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Targeting the tumor microenvironment with anti-neu/anti-CD40 conjugated nanoparticles for the induction of antitumor immune responses

Ana Lucia Dominguez and Joseph Lustgarten

Mayo Clinic College of Medicine, Department of Immunology, Mayo Clinic Arizona

Abstract

Clinical and preclinical data indicate that immunotherapeutic interventions could induce immune responses capable of controlling or retard the tumor growth. However, immunotherapies need to be further optimized. We hypothesized that a more effective strategy for tumor eradication is to directly target the tumor microenvironment in order to generate a proinflammatory response and induce a localized antitumor immune response capable of eliminating the tumor cells. Nanoparticles have been proven to be an effective delivery system. In these studies we evaluated conjugated anti-RNEU and anti-CD40 antibodies onto PLA-(poly DL-lactic acid)-biodegradable nanoparticles (PLA-NP) for the induction of antitumor immune responses. The anti-neu/anti-CD40-NP were functional *in vitro* recognizing RNEU⁺ tumors and activating dendritic cells. The delivery of anti-neu/anti-CD40-NP but not anti-neu-NP or anti-CD40-NP induced an antitumor response resulting in complete tumor elimination and generation of protective memory responses. The anti-neu/anti-CD40-NP specifically activated an antitumor response against RNEU⁺ tumors but not against RNEU⁻ tumors. The antitumor immune responses correlate with the induction of a Th1 proinflammatory response, reduction in the number of Tregs within the tumor and activation of a specific cytotoxic response. These results indicate that anti-neu/anti-CD40-NP with immunomodulatory properties are safe and can be used effectively as cancer vaccines strategy for the specific induction of antitumor immune responses.

Keywords

Nanoparticles; CD40; RNEU; T cell responses; Dendritic cells and Immunotherapy

1. Introduction

T cell immunity is a critical component of the immune response to a growing tumor. The discovery of tumor associated antigens (TAA) [1,2] has been an important breakthrough in tumor immunology, because it is possible to devise immunotherapeutic approaches to promote T cell responses against such antigens and induce a protective immunity against neoplastic malignancies [3,4]. A common practice to induce antitumor immune responses is the use of T cell epitopes derived from specific sequences from TAA [5]. Dendritic cells (DCs) are

Correspondence: Joseph Lustgarten, Mayo Clinic Arizona, 13400 East Shea Boulevard Scottsdale, AZ 85259, Phone: (480) 301-6662, lustgarten.joseph@mayo.edu, fax: 480-301-9162.

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considered the most potent antigen presenting cells (APCs) capable of effectively stimulating a robust immune response [6]. The most common strategy evaluated to effectively induce a TAA-specific antitumor immune response is DCs pulsed with TAA-epitopes [7,8]. Although some preclinical and clinical studies show some antitumor effects following DC-peptide vaccination [9,10], many other studies have not observed similar results [11,12]. For example, we have tested the effect of DCs in the RNEU tumor model. Our results indicate that DC pulsed with RNEU peptides or RNEU soluble proteins delays tumor growth but is not sufficient to control tumor growth [13]. Additionally, there are other drawbacks for implementing the use of DC immunotherapeutic strategy such as: 1) it is necessary to derive large numbers of autologous DC *in vitro*, therefore this is considered an individualized strategy; 2) standardization of how to prepare DCs for *in vivo* use; and 3) it is a labor intensive and an expensive process that requires cell purification. This leads to an important issue of how to prepare a “universal vaccine” that is cell-free and effectively activates APCs capable of inducing an antitumor immune response and achieve tumor elimination. We hypothesized that perhaps a more effective strategy for tumor eradication is to directly target the tumor microenvironment in order generate a proinflammatory response and induce a localized antitumor immune response capable of eliminating the tumor cells.

CD40 is a TNF receptor family member that plays a crucial role in shaping both the cellular and the humoral immune responses [14]. It is expressed on B cells, DCs, and macrophages. Its specific ligand (CD40L) is expressed in a highly restricted fashion by activated T-helper cells [15]. Therefore, CD40 is a key molecule in the instructive activity of T-helper cells [16]. It has been demonstrated that a helper-dependent CD8 T cell response could be converted to a helper independent response by activating APCs with anti-CD40 monoclonal antibody (mAb) [17–19]. Recently, it has been found that CD40 is also expressed by CD8⁺ T cells and plays a key role in the activation of memory but not naive cytotoxic T lymphocyte (CTL) precursors [20]. We tested whether intratumoral (i.t.) injections of anti-CD40 mAb was capable of inducing an antitumor response that would control tumor growth in the RNEU model. Surprisingly the data indicated that this vaccination strategy was ineffective and no antitumor responses were observed. These results are in agreement with previous studies demonstrating that systemic administration of anti-CD40 mAb does not induce an antitumor response [21, 22] and can enhance the deletion of tumor specific CD8⁺ T cells [23].

For biological purposes, nanotechnology can be used for detection, diagnosis, and treatment of cancers [24–26]. Key advantages of many nanoparticles (NP) are their low toxic effects and biocompatibility. Nanoparticles can be conjugated to cancer-specific ligands (e. g. antibodies) for tumor detection using *in vivo* imaging or targeted therapy [27–29]. More than one ligand can be linked into a single nanoparticle. We hypothesize that immobilization of anti-CD40 mAb at the tumor site could be an effective strategy to stimulate and provide an appropriate stimulatory signal to APCs for the induction of antitumor immunity. To target the anti-CD40 antibody to the tumor site, we covalently conjugated anti-RNEU and anti-CD40 antibodies onto PLA-(poly DL-lactic acid)-biodegradable nanoparticles (PLA-NP). Our results indicate that the anti-neu-/anti-CD40-NP are functional molecules *in vitro* and treatment with these conjugated-nanoparticles induce an antitumor response while no antitumor responses were observed following treatment with anti-neu-NP or anti-CD40-NP. This indicates that the delivery mechanism of anti-CD40 mAb is critical for determining therapeutic outcome. Furthermore, these results indicate that the use of NP conjugated with anti-neu and anti-CD40 mAb can be used as a “universal” therapeutic strategy without being patient specific.

2. Material and Methods

2.1. Mice, Cell Lines, and Reagents

Female Balb/c mice were purchased from Harlan (Indianapolis, IM). TUBO is a cell line generated from a spontaneous mammary gland tumor from a BALB-neuT mouse [30]. The mouse renal cell carcinoma RENCA cells of Balb/c origin was used as a negative control. Anti-neu (7.16.4, against the rat neu) was obtained from Dr. Mark Greene (University of Pennsylvania, Philadelphia, PA). Anti-CD40 mAb (clone FGK45) was obtained from Dr. Stephen Schoenberger (La Jolla Institute for Allergy and Immunology, La Jolla, CA). Dendritic cells (DCs) were derived from bone marrow as previously described [13]. Briefly, bone marrow cells were depleted of lymphocytes. The remaining cells were cultured in complete RPMI medium containing 3% GM-CSF (supernatant from J558L cells transfected with murine GM-CSF gene, obtained from Dr. R. Steinman, Rockefeller University, NY). Media was changed every second day, each time applying fresh complete RPMI medium containing 3% GM-CSF. On day eight, DCs were collected. All cell lines were maintained in complete RPMI medium (RPMI 1640) supplemented with 10% FCS, 2mM glutamine, 5×10^{-5} M 2-mercapethanol (ME), and 50µg/mL gentamicin.

2.2. Generation of nanoparticle-conjugates

Biodegradable polylactic acid (PLA) nanoparticles with surface carboxyl groups (PLA-COOH) of 250 nm were purchased from Corpuscular Inc (Cold Spring, NY). Nanoparticles were washed in 25 mM MES (N-morpholino ethane sulfonic acid) buffer, pH 6. Washed nanoparticles (10 mg) were mixed with 1mg of antibody in 25 mM MES buffer, pH 6. Nanoparticles and antibodies were incubated overnight at 4°C. After incubation, nanoparticles were washed three times with PBS by centrifugation to remove excess of antibody. Possible free carboxyl groups were blocked with 1% bovine serum albumin (BSA). After blocking, conjugated-nanoparticles were washed with PBS and resuspended in 1 mL of PBS-Triton-0.01% and stored at 4°C. We estimated that in 1 mL of nanoparticles there is 500 µg of antibody incorporated.

2.3. Flow cytometry analysis

TUBO cells and dendritic cells were first surface stained with anti-neu, anti-CD40, anti-neu-NP, anti-CD40-NP, and anti-neu/anti-CD40 for 30 minutes at 4°C. For staining 5 µg of antibody or 10 µl of conjugated nanoparticle (equivalent as 5 µg of antibody) was used. Cells were washed with FACS buffer (PBS, 0.5%, BSA, 0.02% sodium azide) and then stained with goat-anti-rat-FITC.

2.4. Formation of dendritic cells and tumor cells conjugates

TUBO cells were labeled with the aliphatic green fluorochrome PKH2-GL (Sigma) and dendritic cells were labeled with the aliphatic red fluorochrome PKH2-GL. Labeled tumor cells and DCs were incubated at 3:1 ratio, respectively in the presence of anti-neu-NP+anti-CD40 (5 µl/of each conjugated-nanoparticle) or anti-neu/anti-CD40 (10 µl) for 1 hour at 37°C. Samples were analyzed by confocal microscopy.

2.5. In vivo tumor studies

TUBO or RENCA cells (1×10^6) were implanted subcutaneously (s.c.) in Balb/c. Tumors were allowed to grow for 10 days before treatment was initiated. On day ten after tumor challenge (tumor size $\sim 150\text{--}200\text{mm}^3$), animals were randomly divided into groups of 5–8 mice/group. Animals received i.t. injections of anti-CD40 (100 µg/injection) twice a week for three weeks. Animals injected with the conjugated nanoparticles (50 µl/injection) were treated twice a week for three weeks. Animals injected with isotype-NP (50 µl/injection) served as controls. For the

evaluation of memory responses, animals were challenged 70 days after the first tumor challenge with a second dose of 10^6 TUBO cells. Tumor growth was monitored every two to three days.

2.6. Depletion studies

Anti-CD4 (GK1.5) and anti-CD8 (56-6.37) mAbs were used for *in vivo* depletion of T cell subsets. Anti-asialo GM1 (Wako Pure Chemical Industries, Richmond, VA) was used to deplete NK cells. Animals were injected i.p. with 300 μ g of anti-CD4 and anti-CD8 mAb or 50 μ g of anti-asialo GM1 twice per week, starting 1 week before inoculation of the tumor cells and continuing for the duration of the experiment.

2.7. Multiplex analysis

For determining soluble factors within the tumor microenvironment after treatment with anti-neu/anti-CD40-NP we followed the same protocol as we have previously described [31]. Briefly, a tumor extract was prepared in T-per extraction buffer (Pierce, Rockford, IL) and levels of cytokines were assayed using multiplex luminescent beads (Invitrogen, San Diego, CA). The lower limit of detection was 1.5 pg/mL for each cytokine.

2.8. Analysis of CD4⁺ Foxp3⁺ T-regs

The numbers of T-regs in the tumor microenvironment, spleen, and lymph nodes (LN) in Balb/c tumor bearing mice and after treatment with anti-neu/anti-CD40 was determined by the analysis of CD4⁺Foxp3⁺ cells using a commercially available kit (eBioscience, San Diego, CA) following the manufacturer's protocol.

2.9. Generation of CTL bulk cultures and cytotoxic activity

Balb/c tumor bearing mice were injected i.t. twice a week with anti-neu/anti-CD40-NP or isotype-NP (50 μ l/injection) for two weeks. One week after the last injection with anti-neu/anti-CD40-NP, animals were sacrificed. Spleen cells (6×10^6) from primed animals were restimulated *in vitro* with 5×10^5 irradiated (3000 rads) TUBO cells plus 1×10^6 irradiated Balb/c spleen cells as feeders. After five days, cultures were assayed for lytic activity in a ^{51}Cr release assay against TUBO and RENCA cells. Supernatants were recovered after 6 hours of incubation at 37°C and the percent of lysis was determined by the formula: percent specific lysis = $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$.

2.10. Statistical analyses

Statistical significance of data was determined by Student's t test to evaluate the p value. The relationship between two parameters was tested using regression analysis and $p < 0.05$ was considered significant. Survival analysis used the Breslow modification of the Kaplan-Meier test.

3. Results

3.1. Analysis of intratumoral injections of anti-CD40 mAb

Our previous results indicated that DCs pulsed with RNEU antigens was not sufficient to control tumor growth of TUBO RNEU positive tumor cell line [13]. Previous studies show that ligation of CD40 leads to changes in APC phenotype and function [14–16] and also it can substitute the CD4⁺ T cell help functional response in the priming of naive CD8⁺ T cells [19]. We tested the antitumor effect of injecting anti-CD40 mAb on Balb/c mice implanted with TUBO cells. To our surprise, intravenous (i.v.) injections of anti-CD40 (clone FGK45) did not have any effect in controlling tumor growth (Fig 1). Perhaps under these administration

conditions a sufficient concentration of anti-CD40 mAb did not reach the tumor site. Therefore, we also tested the effect of injecting anti-CD40 at the tumor site (intratumoral) and under these conditions no antitumor responses were observed (Fig 1). Dosage was not an issue because injections of higher concentrations of anti-CD40 (300 or 500 µg/injection) did not induce tumor rejection and, to the contrary, at these concentrations toxic effects were observed (data not shown).

3.2. Generation of anti-CD40/anti-neu-nanoparticles

We were surprised that i.t. injections of anti-CD40 mAb did not delay tumor growth. We hypothesized that if anti-CD40 mAb was immobilized at the tumor site, it would prolong the half life of the antibody within the tumor microenvironment, therefore, it will retain APCs at the tumor site for longer periods of time resulting in activation and generation of an antitumor response. To this end, the anti-neu and anti-CD40 mAb were covalently linked to PLA-nanoparticles (NP) (Fig 2A). First, the ability of conjugated-NP to recognize tumor cells expressing neu (TUBO cells) (Fig 2B) or DCs (Fig 2C) expressing CD40 was evaluated. The anti-neu-NP and anti-neu/anti-CD40-NP were able to stain TUBO cells with the same efficiency as the control anti-neu antibody (Fig 2B). In contrast anti-CD40-NP did not recognize the TUBO cells (Fig 2B). These results indicated that conjugated-NP are specific in recognizing tumor. These results also indicated that this tumor is CD40 negative. The anti-CD40- and anti-neu/anti-CD40-NPs stained the dendritic cells but no staining was observed with anti-neu-NP (Fig 2C). Next, we evaluated the functional capacity of conjugated NP to stimulate DCs. DCs were incubated in the presence of anti-neu-NP, anti-CD40-NP, anti-neu/anti-CD40-NP, anti-neu and anti-CD40 mAb overnight. The next day, supernatants were collected and the secretion of IL-12 was assessed. DCs treated with anti-CD40-NP and anti-neu/anti-CD40-NP produced similar amounts of IL-12 as did DCs treated with anti-CD40 mAb (Fig 2D). However, no production of IL-12 was detected after treatment with anti-neu-NP or anti-neu mAb. These results demonstrated that anti-neu/anti-CD40-NP retained their ability to recognize RNEU⁺ tumor cells and activate DCs.

3.3. Anti-neu/anti-CD40-NPs induces the formation of conjugates between tumor cells and DC

Our hypothesis is based on the premise that anti-neu mAb bound to NPs will recognize the tumor antigen expressed on the tumor cells and anti-CD40 mAb bound to the same solid support will recognize APCs in which these cells could be activated at the tumor site bringing to proximity tumor cells and APCs. Therefore, anti-neu/anti-CD40-NPs could induce the formation of conjugates between tumor cells and APCs. We evaluated whether anti-neu/anti-CD40-NP forms conjugates between TUBO cells and DCs. As shown in Fig 3, the combination of anti-neu-NPs+anti-CD40-NPs did not form conjugates between tumors and DCs (Fig 3A, picture taken in 10x). In contrast, the addition of anti-neu/anti-CD40-NPs brought to proximity TUBO cells (green) and DCs (red) inducing the formation of conjugates between these cells (Figs 3B, C and D, indicated by the white arrows, pictures taken in 40x). These results support our hypothesis that with the use of anti-neu/anti-CD40-NPs, the anti-CD40 mAb could be anchored at the tumor site retaining for longer periods of time APCs within the tumor microenvironment resulting in the induction of an antitumor response.

3.4. Antitumor effect of NP conjugated with anti-neu, anti-CD40 and anti-neu/anti-CD40

Next, we evaluated whether injections of anti-neu-NP, anti-CD40-NP, anti-neu-NP+anti-CD40-NPs, or anti-neu/CD40-NPs induce an antitumor response. As a proof of concept for these experiments, the conjugated-NP were injected intratumorally (i.t.). Our results indicate that 100% of the animals that were treated with anti-neu/anti-CD40-NP rejected the tumor (Fig 4A). No antitumor effect was observed in animals treated with anti-neu-NP, anti-CD40-NP or

the combination of anti-neu-NP plus anti-CD40-NP. Vaccinations with the NP were well tolerated by the animals and no signs of toxicity were observed. These experiments indicate that only NP conjugated to both anti-neu and anti-CD40 mAb were capable of inducing an antitumor response. An important observation from these experiments was that the anti-neu mAb was not the mechanism by which the tumor was rejected since anti-neu-NP or the combination of anti-neu-NP plus anti-CD40-NPs did not have an antitumor effect on the tumor. Our data also indicate that i.t. injections of anti-neu mAb alone did not induce an antitumor response (data not shown).

We also evaluated whether the antitumor response depends on CD4 T cells, CD8 T cells, or NK cells. Animals were treated with intraperitoneal (i.p.) injections of anti-CD4, anti-CD8, or anti-asialoGM1 antibodies (anti-NK Ab) (300 µg/injection) twice a week starting one week prior to tumor implantation and throughout the duration of the experiment. Depletion of CD4⁺ T cells, CD8⁺ T cells, and NK cells abrogated the anti-tumor response indicating that these cells are critical for the rejection of the tumor after anti-neu/anti-CD40-NP injections. Intratumoral injections of anti-neu/anti-CD40-NP on Balb/c athymic mice having grown the TUBO cells did not induce the rejection of tumors (data not shown). These results indicate that the antitumor response is depended on the activation of APCs and T cell response.

3.5. Systemic delivery of anti-neu/CD40-NPs

Having demonstrated that i.t. injections of anti-neu/anti-CD40-NP induce an antitumor immune response, we evaluated next the effect of delivering conjugated-NP systemically. Balb/c mice were implanted with TUBO (Her2/neu⁺) and RENCA (RNEU⁻) tumor cells and animals were treated with anti-neu/anti-CD40-NP. Our results indicate that 5/7 animals having grown TUBO cells, rejected the tumor and those animals that did not reject the tumor, significantly delayed tumor growth when compared to the control group (Fig 5A). No antitumor effect was observed on animals implanted with RENCA cells (Fig 5A). These results demonstrate that the anti-neu/anti-CD40-NP could be used for systemic delivery and specifically target the tumor microenvironment to induce antitumor immune responses. We further tested whether animals that rejected the tumor following treatment with anti-neu/anti-CD40-NP develop protective immune memory responses. As shown in Fig 5B, 5/5 of the animals that rejected the primary tumors were able to reject the challenged tumor indicating the development of a cellular memory response.

3.6. Analysis of the antitumor immune responses elicited by anti-neu/anti-CD40-NP

Next we evaluated whether vaccination with anti-neu/anti-CD40-NP correlates with the activation of a cellular immune response. Our data indicate that following injections of anti-neu/anti-CD40-NP a strong pro-inflammatory response was generated in which the levels of IL-6, IL-12, INF-γ, and TNF-α were significantly elevated ($p < 0.01$) when compared to control animals or those treated with anti-CD40-NP or isotype-NP (Fig 6A). No significant changes in the level of IL-2, IL-4, IL-10 or TGF-β were observed in animals treated with anti-neu/anti-CD40-NP (Fig 6A). We also observed that the levels of Tregs within the tumor were significantly decreased ($p < 0.01$) when compared to control animals but not in spleen or tumor draining lymph nodes (TDLN) (Fig 6B). We did not observe any major differences in cytokines or Tregs levels compared to control or isotype-NP. The reason why anti-CD40-NP does not change the levels of cytokines or Tregs is because this molecule likely does not remain at the tumor site for longer periods while the anti-neu/anti-CD40-NP does, activating an immune response within the tumor. No changes in the levels of cytokines or Tregs were observed following treatment with anti-neu-NP (data not shown). Our results also indicate that following injections with anti-neu/anti-CD40-NP there is an activation of a cytotoxic response in which the CTLs effectively recognize and kill TUBO but no cytotoxic effect was observed against RENCA cells indicating that a RNEU tumor specific immune response was generated. No

cytotoxic response was observed in animals treated with isotype-NP. Taken together, these results suggest that the activation of an antitumor immune response induced by anti-neu/anti-CD40-NP is through the activation of multiple mechanisms used by the immune system.

4. Discussion

In these studies we evaluated a novel strategy to target the tumor microenvironment using nanotechnology. Our results indicate that systemic or intratumoral injections of anti-CD40 do not have an antitumor effect. Considering that CD40 plays an important role in maturation and activation of DCs resulting in the induction of immune responses [32,33] and anti-CD40 could significantly enhance the immune responses of vaccination formulations [34,35], we proposed that direct injections of anti-CD40 is the wrong strategy to stimulate an antitumor response. We hypothesize that if the anti-CD40 could be retained at the tumor site it would be possible to induce an immune response with antitumor effects. To retain the anti-CD40 mAb for longer periods of times at the tumor site we developed nanoparticle-conjugates constituted of an antibody against a tumor antigen (anti-RNEU) and anti-CD40. The rationale for developing this nanoparticle is that the anti-neu antibody will bind the RNEU⁺ tumor anchoring the nanoparticle at the tumor site while the anti-CD40 mAb activates DCs inducing a robust immune response. Our results indicate that the anti-neu/anti-CD40-NP retains its dual function recognizing RNEU⁺ tumors and activating DCs. Furthermore, our data demonstrate that anti-neu/anti-CD40-NP induce the formation of conjugates between dendritic cells and tumor cells supporting our hypothesis. More importantly, treatment of tumor bearing mice with anti-neu/anti-CD40-NP induced an antitumor immune response resulting in tumor rejection. These results demonstrated that it was not the dosage of the anti-neu-CD40 antibody that determines the activation of an immune response since higher concentrations of anti-CD40 or anti-neu did not induce an antitumor response, but it was the delivery mechanism that was critical for determining the therapeutic outcome of the anti-CD40 therapy. These results have important clinical implications: 1) they indicate that low concentrations of anti-CD40 are sufficient to activate APCs; 2) the anti-neu/anti-CD40-NPs most probably remain for longer periods of time at the tumor site when compared to soluble antibodies generating and activating a more robust immune response; and 3) we and others have demonstrated that high doses of anti-CD40 could have toxic side effects. Therefore, the use of anti-neu/anti-CD40-NP will have clinical benefits such as reducing the possible side effects of injecting high doses of anti-CD40 mAb.

The responses induced by anti-neu/anti-CD40-NP are dependent on the activation of a cellular response since the depletion of CD4⁺ and CD8⁺ T cells and NK cells abrogated the antitumor responses and no antitumor effect was observed in Balb/c athymic mice. The antitumor responses induced by anti-neu/anti-CD40-NP correlate with: 1) the induction of a Th1-proinflammatory response where cytokines such as TNF- α , INF- γ , IL-6 and IL-12 were highly produced; 2) a stark reduction in the levels of Tregs within the tumor; and 3) activation of a specific cytotoxic immune response. These results are critical for indicating how anti-neu/anti-CD40-NP might induce an immune response. By activating DCs or other APCs with the anti-CD40 mAb, a proinflammatory response is generated within the tumor that influences the induction of an immune response [31,36,37]. It has been demonstrated that cytokines in particular, IL-6, could influence the suppressive function [38] or inhibit de novo conversion of Tregs [39]. Considering that Tregs could inhibit DCs and T cells [40], lower numbers and less functional Tregs in the tumor strongly correlate with the activation of a T cell response [41,42]. Therefore, it can be argued that by targeting the tumor with anti-neu/anti-CD40-NP it is possible to modulate the tumor microenvironment favoring the activation of an immune response. Furthermore, following injection of anti-neu/anti-CD40-NP a protective memory response was generated indicating that by targeting the tumor microenvironment it is possible not only to generate a localized immune response but also to induce a systemic immune

response. More importantly, the anti-neu/anti-CD40-NP can be delivered systemically specifically targeting the tumor.

Many studies have evaluated the use of nanoparticles to encapsulate antigens [43,44] or cytokines [45,46] to induce immune responses or make vaccines more effective. To the best of our knowledge, this is the first time that a nanoparticle conjugated with multiple antibodies to modulate the tumor microenvironment and activate antitumor responses has been generated. These results are very encouraging and demonstrate the proof of concept that anti-neu/anti-CD40-NP are functional *in vitro* and *in vivo* and that they can serve as a new immunotherapy strategy for fighting cancer. Furthermore, based on the data presented, it can be argued that anti-neu/anti-CD40-NP could be more effective than the application of anti-CD40 based on the dose applied. Although we have only tested the biodegradable polylactic acid (PLA) nanoparticles there are other biodegradable nanoparticles such as poly(lactic-co-glycolic acid) (PLGA) nanoparticles that can be used *in vivo* [47]. Additionally, there are gold nanorods that are biocompatible in which ligands could be conjugated onto these nanoparticles for therapeutic delivery [48]. There is data indicating that the type (material, surface properties) and size of the nanoparticle could influence the efficacy of the therapeutic delivery [49]. We are currently evaluating in other tumor models different biodegradable/biocompatible nanoparticles of different size to identify which nanoparticle is the most effective in inducing an antitumor response. Even though bispecific-antibodies[50] could be generated with the anti-neu and anti-CD40 mAb and might have the same antitumor effect as the anti-neu/anti-CD40-NP in inducing an antitumor immune response, the advantage of using a nanoparticle is that in addition to the anti-neu and anti-CD40 mAb other ligands or antibodies could be conjugated onto the nanoparticle. For example, we have shown that the combination of immunotherapy and antiangiogenic is more efficient than each monotherapy alone [51]. We are currently generating a new generation of nanoparticles to induce an immune response and reduce tumor angiogenesis by including in the anti-neu/anti-CD40-NP and anti-VEGF antibody. The advantage of this nanotechnology approach is that many ligands could be attached to the nanoparticles to deliver the desired therapy. This approach is not exclusively to formulate vaccines against cancer but potentially could be used to treat infection, autoimmune and other diseases. There has been considerable interest in the development of nanotechnology vaccines [52]. These studies show that biodegradable NP with immunomodulatory properties have a potential capacity to stimulate a tumor specific immune response that could be used as cancer vaccines.

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Fig 1. Injection of anti-CD40 does not have an antitumor effect

Balb/c mice were implanted with 10^6 TUBO cells on day zero. On day 10, animals started treatment with intravenous (i.v.) or with intratumoral (i.t.) injections of anti-CD40 mAb (100 μ g/injection) or control isotype match Ab. Animals were immunized twice a week for three weeks. Five animals were included per group. Survival percentages were determined. Data are representative of two experiments.

Fig 2. Generation and characterization of antibody-conjugated nanoparticles

(A) Schematic representation of conjugated nanoparticles. PLA-NP were conjugated with anti-neu, anti-CD40 or anti-neu+anti-CD40 as described in Material and Methods. (B) TUBO cells were stained with anti-neu mAb (red), anti-CD40-nanoparticles (black), anti-neu-nanoparticles (blue) and anti-neu/anti-CD40-nanoparticles (green). (C) Dendritic cells were stained with anti-CD40 mAb (red), anti-neu-nanoparticles (black), anti-CD40 nanoparticles (blue), and anti-neu/anti-CD40-nanoparticles (green). (D) DCs were stimulated with anti-neu, anti-neu-NP, anti-CD40, anti-CD40-NPs, or anti-neu/CD40-NPs for 48 hours. Supernatants were collected and secretion of IL-2 was evaluated by ELISA. Samples were run in triplicates. Data are representative of two experiments.

Anti-neu-NP+anti-CD40-NP **Anti-neu/anti-CD40-NP**

Fig 3. Formation of conjugates between tumor cells and DCs in the presence of anti-neu/anti-CD40-NP

TUBO cells were labeled with the aliphatic green fluorochrome PKH2-GL. Labeled tumor cells and DCs were incubated at 3:1 ratio, respectively in the presence of anti-neu-NP+anti-CD40-NP (5 μ l/of each conjugated-nanoparticle) or anti-neu/anti-CD40-NP (10 μ l) for 1 hour at 37°C. Samples were analyzed by confocal microscopy. (A) Picture of DCs and TUBO cells in the presence of anti-neu-NP+anti-CD40-NP (picture taken in 10x). (B–D) Pictures of DCs and TUBO cells in the presence of anti-neu/anti-CD40-NP (picture taken in 40x). White arrows indicate the formation of conjugates between DCs and TUBO cells.

Fig 4. Analysis of the antitumor effect of conjugated-NP in tumor bearing Balb/c mice
 (A) Balb/c mice were implanted with 10^6 TUBO cells on day zero. On day ten, animals received intratumoral injections of isotype-NP (use as a control), anti-neu-NP, anti-CD40-NP, anti-neu-NP+anti-CD40-NP or anti-neu/anti-CD40-NP (50 μ l/injection) twice a week for three weeks. Animals were monitored for the development of tumors and survival. Six animals were included per group. Survival percentages were determined. Data are representative of two experiments. A significant $P < 0.001$ difference was found between all groups and Balb/c injected anti-neu/anti-CD40-NP. Dose escalation experiments were performed immunizing animals with 10, 25, 50, and 100 μ l/injection of anti-neu/anti-CD40-NP and 50 μ l/injection was found to be optimal (data not shown). (B) Balb/c mice were treated with anti-CD4, anti-CD8, and anti-NK starting one week prior to tumor implantation and throughout the experiment. Animals were s.c. implanted with 10^6 TUBO cells on day zero. On day ten, animals were injected i.t. with anti-neu/anti-CD40-NP as described in Fig A. Animals were monitored

for the development of tumors and survival. Six animals were included per group. Data are representative of two experiments. Survival percentages were determined.

Fig 5. Systemic injections of anti-neu/anti-CD40-NP for the induction of antitumor responses

To evaluate whether systemic injection of anti-neu/anti-CD40-NP induces a specific antitumor response Balb/c mice were implanted s.c. with 10^6 TUBO (RNEU⁺) or RENCA (RNEU⁻) cells on day zero. On day 10, animals started treatment with i.v. injections of anti-neu/anti-CD40-NP or isotype-NP (use as a control) twice a week (50 μ l/injection: equivalent at 25 μ g/injection) for three weeks. (A) Tumor growth of all groups was evaluated. Six animals were included per group. A significant $P < 0.001$ difference was found between anti-neu/anti-CD40-NP and the rest of the groups. (B) Balb/c mice that rejected the tumor after i.v. injection of anti-neu/anti-CD40-NP were challenged with the 10^6 TUBO cells 70 days after the primary tumor was implanted. Survival percentages were determined. Control indicates naïve Balb/c mice implanted with 10^6 TUBO to assure tumor growth.

Fig 6. Analysis of immune responses induced by anti-neu/anti-CD40-NP

To evaluate the induction of immune responses induced by anti-neu/anti-CD40-NP, Balb/c mice were implanted with 10^6 TUBO cells on day zero. Tumor was allowed to grow for two weeks. Animals were not treated or treated with isotype-NP, anti-CD40-NP or with anti-neu/anti-CD40-NP. For treatment, animals were i.t. injected with isotype-NP, anti-CD40-NP or anti-neu/anti-CD40-NP (50 μ l/injection) two times a week for one week. Two weeks after the first injection with the conjugated-NP, animals were sacrificed and analyzed. (A) Tumors were homogenized and levels of cytokines were assayed using multiplex assay as described in Material and Methods. (B) The prevalence of CD4⁺Foxp3⁺ T-regs in LN, spleen, and within the tumor was determined. (C) Cytotoxic activity of restimulated cultures was measured against TUBO and RENCA (haplotype irrelevant control cells) in standard 6-hour 51 Cr release assay

at 30:1 Effector:Target ratio. Four animals were included per group $\pm$ SD. Data are representative of two experiments.