Biodegradable Nanoparticles for In Situ Immunization against Lymphoma

1. Background and significance

1.1 In Situ Immunization: a 3-step approach for inducing immunity

Non-Hodgkin’s lymphoma comprises 4.2% of all cancers in the United States. In 2013, it was estimated that there would be over 69,000 new cases of non-Hodgkin’s lymphoma and that over 19,000 people would die of this cancer [1]. Innovative immunotherapeutic approaches aimed at harnessing the patient’s immune system against their cancer are thus being tested at a fierce pace.

The goal of cancer immunotherapy is to overcome immunological tolerance to tumor antigens and generate potent immune responses in the form of effector CD4 and CD8 T cells [2]. Among various immunotherapy approaches, in situ immunization is attractive because it utilizes the patient’s own tumor antigens by inducing tumor cell death in situ. This provides dendritic cells (DCs) with a wide selection of tumor antigens to be presented to antigen-specific T cells [3].

A 3-step approach to in situ immunization tackles all aspects of generating a potent, long-lasting anti-tumor response. First, agents that induce tumor cell death provide tumor antigen to DCs. Second, agents that enhance T cell activation provide optimal conditions for DCs when presenting tumor antigens to T cells. Finally, agents that overcome immunosuppression allow the activated T cell response to proceed unrestrained [3].

1.2 Doxorubicin, anti-OX40 and anti-CTLA-4

To enhance tumor antigen uptake by DCs, Doxorubicin (Dox) is the ideal candidate given that it is classical chemotherapy for lymphoma patients [4]. A DNA intercalating agent that inhibits DNA replication, Dox also induces immunologic cell death i.e. cell death which stimulates an immune response. This is achieved by inducing the surface expression of calreticulin, an “eat-me” signal that enhances the phagocytosis of dying tumor cells by DCs [5-7].
Following phagocytosis, DCs present tumor-derived antigens to antigen-specific CD4 and CD8 T cells. In order for T cells to be activated, they must also receive a co-stimulatory signal, most notably through OX40 which augments T cell function and survival [8-10]. The second step can thus be achieved through a stimulatory antibody that activates OX40 (α-OX40).

Activated T cells must now overpass the final obstacle of remaining activated to exert their anti-tumor activity [9]. 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 reduces T cell proliferation and activity [11]. In addition, regulatory T cells (Tregs), a suppressive subtype of T cells, employ CTLA-4 to suppress effector T cells [12]. It thus becomes crucial to include checkpoint blockade as a final step of in situ immunization. This is achieved via an antibody that blocks the inhibitory activity of CTLA-4 on both effector T cells and Tregs (α-CTLA-4).

The combination of α-CTLA-4 and α-OX40 has been shown to enhance immune responses against lymphoma [13]. Given that α-CTLA-4 and α-OX40 are delivered systemically, half of the published doses were used to reduce systemic toxicity. Thus, a 3-step approach to in situ immunization would generate systemic immune responses that can target distant tumor sites through local manipulation of a single tumor site.

1.3 The need for biodegradable nanoparticles for delivering Doxorubicin

While the use of Dox to induce immunogenic cell death is very attractive for in situ immunization, it is not feasible due to the risk of its extravasation (leakage into subcutaneous tissues) following injection into the tumor. Dox is one of the most vesicant (skin-blistering) agents when extravasated, resulting in a very high risk for ulcers and tissue necrosis [14]. This represents a critical need for delivering Dox intratumorally while avoiding its serious side effect.
Poly(lactide-co-glycolide) or PLGA is an FDA-approved polymer that is medically used in biodegradable surgical sutures. It is also used to synthesize nanoparticles (NPs) which, when loaded with drugs, degrade slowly and provide sustained release of the drug [15]. Thus, PLGA NPs loaded with Dox (Dox NPs) represent a safe approach for delivering Dox into tumors, as the slow, intratumoral release of Dox would not result in vesication. In addition, PLGA NPs are effective vaccine adjuvants, enhancing antigen-specific immunity by activating DCs [16].

1.4 Significance

Formerly, insufficient understanding of the immune system has been a barrier to advancing immunotherapy for lymphoma. With the immense scientific insight into the pathways that regulate the immune system and cancer, we can intelligently design and combine immunotherapies that work in different ways to overcome deficiencies of single therapies [17]. More importantly, our designed therapy is not limited to lymphoma and may potentially pave the way for improved responses and cures in patients with other types of cancer.

2. Objectives

The main goals of this study are to characterize the activity of Dox NPs in vitro against lymphoma tumor cells and DCs and to evaluate the anti-tumor immune responses induced in vivo using our 3-step design. We hypothesize that a 3-step approach to in situ immunization (Dox NPs given intratumorally combined with systemic α-CTLA-4 and α-OX40) can enhance phagocytosis and elicit a systemic anti-tumor immune response (Figure 1). To test this hypothesis, we propose:

2.1 To characterize the activity of Dox NPs in lymphoma tumor cells and DCs in vitro

2.2 To investigate the induction of curative systemic anti-tumor immune responses in mice treated with the 3-step therapy.
3. **Experimental design and methods**

3.1 **Specific Aim 1: To characterize the activity of Dox NPs in tumor cells and DCs in vitro**

Since Dox NPs are injected into the tumor where there are infiltrating immune cells (most importantly DCs), it is crucial to evaluate the cytotoxic activity of Dox NPs against both tumor cells and DCs populations. To do this *in vitro*, a lymphoma tumor cell line (A20) and DCs cultured from the bone marrow of mice were used. Furthermore, the internalization of Dox NPs into either cell type was examined. Lastly, the ability of Dox NPs to enhance phagocytosis of A20 lymphoma tumor cells by DCs was assessed.

3.1.1 **Preparation and characterization of Dox NPs**

Doxorubicin nanoparticles (Dox NPs) were prepared using the double emulsion solvent evaporation method. Briefly, the water phase (doxorubicin hydrochloride dissolved in 1% poly(vinyl alcohol)) was sonicated in the oil phase (PLGA in dichloromethane). The resulting emulsion was poured into 1% PVA solution to form the double emulsion. Dox NPs were collected by centrifugation, washed, frozen and lyophilized. Blank nanoparticles (Blank NPs) were prepared in the same manner as Dox NPs with the omission of Dox. Dox NPs at loadings of 13 µg/mg were used in all experiments except for MTS assays (8 µg/mg).

3.1.2 **Evaluating the cytotoxicity of Dox NPs in tumor cells and DCs in vitro**

The A20 cell line, a BALB/c B cell lymphoma, was purchased from ATCC (Manassas, VA). Tumor cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 50 µM 2-ME, as complete medium.

To generate bone-marrow-derived DCs, bone marrow cells were flushed from the tibias and femurs of BALB/c mice with complete medium, and mononuclear cells were isolated using Ficoll gradient separation (Fico/Lite-LM, Atlanta Biologicals, Flowery Branch, GA). Cells were
cultured in medium supplemented with 20 ng/ml each GM-CSF and IL-4 (PeproTech, Rocky Hill, NJ) for 6 days to enrich for DCs. After 6 days, the non-adherent cells were harvested and used. Cells were > 70% DCs as determined by CD11c staining.

**Viability assay**

To determine the cytotoxic activity of Dox NPs against A20 and DCs, an MTS assay for viability was conducted (Promega, Madison, WI). Briefly, 5 x 10^3 A20 cells and DCs each were separately incubated with Dox NPs in 96-well plates for 24, 48 and 72 hr (4 wells per group) at final Dox concentrations ranging 0.28125-4.5 µg/ml. Blank NPs of equivalent weights were used as negative controls. The MTS/PMS reagent was then added for 4 hr at 37°C. Absorbance was read at 490 nm using Thermomax Microplate Reader (Molecular Devices, Sunnyvale, CA). Percent 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 NP concentrations in absence of cells were used as blanks.

**3.1.3 Evaluating the uptake of Dox NPs in tumor cells and DCs in vitro**

To assess the uptake of Dox NPs, transmission electron microscopy was utilized. Briefly, A20 and DCs were incubated for 24 hr with Dox NPs (1 µg/ml) or blank NPs (equivalent weight) as control. Following 24 hr incubation, the cells were washed, fixed, embedded in Epon resin, and sectioned. Ultrathin sections were counterstained with uranyl acetate and lead citrate. TEM images were taken by JEOL JEM-1230 transmission electron microscope provided with Gatan UltraScan 1000 2k x 2k CCD camera (JEOL USA, Inc., Peabody, MA).

**3.1.4 Evaluating the induction of phagocytosis of lymphoma tumor cells by Dox NPs**

To quantitate phagocytosis, A20s were first labeled with CellTrace Violet (Invitrogen, Carlsbad, CA), a DNA-dye that is compatible with Dox fluorescence. After washing, labeled
A20 were left untreated or treated with Dox NPs for 48 hr (3 wells per group) at final concentrations ranging 0.1-9 µg/ml. Treated A20 were washed and co-incubated with DCs at a 1:1 ratio for 2 hr to allow for phagocytosis. The cell mix was then stained with anti-CD11c-APC-Cy7 (Becton Dickinson, Franklin Lakes, NJ). Data was acquired on a LSR II flow cytometer (Becton Dickinson) and analyzed using FlowJo Software (TreeStar, Ashland, OR). As controls, soluble Dox (at the same concentration) and blank NPs (at the equivalent weight) were used. The percentage of double-positive cells (CD11c and CellTrace Violet) reflects phagocytosis.

3.2 Specific Aim 2: To investigate the induction of curative systemic anti-tumor immune responses in mice treated with the 3-step therapy

To examine the induction of systemic antitumor immune responses through local *in situ* immunization, the two-tumor lymphoma model was used [13]. Tumor cells were injected subcutaneously on opposite sides of the animal, with one site used for *in situ* immunization (injection of Dox NPs) and the contralateral site observed to assess systemic immune responses.

**Two-tumor lymphoma model**

Mice (BALB/c females, 6-8 weeks old) were purchased from Harlan Laboratories (Indianapolis, IN). All animal protocols used in these studies were approved by the Institutional Animal Care and Use Committee at the University of Iowa and complied with NIH Guidelines.

For tumor challenge, mice were subcutaneously implanted with A20 at a dose of 7-8 × 10^6 cells in 100 µL sterile PBS in the right and left flanks. Treatment began when tumors reached 5-7 mm in largest diameter, which typically occurred at days 6 to 11 after tumor inoculation.

3.2.1 Evaluating the efficacy of 3-step therapy in inducing systemic immunity

Dox NPs (2 µg Dox in 100 µL PBS) were injected into the left tumor. Blank NPs (equivalent weight) or PBS (100 µL) were given to control groups. Antibodies were administered by
intraperitoneal injections of three doses given every 3-4 days, starting from Day 1 of treatment. Anti-CTLA4 (hamster IgG, clone UC10-4F10-11) and anti-OX40 (rat IgG1, clone OX86) were purchased from BioXCell (West Lebanon, NH) and given at half the published doses [13]: 50 μg for α-CTLA4 and 200 μg for α-OX40 per injection (collectively referred to as Ab). 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 diameter in any direction.

3.2.2 Evaluating the efficacy of 3-step therapy over single therapies

Seven groups (7-8 mice per group) were used: PBS, PBS+α-CTLA-4, PBS+α-OX40, Dox NP, Dox NP+α-CTLA-4, Dox NP+α-OX40 and Dox NP+α-CTLA-4+α-OX40. Mice were treated and monitored as previously mentioned.

All statistical analyses were performed using GraphPad Prism software, version 5.00 (San Diego, CA). Data were analyzed using one or two way analysis of variance (with a Bonferroni post test). Analysis of survival curves was performed using log-rank (Mantel-Cox) test.

4. Results

4.1 Specific Aim 1: To characterize the activity of Dox NPs in tumor cells and DCs in vitro

4.1.1 Dox NPs are more cytotoxic to lymphoma tumor cells than to DCs

Upon injection into the tumor microenvironment, both tumor cells and immune cells are exposed to Dox released from degrading NPs. Therefore, it is vital that Dox NPs are not toxic to DCs. Using A20 (a B-lymphoma cell line syngeneic with BALB/c mice) and DCs derived from the bone marrow of BALB/c mice, we found that increasing concentrations of Dox NPs led to a dramatic decrease in A20 viability that was significant within 24 hr of incubation with Dox NPs (Figure 2A). This indicates that Dox NPs are cytotoxic to A20. More importantly, Dox NPs were less cytotoxic to DCs than to A20, as reflected by their higher % survival that was significant
even at 24 hr incubation (23% survival for A20 versus 81% for DCs at 1.125 µg/ml Dox; p < 0.001). By 72 hr, lower concentrations of Dox were still significantly more cytotoxic to A20 than DC (29% survival for A20 versus 74% for DCs at 0.5625 µg/ml Dox; p < 0.001), while higher concentrations were detrimental to both (0% survival for A20 versus 10% for DCs at 2.25 µg/ml Dox; not significant). This merits careful titration of Dox NP doses to be injected, as high amounts can impair DCs that are crucially needed to induce the immune response.

4.1.2 Dox NPs do not require internalization by tumor cells for their cytotoxic activity

To evaluate whether Dox NPs require internalization for their cytotoxic activity, A20 and DCs were incubated with blank NPs as control or Dox NPs for 24 hr, and uptake was assessed by TEM (Figure 2B). While blank NPs were internalized by DCs (in agreement with their phagocytic nature), they were not taken up by A20. However, this does not hinder their cytotoxic activity, since A20 incubated with Dox NPs showed signs of cytotoxicity (dissolution of cellular organelles, increased chromatin clumping and nuclear fragmentation, and blebbing of nuclear and plasma membranes). Conversely, DCs readily took up Dox NPs but were less susceptible to their cytotoxic activity (no differences between DCs exposed to blank NPs or Dox NPs).

4.1.3 Dox NPs enhance phagocytosis of tumor cells by DCs

It has already been established that Dox enhances surface calreticulin expression by tumor cells and their phagocytosis by DCs [7]. To evaluate whether Dox NPs are similar to Dox (referred to as soluble Dox), A20 were labeled with CellCycle Violet then left untreated or treated with soluble Dox, blank NPs or Dox NPs for 48 hr. Cells were washed, co-incubated with DCs (1:1 ratio) for 2 hr to allow for phagocytosis, stained for CD11c (a surface marker for DCs) and evaluated by flow cytometry (Figure 3). CD11c⁺ cells that are also CellCycle Violet⁺ reflect phagocytosis. We found that increasing concentrations of Dox enhanced phagocytosis of A20 by
DCs in both (soluble Dox) and (Dox NP) groups. More importantly, Dox NPs were superior to soluble Dox in inducing phagocytosis at all concentrations utilized (p < 0.001), indicating that Dox NPs are fully capable of substituting soluble Dox for inducing phagocytosis of tumor cells.

4.2 Specific Aim 2: To investigate the induction of curative systemic anti-tumor immune responses in mice treated with the 3-step therapy

4.2.1 3-step therapy induces systemic anti-tumor responses that eradicate tumors

To examine systemic antitumor immune responses induced by local in situ immunization, the two-tumor lymphoma model was used in mice. The principle is that regression of the contralateral tumor can only be due to systemic immune responses induced at the treated tumor. Mice were inoculated subcutaneously with A20 cells on both flanks, and only one tumor received a single intratumoral (i.t.) injection of Dox NPs at a very low dose (2 µg). Mice also received three systemic injections of α-CTLA4 and α-OX40 (referred to as Ab) at half the published doses [13] to complete 3-step therapy (Dox NP+Ab). All of the mice receiving 3-step therapy survived and completely eradicated their contralateral tumors (Figure 4A). This is significantly different from control mice receiving only i.t. PBS, which succumbed to tumors within 30 days (p = 0.001). Interestingly, Ab alone (without Dox NP) or combined with blank NPs induced strong immune responses as well that led to 80% and 90% survival, respectively.

4.2.2 Dox NPs, α-OX40 and α-CTLA-4 are all required for maximum efficacy

To evaluate which of the three components is essential for efficacy, we administered antibodies individually with i.t. PBS or Dox NPs. We also injected i.t. Dox NPs without antibodies (Figure 4B), and found that Dox NPs alone are incapable of inducing efficient immune responses, as is deduced from the unrestrained growth of tumors and poor survival. Similarly, α-CTLA-4 alone or in combination with Dox NPs was insufficient to cure distal
tumors. While $\alpha$-OX40 initially delayed tumor growth, tumors progressed with time and survival was not enhanced beyond 30% even when combined with Dox NPs ($p > 0.05$). In contrast, the 3-step therapy significantly reduced tumor growth as compared to all other groups (6 out of 8 mice became tumor-free) and enhanced survival to 75% ($p = 0.0151$). This establishes the requirement for all three components of the therapy for maximum efficacy.

5. Conclusions

In this study, we designed a three-step approach to in situ immunization using biodegradable Dox NPs, $\alpha$-CTLA-4 and $\alpha$-OX40. We show that Dox NPs are less cytotoxic to DCs than to A20 lymphoma cells and do not require internalization. Moreover, Dox NPs enhanced phagocytosis of tumor cells by DCs. When injected into the tumor and combined with systemic $\alpha$-CTLA-4 and $\alpha$-OX40 at reduced doses, Dox NPs were able to induce systemic immune responses that eradicated distant tumors and improved survival. Finally, all three members of the designed approach were crucial for maximum immunological efficacy. Our findings demonstrate that antitumor immune responses elicited by the 3-step therapy can eradicate tumor cells at distant sites and merit further investigation into its use as an immunotherapy for lymphoma patients.

6. Future work

Further experiments are needed to fully characterize the immune response generated by the 3-step therapy and the immune effectors that deliver it. Preliminary results indicate that the combination therapy, but not Ab alone, induces significant inflammatory infiltration into distant tumors. Ongoing experiments are aiming at characterizing the number of infiltrating immune cells, their type, and their activity over time. Furthermore, the dependence of the generated immune response on effector CD4 and CD8 T cells will be tested through depletion studies.
Figure 1. A systemic immune response is generated through local tumor manipulation. Dox NPs injected intratumorally upregulate surface calreticulin expression on dying tumor cells, enhancing their phagocytosis by DCs which migrate to draining lymph nodes and present tumor antigen to antigen-specific T cells. α-OX40 enhances T cell activation while α-CTLA-4 blocks immunosuppression imposed by CTLA-4, thus allowing tumor-specific T cells to proceed unrestrained to distant tumor sites.

Figure 2. 2A. Dox NPs are less cytotoxic to DCs than to tumor cells. A20 and DCs were cultured for 24, 48 and 72 hr with increasing concentrations of Dox NPs (8 µg/mg). Viability was assayed by MTS. Results are mean ± SEM (n = 4). Statistical analysis is shown for Day 3. 2B. Dox NPs do not require internalization by tumor cells for their cytotoxic activity. A20 and DCs were cultured for 24 hr with blank NPs or Dox NPs (1 µg/ml). Cells were washed, fixed and analyzed by TEM.
Figure 3. Dox NPs enhance phagocytosis of tumor cells by DCs. A20 were labeled with CellCycle Violet then left untreated or treated with soluble Dox, blank NPs or Dox NPs for 48 hr. Cells were washed, co-incubated with DCs (1:1 ratio) for 2 hr, stained for CD11c and evaluated by flow cytometry. Results are mean ± STD (n = 2).

Figure 4. 4A. 3-step therapy eradicates distant tumors and enhances survival. Eight million A20 were injected subcutaneously into each flank of BALB/c mice (10/group). After six days, left-side tumors were injected with PBS, Blank NPs (equivalent weight) or Dox NPs (2 µg Dox). Mice also received 3 intraperitoneal injections of α-CTLA-4 (50 µg) and α-OX40 (200 µg) over 10 days (collectively referred to as Ab). Mice were observed for tumor growth and survival. Tumor areas are mean ± SEM. 4B. Dox NPs, α-CTLA-4 and α-OX40 are all required for maximum efficiency. Mice (7-8/group) were treated and observed similarly to 4A except that different combinations among the three therapy components were used to observe the contribution of each.
References


