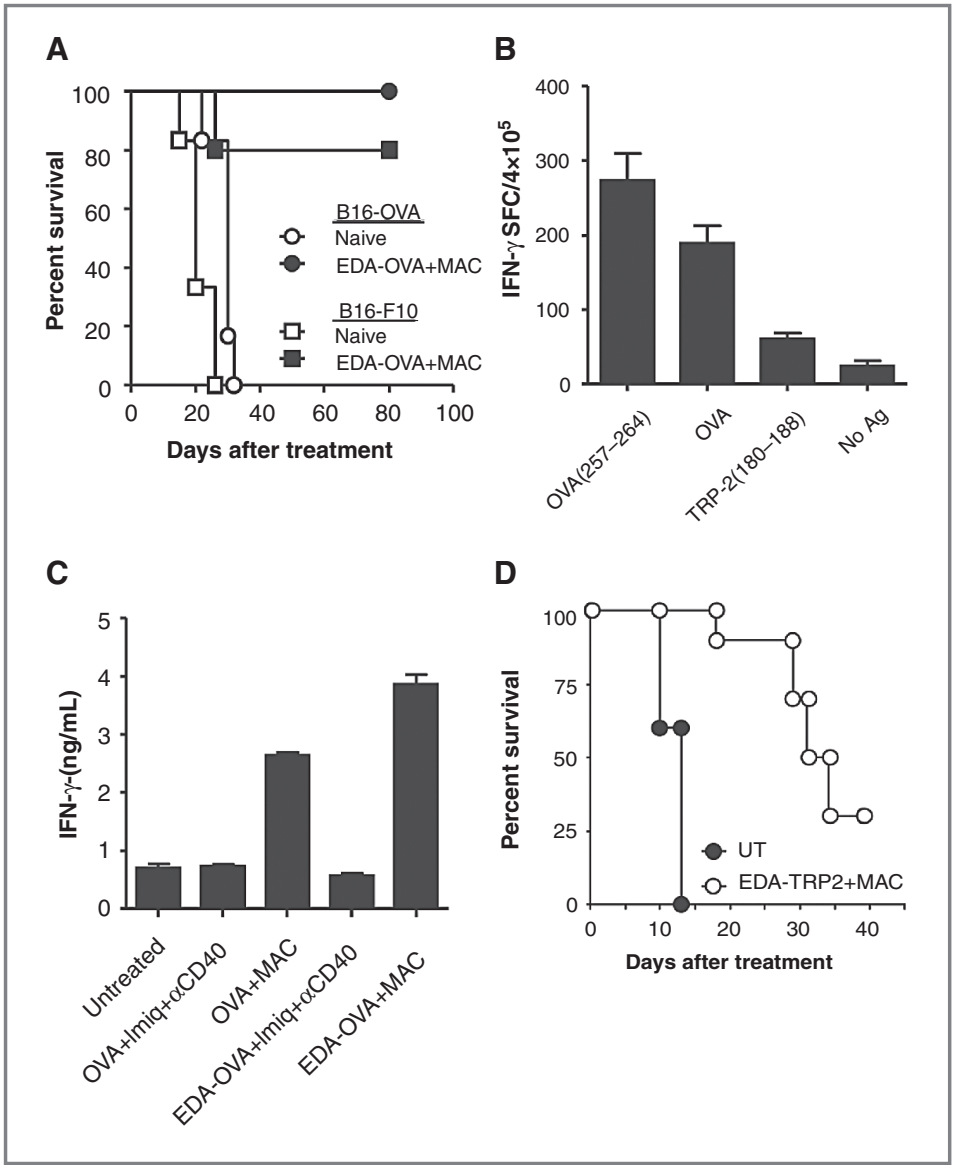

Figure 6. Administration of EDA-OVA+MAC to tumor-bearing mice induces T-cell responses against different tumor antigens. A, mice ($n = 5$–6 per group) that rejected B16-OVA tumors after immunotherapy with EDA-OVA+MAC or naïve mice were challenged with 10^5 B16-OVA or B16.F10 tumor cells 60 days after completing the treatment and survival was monitored twice a week. B, immune responses against tumor antigens were measured by IFN-γ ELISPOT in mice cured after immunotherapy with EDA-OVA+MAC. C, immune responses against TRP-2(180–188) peptide were measured in B16-OVA tumor-bearing mice treated for 10 days with different adjuvant combinations. D, C57BL/6 mice (10 per group) were injected s.c. with 10^5 B16.F10 tumor cells and when tumor diameter reached 5 mm they were treated during a 20-day period with EDA-TRP2+MAC or left untreated. Mice survival was monitored twice a week.

immunity, whereas in the latter, only innate immune responses can be detected. The low levels of tumor antigen expressed and/or released by B16-OVA cells which are available for presentation to T cells after activation of innate immune cells, probably account for the lack of induction of T-cell responses. This hypothesis is reinforced by the finding that tumor growth is slower only during the treatment period, probably due to innate immunity primed by adjuvants, and increases after treatment.

Inclusion of the antigen OVA with Imiquimod and anti-CD40, or with poly(I:C) and anti-CD40, induced T-cell responses in naïve and tumor-bearing mice, and tumor growth was delayed several days beyond the end of treatment, suggesting that the induction of T-cell responses has additional but not sufficient antitumor effect. To overcome the poor efficacy of these protocols, we used a multiple adjuvant combination containing Imiquimod, poly(I:C), anti-CD40 as well as EDA coupled to OVA, which acts as adjuvant through TLR4 and targets the antigen to antigen presenting cells. In this case, tumor rejection was observed in half of treated animals, associated with a clear improvement of the immune response induced by treatment. A synergy between different adjuvants has been reported *in vitro* and *in vivo* for DC (7, 8, 10, 11, 24), mainly for those receptors which do not share signaling pathways. Thus, we used poly(I:C), which binds to TLR3 (25) and triggers the TRIF pathway, Imiquimod, which uses TLR7 with Myd88 as adaptor molecule (26), anti-CD40, which triggers a TRAF-dependent pathway (27), and EDA, able to bind to TLR4, a receptor connected to the TRIF and Myd88 pathways (28). Although EDA+MAC induced the highest upregulation of costimulatory molecules as well as cytokines associated with T-cell activation on DC *in vitro*, both MAC and

EDA+MAC induced the highest DC activation *in vivo* in tumor-bearing mice. Moreover, enhancing effects were observed not only on antigen-presenting cells, which concomitantly affect T-cell responses, but also on NK cells, which in turn may also help the induction of Th1 T-cell responses. Indeed, when using this adjuvant combination, NK cells are partially responsible for the induction of IFN-γ–producing CD8 T cells. The lack of tumor rejection in mice treated at a distal site suggests that local effect of adjuvants is necessary for tumor rejection, because it may activate innate immunity with direct inflammatory and antitumor effects which also collaborate by triggering adaptive immunity.

Regarding adaptive immunity, although MAC induced similar innate immunity *in vivo*, EDA-OVA + MAC activated stronger T-cell responses, probably due to the targeting effect of EDA. Moreover, EDA-OVA + MAC also induced qualitatively better responses, related to antigen avidity and polyfunctional T cells. Concerning avidity of CD8 T cells, which has been related to the efficacy of antiviral (29, 30) and antitumor immunity (31, 32), it was found that CD8 T cells primed by EDA-OVA+MAC strongly recognized OVA(257–264) peptide at low antigen concentrations. The high IFN-γ production induced by these low antigen concentrations (a situation similar to that found in poorly immunogenic tumors) probably helps upregulating MHC molecules on tumor cells, and concomitantly in their recognition by T cells. Indeed, EDA-OVA+MAC induced T cells that efficiently recognized B16-OVA tumors *in vitro*, and is probably responsible for its antitumor efficacy *in vivo*. In line with this result, it has been recently published that an adjuvant combination containing ligands of TLR2/6, TLR3 and TLR9 qualitatively enhanced T-cell responses, by increasing their functional avidity in a viral protection model (33). Interestingly, that triple combination, which only shares with EDA + MAC the TLR3 ligand poly(I:C), also increased IL-15 and IL-15R expression, a cytokine which mediates CD8 T-cell avidity maturation (34), suggesting that induction of this cytokine by adjuvant combinations might be related to their ability to activate high-avidity T cells. Finally, activation of polyfunctional CD8 T cells, those producing several cytokines and displaying different effector functions, was higher in response to low antigen concentrations after EDA-OVA + MAC administration. Polyfunctional T cells produce more cytokine per cell (35, 36) and they are associated with control of viral infections (37, 38) and tumor regression (39). Thus, parameters associated with better T-cell responses such as higher IFN-γ production, induction of high-avidity T cells which recognize lower antigen levels expressed by poorly immunogenic tumor cells, or induction of polyfunctional T cells, may explain the higher efficacy of this strategy.

Rechallenge experiments using cured mice have shown that they are not only completely protected against the same tumor, but also show partial protection against a tumor lacking the antigen used in the therapeutic adjuvant combination, suggesting that epitope spreading takes place during therapy-mediated tumor rejection. Immunity against tumor antigens not used in immunization (mainly TRP-2) was induced when administering EDA-OVA + MAC, making these tumor antigens immunogenic, which in the absence of activating signals, did not trigger T-cell responses. Thus, this strategy has the advantage of inducing additional protective immunity against other unknown tumor antigens not used in the therapy.

In summary, a poorly immunogenic tumor not responding to therapies based on single or double adjuvant administration can be made to respond using a multiple adjuvant combination and tumor antigen targeting strategy, associated with the induction of quantitatively and qualitatively better T-cell responses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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