Three Steps to Breaking Immune Tolerance to Lymphoma: A Microparticle Approach

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Abstract

In situ immunization aims at generating antitumor immune responses through manipulating the tumor microenvironment. On the basis of recent advances in the understanding of antitumor immunity, we designed a three-step approach to in situ immunization to lymphoma: (i) inducing immunogenic tumor cell death with the chemotherapeutic drug doxorubicin. Doxorubicin enhances the expression of "eat-me" signals by dying tumor cells, facilitating their phagocytosis by dendritic cells (DC). Because of the vesicant activity of doxorubicin, microparticles made of biodegradable polymer poly(lactide-co-glycolide) or PLGA can safely deliver doxorubicin immunization and are effective vaccine adjuvants, (ii) enhancing T-cell activation using anti-OX40 and (iii) sustaining T-cell responses by checkpoint blockade using anti-CTLA-4. In vitro, doxorubicin microparticles were less cytotoxic to DCs than to B lymphoma cells, did not require internalization by tumor cells, and significantly enhanced phagocytosis of tumor cells by DCs as compared with soluble doxorubicin. In mice, this three-step therapy induced CD4- and CD8-dependent systemic immune responses that enhanced T-cell infiltration into distant tumors, leading to their eradication and significantly improving survival. Our findings demonstrate that systemic antitumor immune responses can be generated locally by three-step therapy and merit further investigation as an immunotherapy for patients with lymphoma.

Introduction

The goal of many forms of cancer immunotherapy is to overcome immunologic tolerance to tumor antigens and generate immune responses in the form of effector T cells (1). In situ immunization is attractive because it uses the patient's unique tumor antigens by inducing tumor cell death in situ. This limits systemic drug toxicity and provides dendritic cells (DC) with a wide selection of tumor antigens to be presented to antigen-specific T cells (2, 3).

Recent advances in our understanding of antitumor immunity suggest that generating a potent, long-lasting antitumor response might benefit from a three-step approach. Step one—treatment would be delivered locally to induce tumor cell death and provide tumor antigens to DCs. Step two—activation of tumor-specific T cells by DCs would be enhanced. Step three—the activated T-cell response would be maintained so that the systemic response can proceed unrestrained (2).

Doxorubicin is an excellent candidate drug for enhancing tumor antigen uptake by DCs and is routinely used for lymphoma (4). Doxorubicin induces immunogenic cell death, which stimulates an immune response, in part, by inducing surface expression of calreticulin, an "eat-me" signal that enhances phagocytosis of dying tumor cells by DCs (5–7).

In order for T cells to be activated by DCs, they must also receive a costimulatory signal, which can be supplied by Toll-like receptor (TLR) agonists (such as TLR9 agonist CpG), cytokines (such as IL2), and stimulatory antibodies that target members of the tumor necrosis factor receptor (TNFR) superfamily (such as OX40; refs. 8–10). OX40 augments T-cell function and survival (10–12). A stimulatory antibody that activates OX40 (anti-OX40) could thus be used to further activate tumor-specific T cells. We chose to focus on anti-OX40 due to its demonstrated synergistic activity with anti–CTLA-4, which enhances antitumor immune responses in murine lymphoma models (13).

The activity of T cells is tightly regulated by checkpoints that control the magnitude of the immune response, exemplified by cytotoxic T-lymphocyte antigen 4 (CTLA-4). CTLA-4 is upregulated on activated T cells, and signaling via CTLA-4 reduces T-cell proliferation and activity (14). In addition, CTLA-4 plays a central role in the suppressive effect of regulatory T cells (Treg; ref. 15). This provides a strong rationale for including checkpoint blockade as a final step of in situ immunization.

Although the use of doxorubicin to induce immunogenic cell death is attractive for in situ immunization, an intratumoral injection of the soluble drug is not feasible due to its potent vesicant effects (16). Poly(lactide-co-glycolide), or PLGA, is an FDA-approved biodegradable polymer that is clinically used in surgical sutures and for controlled delivery of therapeutic drugs (17). Following intratumoral injection, PLGA microparticles can provide sustained release of encapsulated molecules (18) into the
tumor microenvironment without a vesicant effect. In addition, PLGA microparticles are effective vaccine adjuvants. They activate the NALP3 inflammasome in DCs, which leads to IL-1β secretion and the enhancement of innate and antigen-specific cellular immune responses (19).

On the basis of this background, we hypothesized that a three-step approach to in situ immunization (doxorubicin microparticles given intratumorally combined with systemic anti–CTLA-4 and anti-OX40) can elicit a systemic curative adaptive immune response.

Materials and Methods

Mice and cell lines

Mice (BALB/c and C57BL/6 female, 6–8-week-old) were purchased from Harlan Laboratories. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Iowa (Iowa City, IA) and complied with NIH Guidelines.

A20 (murine BALB/c B-cell lymphoma), Raji (human Burkitt lymphoma B), and EL4 (murine C57BL/6 T-cell lymphoma) were purchased from the American Type Culture Collection (ATCC). Epstein–Barr virus (EBV)–transformed B cells were previously generated per standard protocols (20, 21). Subjects’ informed consent was obtained in accordance with the Declaration of Helsinki under protocols approved by the Institutional Review Board. Cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% heat-inactivated FCS (HyClone), 100 U/mL penicillin, 100 µg/mL streptomycin, and 50 µmol/L 2-ME (Gibco). All cell lines used were confirmed to be Mycoplasma free. No additional validation assays were performed.

Therapeutic antibodies

Anti–CTLA-4 (hamster IgG, clone UC10-4F10-11) and anti-OX40 (rat IgG1, clone OX86) were purchased from BioXCell. A20 were previously shown to lack surface expression of CTLA-4 and OX40 (13).

Generation of DCs

To generate murine bone marrow–derived DCs (BMDC), bone marrow cells were flushed from tibias and femurs of BALB/c mice, and mononuclear cells isolated using Ficoll gradient separation (Fico/Lite-LM; Atlanta Biologicals). Cells were cultured in medium supplemented with 20 ng/mL each of GM-CSF and IL4 (PeproTech) for 7 days. Nonadherent cells were harvested. Cells were >70% DCs as determined by CD11c staining.

Viability assays

The MTS assay for viability was used to determine the cytotoxic activity of doxorubicin microparticles against A20 and DCs (Promega). Briefly, 5 × 10⁴ A20 cells or DCs were incubated with doxorubicin microparticles (8 µg/mg) for 24, 48, or 72 hours (4 wells/group) at a range of doxorubicin concentrations. Blank microparticles were used as negative controls. MTS was added for 4 hours at 37°C. Following centrifugation, 90 µL of supernatant was removed. Absorbance was read at 490 nm using a Thermomax Microplate Reader (Molecular Devices).

Rhodamine particle uptake

Uptake of particles by A20 and DCs was determined using rhodamine-loaded microparticles prepared similarly to doxorubicin microparticles. A20 and DCs were incubated for 24 hours either alone or in a 1:1 mix with rhodamine-loaded microparticles (0.5 µg/mL). Cells were washed and stained for CD11c–APC-Cy7 and CD19–APC (BD Biosciences). Uptake was assessed by flow cytometry using an LSRII flow cytometer (BD Biosciences) by gating on rhodamine⁺ DCs (CD11c⁺) and A20 (CD19⁺).

Transmission electron microscopy

Uptake of doxorubicin microparticles was assessed using transmission electron microscopy (TEM). Briefly, A20 and DCs were incubated for 24 hours with doxorubicin microparticles (1 µg/mL) or blank microparticles (equivalent weight), washed with PBS, and fixed in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate. Postfixation was carried out in 1% osmium tetroxide with 1.5% potassium ferrocyanide for 2 hours, 2.5% uranyl acetate for 20 minutes, followed by dehydration in graded ethanol and embedding in Epon resin (Electron Microscopy Sciences). Ultrathin sections were counterstained with uranyl acetate and lead citrate. TEM images were taken by JEOL JEM-1230 transmission electron microscope provided with a Gatan Ultrascan 1000 2k × 2k CCD camera (JEOL USA, Inc.). Micrographs were processed with the Image software.

Phagocytosis assay

A20 phagocytosis was quantified using A20 cells labeled with CellTrace Violet (Invitrogen). Labeled A20 cells were left untreated or treated with doxorubicin microparticles for 48 hours (3 wells/group) at various concentrations. Controls included soluble doxorubicin (at the same concentrations) and blank microparticles (at equivalent weights). Treated A20 cells were washed and coincubated with DCs at a 1:1 ratio for 2 hours, stained with anti-CD11c–APC–Cy7 (BD Biosciences), and analyzed by flow cytometry. The percentage of double-positive cells (CD11c and CellTrace Violet) was determined.

Confocal microscopy

Microparticle uptake by A20 cells was visualized by culturing cells for 24 hours with doxorubicin microparticles at a final doxorubicin concentration of 2.25 µg/mL. Cells were washed, incubated at 37°C for 2 hours with the nucleic acid dye Cyto16 (Invitrogen), washed, fixed, cytopsined, and mounted on Vector Sheild (Vector Laboratories). DCs cultured on dishes with cover slides bottom were treated for 3 hours together with Cyto16, then stained with anti-CD11c–APC (BD Biosciences) for 2 hours (4°C) and visualized. Images were acquired with a Zeiss LSM510 confocal microscope (Carl Zeiss Co.) equipped with a 63× oil-immersion objective and controlled by ZEN 2009 software (Zeiss). Images were processed with the Image software.

In vivo A20 tumor transplantation and assessment

BALB/c mice were subcutaneously inoculated with A20 at a dose of 6.7 to 9 × 10⁶ A20 cells in 100-µL sterile PBS on the right and left flanks. Treatment began when tumors reached 5 to 7 mm in the largest diameter (days 6–11 after inoculation). Tumor growth was monitored by calipers and expressed as length by width in square millimeters. Mice were euthanized when either tumor reached 20 mm in any direction or when tumor sites ulcerated.

A20 tumor immunotherapy

Doxorubicin microparticles (2 µg doxorubicin in 100 µL PBS) or PBS (100 µL) were injected into the left flank tumor. Three
doses of anti–CTLA-4 and anti–OX40 (collectively referred to as Ab) were administered by intraperitoneal injections every 3 to 4 days. Half of the published doses were used (13): 50 μg for anti–CTLA-4 and 200 μg for anti–OX40 per injection. Treatment groups included PBS, PBS + anti–CTLA-4, PBS + anti–OX40, doxorubicin microparticle, doxorubicin microparticle + anti–CTLA-4, doxorubicin microparticle + anti–OX40, and doxorubicin microparticle + anti–CTLA-4 + anti–OX40. Mice were treated and monitored as before. Additional studies were done with mice receiving lower doses of Ab.

CD4 and CD8 depletion
Antis-CD4 (rat IgG2b, clone GK1.5) and anti-CD8 (rat IgG2b, clone 2.43) were purchased from BioXCell. Rat IgG (MP Biomedicals LLC) was used as an isotype control. Antibodies (200 μg/injection) were administered 1 day before therapy and on days +1, +4, +8, +12, and +18. CD4 and CD8 T-cell depletion was validated by flow cytometry (>99% depletion).

Flow cytometric analysis of tumors and lymphoid tissue
Tumor and lymph node immune infiltrates were evaluated on day 5 after therapy. Injected tumors, contralateral tumors, and draining lymph nodes were harvested and single-cell suspensions surface stained with CD3-APC, CD4-FITC, CD8-PE-Cy7, IFNγ-PE, CD11b-PE, CD11c-APC-Cy7, CD44-APC, CD62L-PE (BD Biosciences), Foxp3-APC, and Gr-1-FITC (eBioscience) and fixed using BD Cytofix/Cytoperm.

Statistical analysis
GraphPad Prism software, version 6.0, was used to analyze tumor growth and to determine differences between groups using unpaired two-tailed Student t tests or ANOVA (Bonferroni correction) where appropriate. Survival curves were compared using the log-rank (Mantel–Cox) test.

Supplementary methods are detailed in the Supplementary Data.

Results
Doxorubicin microparticles provide sustained release of doxorubicin
Doxorubicin microparticles were prepared by the double emulsion-solvent evaporation method (Supplementary Fig. S1A; ref. 22). The target particle size was 1 μm based on the ability to promote inflammasome activation in DCs (19). Scanning electron microscopy revealed a smooth morphology and spherical shape (Supplementary Fig. S1B). Particle size was 1.2 ± 0.4 μm, which is comparable with the size of blank microparticles (empty microparticles) of 1.4 ± 0.3 μm. Kinetic release studies showed 13% burst release of doxorubicin within 1 hour followed by sustained release as the polymer underwent degradation (Supplementary Fig. S1C).

Doxorubicin microparticles kill tumor cells more slowly than soluble doxorubicin and are less cytotoxic to DCs
Doxorubicin microparticles and soluble doxorubicin were compared for their ability to kill A20 lymphoma cells. Increasing concentrations of soluble doxorubicin led to a significant decrease in A20 viability within 24 hours of exposure (87% at 0.5625 μg/mL vs. 46% at 1.125 μg/mL on day 1; P < 0.0001; Fig. 1A). A less pronounced decrease in A20 viability was seen with doxorubicin microparticles (97% at 0.5625 μg/mL vs. 84% at 1.125 μg/mL doxorubicin on day 1; not statistically significant). This was confirmed when comparing doxorubicin microparticles to soluble doxorubicin (46% with soluble doxorubicin vs. 84% with doxorubicin microparticles at 1.125 μg/mL, P < 0.0001) and also after 48 hours of exposure (5% with soluble doxorubicin vs. 24% with doxorubicin microparticles at 1.125 μg/mL on day 2; P < 0.0001). These data indicate that doxorubicin microparticles kill A20 cells

Figure 1.
Doxorubicin (Dox) microparticles (MP) kill tumor cells more slowly than soluble doxorubicin and are less cytotoxic to DCs. A20 B lymphoma tumor cells (A and B) and DCs (B) were cultured for 24, 48, and 72 hours with increasing concentrations (μg/mL) of soluble doxorubicin or doxorubicin microparticles. Media or blank microparticles (equivalent weight) were used as controls. Viability was assayed by MTS. Percentage survival was expressed as the ratio of absorbance of treated cells relative to that of untreated cells (after subtracting the absorbance of the blank from each) multiplied by 100. Wells with equivalent microparticle concentrations in the absence of cells were used as blanks. Results are mean ± SEM (n = 4).
more slowly than soluble doxorubicin. Moreover, tumor cells had an average survival rate of 82% following 3 days of incubation with blank microparticles, indicating that PLGA microparticles are not toxic to tumor cells.

Upon injection into the tumor microenvironment, both tumor cells and immune cells would be exposed to doxorubicin released from degrading microparticles. Therefore, we evaluated the effect of doxorubicin microparticles and soluble doxorubicin on DCs. Doxorubicin microparticles were less cytotoxic to DCs than to A20 (24 hours of survival at 1.125 μg/mL doxorubicin—23% for A20 vs. 81% for DCs; \( P < 0.05 \)). On the other hand, soluble doxorubicin was equally cytotoxic to both (26% survival for A20 vs. 19% for DCs; not statistically significant; Fig. 1B). By 72 hours, lower concentrations of doxorubicin microparticles were still significantly more cytotoxic to A20 than to DCs (0% survival for A20 vs. 74% for DCs at 0.28125 μg/mL; \( P < 0.001 \)), whereas higher concentrations were cytotoxic to both (0% survival for A20 vs. 10% for DCs at 2.25 μg/mL; not statistically significant). This suggests that careful titration of doxorubicin microparticle doses will be important to identify the window where doxorubicin microparticles are toxic to malignant cells but not to DCs in the tumor.

**Doxorubicin microparticles are cytotoxic despite limited internalization**

To evaluate whether microparticles are internalized by cells, we used rhodamine-labeled microparticles and tracked their uptake by flow cytometry (Fig. 2A). A20 tumor cells did not internalize microparticles, which is in agreement with published reports (23). In contrast, DCs readily took up the particles even when cocultured with tumor cells. Similar results were found with doxorubicin microparticles, taking advantage of the natural fluorescence of doxorubicin (Fig. 2B; ref. 24). Internalization and cytotoxicity were not strongly linked. A20 tumor cells incubated with doxorubicin microparticles showed signs of cytotoxicity (dissolution of cellular organelles, increased chromatin clumping and nuclear fragmentation, and blebbing of nuclear and plasma membranes) despite limited internalization, whereas DCs readily took up microparticles (red arrows) but showed little toxicity (Fig. 2C). Collectively, these data show that
doxorubicin microparticles do not require internalization for their cytotoxic activity but rather release the encapsulated drug locally, which is then taken up by tumor cells. In addition, various cells in the tumor microenvironment can have different levels of sensitivity to the slow release of doxorubicin by the microparticles.

**Doxorubicin microparticles enhance phagocytosis of tumor cells by DCs**

We next evaluated whether doxorubicin microparticles enhance phagocytosis of tumor cells by DCs in a manner similar to that seen with soluble doxorubicin (7). Increasing concentrations of doxorubicin enhanced phagocytosis of A20 cells treated with both soluble doxorubicin and doxorubicin microparticles. However, doxorubicin microparticles were superior to soluble doxorubicin at all concentrations tested ($P < 0.001$; Fig. 3A). Phagocytosis was also visualized by confocal microscopy (Supplementary Fig. S2). Together, these results show that doxorubicin microparticles are superior to soluble doxorubicin in inducing phagocytosis of tumor cells by DCs.

**Doxorubicin microparticles exert similar effects to soluble doxorubicin in human cell lines**

We also evaluated the effect of doxorubicin microparticles on human cell lines using doxorubicin concentrations comparable with peak plasma concentrations achieved in patients with lymphoma (278 ng/ml at 30 mg/m$^2$ doxorubicin; ref. 25). Doxorubicin microparticles were similar to soluble doxorubicin in their killing efficiency of EBV-transformed and Raji B cells (Supplementary Fig. S3A). Doxorubicin microparticles were also similar to soluble doxorubicin at inducing the phagocytosis of EBV-transformed B cells by autologous myeloid-derived DCs (MDDC) when EBV-transformed B cells and MDDCs were simultaneously incubated with doxorubicin or doxorubicin microparticles (Supplementary Fig. S3B and S3C).

**Three-step therapy eradicates distant tumors and enhances survival**

To examine the induction of systemic immune responses, we used a two-tumor lymphoma model similar to that established by Houot and Levy (13). Mice were inoculated subcutaneously with A20 cells on both flanks, with one site used for *in situ* immunization (injection of doxorubicin microparticles) and the contralateral site observed to assess the systemic antitumor response. In this model, regression of the contralateral tumor can only be due to systemic immune responses.

We first evaluated the effect of doxorubicin microparticles alone. No mice receiving doxorubicin microparticles showed any signs of skin ulceration/necrosis even at doses as high as 100 $\mu$g of doxorubicin, confirming that the sustained release properties of the microparticles protect mice from the vesicant effect of doxorubicin. Although local tumors regressed following treatment with intratumoral doxorubicin microparticles, no systemic antitumor response was observed (as measured by regression of contralateral tumors). We then evaluated the combination of doxorubicin microparticles plus antibody therapy. Mice received a single intratumoral injection of doxorubicin microparticles and three systemic injections of anti-CTLA-4 and anti-OX40 (collectively referred to as Ab). Control group mice received intratumoral PBS with or without Ab. Initial studies revealed that systemic immune responses were not generated when doxorubicin microparticles were used at a dose of 100 $\mu$g of doxorubicin. Dose titration revealed systemic antitumor responses were generated with a lower dose of doxorubicin microparticles (2 $\mu$g; Fig. 4). In agreement with our *in vitro* data demonstrating that high doses of doxorubicin microparticles are detrimental to both tumor cells and DCs. These data also demonstrate that the systemic antitumor response is not due to systemic release of doxorubicin into the circulation, which would have resulted in a greater therapeutic effect on the contralateral tumor with higher doses of doxorubicin microparticles.

Mice treated with the optimized dose of doxorubicin microparticles (2 $\mu$g doxorubicin) combined with Ab had significantly enhanced tumor-free survival as compared with mice receiving Ab therapy only (87% vs. 67%; $P < 0.05$; Fig. 4A). This therapy generated a potent systemic immune response that eradicated most of the contralateral tumors (Fig. 4A and B). Mice that received doxorubicin microparticles + Ab and became tumor free were rechallenged with 10 million A20 tumor cells implanted subcutaneously at a different site from the microparticle-injected tumor at day 51 after tumor challenge (n = 5). These mice remained tumor free, demonstrating a long-term memory response (data not shown).

**Three-step therapy induces CD4- and CD8-dependent immune responses and requires all therapy components for maximum efficiency**

We next evaluated the contributions of the various components of therapy. Doxorubicin microparticles alone were incapable of inducing efficient immune responses, as indicated by unrestrained growth of contralateral tumors (Fig. 5A) and poor survival (Supplementary Fig. S4). Similarly, anti–CTLA-4 alone or in combination with doxorubicin microparticles was insufficient to cure contralateral tumors. Although anti-OX40 alone initially delayed tumor growth, tumors progressed with time, and survival was not enhanced beyond 30% even when combined with...
doxorubicin microparticles (P > 0.05; Supplementary Fig. S4). In contrast, all three components significantly reduced tumor growth as compared with all other groups (6 out of 8 mice became tumor-free; Fig. 5A), confirming all components are needed for maximum efficacy.

We further examined the dose of Ab used in three-step therapy (referred to as full dose) by comparing it with Ab doses that were one fourth and one sixteenth of the established dose (Supplementary Fig. S5). Efficacy was reduced with both lower doses, confirming that our established Ab dose (which is 50% of the reported dose; ref. 13) was optimal in this model.

To confirm the role of T-cell subsets in the therapeutic response, CD4 or CD8 T cells were depleted. Depletion of either CD4 or CD8 T cells abolished the therapeutic effect (Fig. 5B), confirming that the systemic antitumor effect was T cell mediated.

Three-step therapy enhances T-cell infiltration into contralateral tumors

We next evaluated the tumor microenvironment histologically 5 days after initiation of therapy (Supplementary Fig. S6). Although all tumors showed necrosis, mice that received Ab therapy had significantly more tumor necrosis than PBS control mice. Doxorubicin microparticle + Ab therapy and Ab therapy alone induced comparable necrosis, suggesting that the necrosis seen was due to antibody therapy rather than doxorubicin microparticles.

Although doxorubicin microparticles + Ab had no detectable effect on necrosis in the contralateral tumor, it did affect T-cell infiltration (Fig. 6). Mice treated with doxorubicin microparticles + Ab had an increased percentage of T cells infiltrating contralateral tumors. Although CD4 T-cell infiltration was significantly enhanced, CD8 T cells showed a trend toward enhancement. These data are in agreement with T-cell depletion data, indicating...
Three-step therapy is effective in a murine EL4 lymphoma tumor model

The ability of doxorubicin microparticles to enhance the effect of Ab therapy was also evaluated in the EL4 T-cell lymphoma model. Our prior studies demonstrated that microparticles containing higher doses of doxorubicin are needed to effectively treat EL4, and that EL4 grows too rapidly for a two-sided model to be valuable (26). We therefore evaluated the effect of three-step therapy on EL4 by treating a single tumor with doxorubicin microparticles at a dose of 25 μg of doxorubicin. Using this approach, doxorubicin microparticles + Ab significantly reduced EL4 tumor burdens and resulted in a 50% rate of long-term survival. None of the mice receiving Ab therapy alone survived (Supplementary Fig. S11).

Discussion

With the growth of scientific insight into pathways that regulate the immune system and cancer, we can now more intelligently design and combine immunotherapies that work in different ways to overcome deficiencies of single therapies (27). One attractive approach is to use in situ therapy with microparticles to manipulate the tumor microenvironment in a manner that breaks tolerance and allows development of a robust immune response. The number of variables that needs to be evaluated when trying to optimize the promise of such a multistep approach is considerable. Here, we address many of these variables, and demonstrate that this approach has promising immunologic and therapeutic effects.

An ideal in situ immunization approach would deliver localized and effective drug concentrations into the tumor with low systemic toxicity (23). Given its long history of FDA-approved use for biomedical applications and its biocompatibility (28), PLGA was seen as an excellent candidate polymeric vehicle for controlled release of doxorubicin from microparticles into the tumor.

We first optimized formulation parameters affecting particle size, loading, and release. One-μm PLGA microparticles has been shown to be more effective than 200-nm, 500-nm, and 5-μm particles as vaccine adjuvants (29). The relatively low dose of doxorubicin microparticles, coupled with their sustained release, proved to be a safe combination for DCs that survived well in vivo and in situ despite internalizing the particles.

A20 tumor cells were killed more slowly by doxorubicin microparticles than by soluble doxorubicin, as previously seen with chemotherapy-loaded PLGA particles (23). However, doxorubicin microparticles resulted in more efficient phagocytosis. Doxorubicin microparticles and soluble doxorubicin were compared based on equivalent total amounts of doxorubicin. However, at any given time point and at equivalent “doses,” the amount of doxorubicin released by doxorubicin microparticles was likely lower than that of soluble doxorubicin. As such, one explanation for why doxorubicin microparticles were superior to

Figure 5.

Three-step therapy induces CD4- and CD8-dependent immune responses and requires all therapy components for maximum efficiency. A, mice (7–8/group) were treated and observed in a manner similar to that described in Fig. 4, except that different combinations among the three therapy components were used to observe the contribution of each. Tumor areas are mean ± SEM. B, mice (9–13/group) were treated and observed as before. Treatments consisted of PBS as control or doxorubicin (Dox) microparticles (MP) + Ab. Mice receiving three-step therapy additionally received multiple injections of either anti-CD4, anti-CD8, or both according to the schematic shown.
soluble doxorubicin is that the exposure of malignant cells to a lower doxorubicin concentration enhanced the expression of calreticulin as compared with the bolus dose of soluble doxorubicin (30). Because of technical difficulties associated with doxorubicin microparticles adhering to tumor cells, we were unable to demonstrate that doxorubicin microparticles enhanced calreticulin expression by tumor cells. Shurin and colleagues (31) have shown that ultra-low concentrations of doxorubicin regulate the activity of small Rho GTPases that control the endocytic activity of DCs. Thus, it is also possible that doxorubicin microparticles adherent to doxorubicin microparticle–treated A20 cells may be contributing to the enhanced phagocytosis by exposing DCs to very low concentrations of doxorubicin.

Three-step therapy was superior to Ab therapy in inducing curative immune responses. The depletion of CD4 and CD8 T cells abrogated the therapeutic immune response, indicating that it was T cell mediated. Several studies have confirmed the role of CD8 T cells in doxorubicin-mediated antitumor immune responses (32, 33) and of both CD4 and CD8 T cells in anti–CTLA-4– and anti-OX40–mediated immune responses (13, 34, 35). Thus, the finding that both CD4 and CD8 cells are needed for an optimal antitumor effect in our studies was not surprising.

To investigate how the addition of doxorubicin microparticles to Ab therapy is modulating the intratumoral T-cell response, we examined the Treg population and found no effect of three-step therapy on the percentage of Tregs. On the other hand, we found enhanced CD4 T-cell infiltration in contralateral but not injected tumors. These results suggest changes induced by therapy enhanced the ability of CD4 T cells to contribute to the immune response and overcome effects of Tregs.

The combination of doxorubicin microparticles, anti–CTLA-4, and anti-OX40 was required for the most efficient immune response. Given that doxorubicin microparticles alone were incapable of generating immune responses and the combination of anti–CTLA-4 and anti-OX40 without doxorubicin microparticles was not as efficient at reducing tumor burden as three-step therapy, it is likely that anti-OX40 is amplifying the primed T-cell response generated with doxorubicin microparticles and that anti–CTLA-4 is allowing for that response to be maintained. This contention is supported by enhanced T-cell infiltration in tumors following the three components as compared with Ab therapy. The increase in pathologically detectable destruction of established tumors, as reflected by tumor necrosis, was seen with both the three-step therapy and Ab therapy. Quantifying necrosis
Three Steps to Breaking Immune Tolerance: Microparticle Approach

Figure 7.
A systemic immune response is generated through local tumor manipulation—a proposed schematic. Doxorubicin (Dox) microparticles (MP) injected intratumorally upregulate surface calreticulin expression on dying tumor cells, enhancing their phagocytosis by DCs that migrate to draining lymph nodes and present tumor antigen to antigen-specific T cells. Anti-OX40 enhances T-cell activation while anti–CTLA-4 blocks immunosuppression imposed by CTLA-4, thus allowing tumor-specific T cells to proceed unrestrained to distant tumor sites.

Within a tumor sample is difficult, and it was not possible to determine definitively whether three-step therapy enhanced necrosis; however, the improved overall outcome suggests this is the case. Similar responses were seen in tumor samples from patients receiving immunotherapy, and may thus be reflective of the ongoing antitumor immune response (36).

We also validated the efficiency of three-step therapy in the EL4 tumor model. There are clear differences between mouse models of malignancy such as A20 or EL4 and human tumors. An example is potential retroviral contamination of cell lines that could serve as target antigens. Irrespective of the target antigen or immunogenicity of the model, our studies demonstrate that in situ treatment with doxorubicin microparticles can enhance the immunotherapeutic effects of immunostimulatory and checkpoint blockade antibodies. Further studies will be needed to assess the efficacy of our design in other tumor models in mice, and eventually in clinical trials.

The three-step design is complex, and the number of agents that could be evaluated for each step is extensive. Other approaches, such as local radiation, could be used to induce local immunogenic cell death (37). Anti-OX40 can be substituted with other immunostimulatory antibodies targeting TNFR costimulatory molecules, including CD40 and CD137 or TLR agonists such as CpG (37). Anti–CTLA-4 can be substituted with other antibodies that mediate checkpoint blockade such as anti-PD-1 (38). Alternatives related to the dosing and timing of how these agents could be used together lead to an almost endless number of possible combinations. The studies reported here highlight the value of the three-step approach rather than demonstrate that the specific agents or regimen used is superior to other regimens.

Indeed, given that doxorubicin microparticles are not clinically approved, a faster translation to the clinic could require approximation of our design using readily available reagents such as liposomal doxorubicin. In preliminary studies, we found doxorubicin microparticles were more effective than liposomal doxorubicin in inducing systemic immune responses in mice (data not shown), and this is why we used them, PLGA particles were shown to be superior to liposomal formulations in inducing cellular immune responses, which was attributed to their sustained release rather than an adjuvant effect of the synthesizing material (39). Indeed, liposomal doxorubicin is currently under consideration for a clinical trial exploring a combination similar to that outlined in this report. Although liposomal doxorubicin would provide a more direct path toward clinical evaluation, studies comparing doxorubicin microparticles with liposomal doxorubicin in human cell lines would also be informative.

In conclusion, recent advances in our understanding of cancer immunotherapy suggest that rational combined approaches will be keys to enhancing efficacy. We evaluated a three-step approach to in situ immunization using biodegradable doxorubicin microparticles, anti–CTLA-4, and anti–OX40 (Fig. 7). Further preclinical evaluation of this promising therapeutic strategy in other types of cancer is ongoing, as are plans to translate these findings to the clinic.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
The authors thank Scott Coury for help with tumor freezing, Justin Fishbaugh of the Flow Cytometry Facility, and Chantal Allamargot and Katherine Walters of Central Microscopy Research Facility.

Grant Support
This work was supported by grants from the National Cancer Institute at the NIH (P50 CA97274/A1i Mayo Clinic Lymphoma SPORE grant and P30 CA68682 Cancer Center support grant).

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Received September 17, 2014; revised December 23, 2014; accepted January 12, 2015; published OnlineFirst January 27, 2015.
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