Targeted Therapeutic Remodeling of the Tumor Microenvironment Improves an HER-2 DNA Vaccine and Prevents Recurrence in a Murine Breast Cancer Model

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Abstract
The tumor microenvironment (TME) mediates immunosuppression resulting in tumor cell escape from immune surveillance and cancer vaccine failure. Immunosuppression is mediated by the STAT3 transcription factor, which potentiates signaling in tumor and immune cells. Because immunosuppression continues to be a major inhibitor of cancer vaccine efficacy, we examined in this study whether therapeutically targeted delivery of a synthetic STAT3 inhibitor to the TME, combined with an HER-2 DNA vaccine, prevented HER-2+ breast cancer and prevent its recurrence. To this end, we developed a novel ligand-targeted nanoparticle (NP) encapsulating a CDDO-Im payload capable of specific delivery to the TME, which showed an effective therapeutic inhibition of STAT3 activation in primary tumors. Furthermore, we showed that treatment with these NPs resulted in priming of the immune TME, characterized by increased IFN-γ, p-STAT1, GM-CSF, IL-2, IL-15, and IL-12b and reduced TGF-β, IL-6, and IL-10 protein expression. In addition, we found significantly increased tumor infiltration by activated CD8+ T cells, M1 macrophages, and dendritic cells. These changes correlated with delayed growth of orthotopic 4T17 breast tumors and, when combined with an HER-2 DNA vaccine, prevented HER-2+ primary tumor recurrence in immunocompetent mice. Furthermore, antitumor T-cell responses were enhanced in splenocytes isolated from mice treated with this combination therapy. Together, these data show effective protection from cancer recurrence through improved immune surveillance against a tumor-specific antigen.

Introduction
The tumor microenvironment (TME) is a key mediator of solid tumor growth. Tumor and stromal cells, including immune cells, mediate disease progression by secreting factors promoting angiogenesis and tumor cell proliferation and survival. In addition, through cytokine production and release, these cells create an immunosuppressive TME that facilitates tumor cell escape from clearance by the immune system, thus mediating tumor maintenance. The role of immunity in cancer was shown by studies showing that chemically induced carcinomas in mice require an inflammatory response. For example, Rag-1-/- mice, lacking natural killer, B, and T cells, developed sarcomas faster and more frequently following methylcholanthrene injection than wild-type mice with intact immune systems. Similar results were reported in mice with functional ablation of natural killer cells, γδ T cells, αβ T cells, IFN-γ, or interleukin (IL) 12. In humans, immunosuppression of transplant patients and disease-associated immunodeficiencies correlated with significantly higher risk for cancer development. Furthermore, clinical studies have associated chronic inflammation with increased risk of colon and breast cancers. These studies indicate that immunity plays a major role in cancer, in both mice and humans.

The STAT3 transcription factor, a key regulatory molecule in cancer immunity, is a potent suppressor of T helper 1 (Th1) cell-mediated inflammation, which is essential for antitumor immune responses. STAT3 inhibits dendritic cell (DC) maturation and macrophage function by suppressing their expression of antigen presentation and costimulatory molecules. Furthermore, STAT3 signaling promotes proliferation and survival of regulatory T cells that inhibit CD8+ T-cell
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responses (8, 9). Conversely, disruption of STAT-3 in macrophages and DCs restored their ability to present antigens and prime naive antigen-specific T cells (10).

Synthetic triterpenoids are a class of multifunctional inhibitors shown to suppress solid tumor growth through inactivation of STATs (11). In particular, the imidazole derivative of the synthetic oleanane triterpenoid (CDDO-Im) was reported to inhibit STAT-3 phosphorylation at Y705 (11), which is critical for STAT-3 transcriptional activity (6). Importantly, CDDO-Im inhibits STAT-3 activation at nanomolar concentrations, thus making it an attractive compound for use in cancer therapy.

Our laboratory recently developed a novel nanoparticle (NP) targeting strategy utilizing legumain as a functional target for the TME (12). Legumain, an asparaginyl endopeptidase, is overexpressed on tumor cells under hypoxic stress (12), a hallmark of solid tumors, and on tumor-associated macrophages (13, 14). We showed that legumain targeting dramatically improved NP drug delivery to solid tumors whereas preventing nonspecific accumulation in the reticuloendothelial system (12). Therefore, we hypothesized that combination therapy inhibiting STAT-3 specifically in the TME by targeted NP delivery of CDDO-Im would improve the effects of a tumor-specific vaccine and prevent cancer recurrence. In this study, we describe a novel loading strategy to encapsulate CDDO-Im into legumain-targeted NPs for delivery to the TME in vivo and delineate the consequences of TME-specific STAT-3 inactivation on tumor growth in murine models of breast cancer. Finally, we show that this novel combination therapy improved the antitumor effects of an anti-HER-2 DNA vaccine and elucidate the mechanism responsible for enhanced protection against HER-2+ breast cancer recurrence.

Materials and Methods

Animals and cell lines

BALB/c mice were purchased from The Scripps Research Institute (TSRI) Rodent Breeding Facility, FVB/NJ mice from Charles River Laboratories, and housed in our AAALAC-accredited facility. Animal protocols, approved by TSRI Animal Care Committee, were conducted according to NIH Guides for the Care and Use of Laboratory animals.Authenticated 4T07/4T1 murine breast carcinoma cells were provided by Suzanne Ostrand-Rosenberg (University of Maryland, College Park, MD) and maintained in RPMI-1640 medium (Gibco) supplemented with 10% FBS, 1% HEPES, 1% sodium bicarbonate, and 1% sodium pyruvate. Cell lines are authenticated by MycoAlert (2008; Lonza). MMTV-Neu primary tumor was provided by Michael Karin (University of California, San Diego, CA) and maintained by serial passage in syngeneic FVB/NJ mice. Briefly, MMTV-Neu primary tumors were minced and digested under sterile conditions with Type 3 collagenase (Worthington) in RPMI-1640 medium supplemented with 2.5% FBS and 10 mmol/L HEPES. Cells (1 × 106) were resuspended in PBS and injected into the mammary fat pad of syngeneic female FVB/NJ mice. This procedure was repeated once primary tumors reached a size of approximately 500 mm3.

Nanoparticle formulation

Synthesis of the legumain-specific inhibitor RR-11a was previously described (15). Phospholipids (Avanti Polar Lipids) were dissolved in chloroform. RR-11a was conjugated to 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), as previously described (12). The resulting compound was combined with 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), DOPE, cholesterol, and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000], DOPE-PEG, at molar ratios of 1.16:7.6:7:2:2:1, as previously described (16). CDDO-imadazolide (CDDO-Im), provided by Michael Sporn (Dartmouth Medical School, Hanover, NH), was added to the lipids prior to rotoevaporation. Unencapsulated CDDO-Im was removed by ultrafiltration using 100 nm pore size polycarbonate filters. Size distributions and zeta potentials were determined by dynamic light scattering on a Zetasizer Nano (Malvern) and transmission electron microscopy (TEM) carried out, as previously described (17). Loaded concentrations of CDDO-Im were determined by NP sonication in 2% Tween and UV spectrometry analysis.

Western blotting

Protein extracts were prepared as previously described (18). Western blots were probed with the following antibodies: rabbit anti-phospho-STAT-3 (Cell Signaling); goat-anti-β-actin, IL-6, and IL-10, rabbit-anti-phospho-STAT-1, STAT-3, IL-2, Bcl-xL, Bcl-2, and TGF-β, rat-anti-IL-12b, granulocyte macrophage colony-stimulating factor (GM-CSF), IFN-γ, and mouse-anti-IL-15 (all Santa Cruz Biotechnology); and anti-EBRB2 (Abcam). Protein band intensities were quantified using ImageJ software and normalized to β-actin.

In vivo tumor studies

4T07 (5 × 104) cells or MMTV-Neu (1 × 105) primary tumor cells were injected orthotopically into female BALB/c or FVB/NJ mice, respectively. NPs in 200 µL of PBS (~1.36 × 1013 particles) were administered intravenously and tumor dimensions measured using digital microcalipers. Tumor volume was calculated using the formula \(\frac{a^2b}{2}\), where \(a\) is the larger of 2 perpendicular diameters. For recurrence studies, primary tumors were surgically removed and mice rechallenged orthotopically in the contralateral mammary fat pad. Mice were vaccinated 3 times orally at 1-week intervals by gavage with attenuated Salmonella typhimurium (1 × 108 colony-forming units per mouse) transduced with either pNeuTm (provided by Wei-Zen Wei, Karmanos Cancer Center, Detroit, MI) or empty vector, as previously described (19).

Flow cytometry

Splenocytes and tumor-infiltrating lymphocytes were isolated, as previously described (19) and incubated (1 × 106 cells per tube) with fluorescein-conjugated antibodies (0.25 µg antibody per 106 cells in 100 µL volume) against mouse CD8, CD25, CD14, CD11c, CD11b, CD80, CD45, F4/80 (Biolegend) and/or
granzyme B (0.125 μg antibody per 10⁶ cells in 100 μL volume; eBioscience). Data were collected on a digital LSR-II (Becton Dickinson) and analyzed with Flowjo software (Tree Star, Inc.).

Immunohistochemistry

Tumor sections fixed in acetone were stained with the following primary antibodies: rat anti-mouse F4/80 (1:50 dilution; AbD Serotec) and rabbit anti-mouse Nos2 (1:50 dilution; Santa Cruz Biotechnology) and detected with the following secondary antibodies: goat anti-rat IgG Alexa Fluor 568 or goat anti-rabbit IgG Alexa Fluor 488 (both at 1:200 dilution; Molecular Probes), respectively. For staining controls, tissue sections were incubated with secondary antibodies only. Cell nuclei were stained with DAPI dilactate (Sigma).

Statistical analysis

Statistical significance, set at \( P < 0.05 \), of differential findings between experimental groups was determined by 2-tailed Student’s \( t \) test using Prism software (GraphPad).

Results

Formulation of targeted nanoparticles encapsulating CDDO-Im

In the present study, we employed a novel strategy to load CDDO-Im into legumain-targeted NPs (12). We capitalized on the physical characteristics of CDDO-Im, namely, its hydrophobicity and chemical similarity to cholesterol, to assure spontaneous incorporation of CDDO-Im into the lipid bilayer upon rehydration of the lipid film. Addition of a 0.6 molar ratio of CDDO-Im to DOPE: DOPC: cholesterol:DOPE-PEG: DOPE-RR-11a at molar ratios of 6.7:6.7:2.2:1:1.1, respectively, resulted in effective loading of CDDO-Im. Analysis by UV spectrometry of free CDDO-Im and encapsulated CDDO-Im, after release by NP disruption, showed a loaded concentration of 45 μmol/L CDDO-Im (data not shown), which is approximately 450-fold more concentrated than the dose of 100 nmol/L required for effective STAT-3 inhibition. Analysis of NPs by dynamic light scattering and TEM showed an optimal average NP diameter of 100 nm and a \( \zeta \) potential close to 0 (Fig. 1A–D), indicating uniform composition.

CDDO-Im inhibits STAT-3 activation in murine breast cancer cells

We first confirmed that CDDO-Im was able to inhibit IL-6–induced STAT-3 activation in murine breast cancer cells. Thus, when 4T1 tumor cells were incubated with IL-6 and increasing concentrations of free CDDO-Im, Western blot analysis revealed that CDDO-Im blocked STAT-3 phosphorylation and suppressed expression of total STAT-3 protein at 100 nmol/L to 1 μmol/L concentrations (Fig. 2A). We next confirmed the ability of encapsulated CDDO-Im to inhibit...
CDDO-Im inhibits STAT-3 phosphorylation in murine breast tumors (Fig. 2C). Collectively, these data show that CDDO effectively inhibited STAT-3 phosphorylation in primary tumors derived from 4TO7 cell lines.

To evaluate the *in vivo* effects of Leg-NP-CDDO, we orthotopically challenged BALB/c mice with $5 \times 10^3$ 4TO7 tumor cells and 4 days later, treated them with 8 intravenous injections of Leg-NP-CDDO or controls (Fig. 3A). Primary tumor growth was significantly suppressed by Leg-NP-CDDO when compared with controls (Fig. 3B). Importantly, treatment with free CDDO-Im or CDDO-Im encapsulated in nontargeted particles was markedly less effective at suppressing tumor growth when compared with Leg-NP-CDDO. In addition, mice treated with Leg-NP-CDDO showed a significant decrease in tumor burden compared with untreated controls (Fig. 3C).

Finally, we tested the ability of Leg-NPs to deliver a CDDO-Im payload to MMTV-Neu primary tumors in a therapeutic setting. To this end, mice bearing orthotopic breast tumors were given 8 intravenous injections at 3-day intervals with either saline (PBS), Leg-NP, or Leg-NP-CDDO. Western blot analysis of MMTV-Neu primary tumor protein extracts obtained 1 day after the last injection showed that Leg-NP-CDDO effectively inhibited STAT-3 phosphorylation in primary tumors (Fig. 2C). Collectively, these data show that CDDO-Im inhibits STAT-3 phosphorylation in murine breast cancer cells. In addition, we showed successful encapsulation of CDDO-Im into liposomal NPs for targeted delivery to the TME and effective therapeutic inhibition of STAT-3 phosphorylation in vivo.

**Leg-NP-CDDO suppresses growth of murine breast tumors**

To evaluate the *in vivo* effects of Leg-NP-CDDO, we orthotopically challenged BALB/c mice with $5 \times 10^3$ 4TO7 tumor cells and 4 days later, treated them with 8 intravenous injections of Leg-NP-CDDO or controls (Fig. 3A). Primary tumor growth was significantly suppressed by Leg-NP-CDDO when compared with controls (Fig. 3B). Importantly, treatment with free CDDO-Im or CDDO-Im encapsulated in nontargeted particles was markedly less effective at suppressing tumor growth when compared with Leg-NP-CDDO. In addition, mice treated with Leg-NP-CDDO showed a significant decrease in tumor burden compared with untreated controls (Fig. 3C).

However, compared with primary tumor cells, established tumor cell lines, such as 4TO7, that have been in long-term culture *ex vivo* may acquire genetic and phenotypic changes which may affect their therapeutic response (20, 21). Therefore, to critically test the efficacy of Leg-NP-CDDO, we treated mice with orthotopic tumors derived from MMTV-Neu primary cells with 8 intravenous injections of Leg-NP-CDDO, Leg-NP, or PBS (Fig. 4A). Calculation of tumor volumes revealed that mice treated with Leg-NP-CDDO showed only marginally reduced tumor size compared with controls (Fig. 4B), despite significantly reduced tumor burden (Fig. 4C). Therefore, Leg-NP-CDDO was markedly less effective at suppressing *in vivo* growth of primary tumor cells compared with tumors derived from 4TO7 cell lines.
Leg-NP-CDDO or controls

Primary tumors of Leg-NP-CDDO represent mean

Increased in antigen-presenting cells and CD8+ T cells in primary tumors of Leg-NP-CDDO–treated mice

Intriguingly, these results indicate a Th1 cytokine polarization of the TME as a result of Leg-NP-CDDO therapy.

Figure 4. Therapeutic treatment of MMTV-Neu primary tumors with Leg-NP-CDDO delays tumor growth. A, treatment schematic of mice challenged with $1 \times 10^4$ MMTV-Neu primary tumor cells and treated with Leg-NP-CDDO or controls (PBS or Leg-NP; n = 8 mice per group). B, tumors were palpated every 3 days and tumor size calculated. Data represent mean ± SEM. *, P < 0.05.

Combination therapy improves the antitumor effects of an HER-2 DNA vaccine

Thus far, our findings suggested that treatment with Leg-NP-CDDO blocks TME-mediated immunosuppression. Furthermore, on the basis of cytokine expression profiles and immune effector cell infiltration, the immune TME seemed sufficiently primed for an antitumor response. Therefore, we determined whether combination therapy with Leg-NP-CDDO could improve vaccine-induced immune responses against HER-2+ breast cancer and prevent tumor recurrence. To this end, FVB/NJ mice were challenged orthotopically with $1 \times 10^4$ MMTV-Neu primary tumor cells and treated with a combination of Leg-NP-CDDO and a DNA vaccine against the extracellular domain of HER-2 (pNeuTm; Fig. 7A). Alternatively, mice were also treated with empty targeted NPs (Leg-NP) or a control vaccine (pVector). Primary tumors were surgically removed after reaching a volume of 500 mm3, and after 4 weeks of recovery, mice were rechallenged with $1 \times 10^5$ MMTV-Neu primary tumor cells in the contralateral fat pad for experimental recurrence. Tumor recurrence was significantly suppressed in mice treated with the Leg-NP-CDDO/pNeuTm combination therapy, compared with controls, and resulted in complete tumor rejection in 40% (2 of 5) of mice (Fig. 7A). In contrast, vaccination with pNeuTm or treatment with Leg-NP-CDDO alone did not protect against tumor recurrence. These results suggest that combination therapy–mediated protection against tumor recurrence results from Leg-NP-CDDO, which Th1 primes the immune TME, thus improving antitumor immune responses following pNeuTm vaccination.

To further validate this hypothesis, splenocytes from pNeuTm-vaccinated mice, combined with Leg-NP-CDDO, Leg-NP, or PBS, were cultured with irradiated MMTV-Neu.
primary tumor cells and their cytotoxic T lymphocyte (CTL) response measured by flow cytometry. Results showed that pNeuTm-vaccinated mice treated with Leg-NP-CDDO had a 2.3-fold increase in the percentage of CD8<sup>+</sup>/granzyme B<sup>+</sup> splenocytes compared with controls (Fig. 7B). In addition, to determine whether this boost in CTL responses was tumor cell specific, we compared the CTL response of splenocytes from Leg-NP-CDDO/pNeuTm<sup>–</sup>-treated mice cultured with either HER-2<sup>high</sup> MMTV-Neu tumors cells or HER-2<sup>low</sup> HEVc mouse endothelial cells (Fig. 7C). Flow cytometric analysis of these splenocytes revealed a 4-fold increase in percentage of CD8<sup>+</sup>/granzyme B<sup>+</sup> cells in response to HER-2<sup>high</sup> cells versus HER-2<sup>low</sup> cells (Fig. 7C), thus showing that the immune response of mice treated with the combination therapy was indeed tumor antigen specific.

**Discussion**

Inflammation and immunity carry out unequivocal roles in cancer, as shown in part by clinical studies showing that chronic inflammation increases the risk of developing cancer (24). In contrast, positive correlations between increased T-cell numbers and prolonged survival have been observed in patients with breast, colon, prostate, and ovarian cancers (2). Furthermore, ablation of key immune stimulatory molecules, including IFN-γ, IL-12, or STAT-1, in genetically engineered mice significantly increased the occurrence of chemically induced cancers, thus showing the important relationship between immunity and cancer (2).}

The complexity of the TME and the difficulty of manipulating the fine balance between antitumor and autoimmunity...
in vivo, without prohibitive toxicities, are evidenced by the relatively low clinical success rates of cancer immunotherapies for solid tumors. Because cytokines are the “master regulators of immunity,” many therapeutic approaches attempted to use cytokines as adjuvant or single therapies. However, systemic administration of cytokines often resulted in significant toxicities, for example by IL-2, which caused vascular leak syndrome, or IFN-γ, a neurotoxin when administered at higher doses (25, 26). These findings emphasize the yet unmet need to refine the strategy of immunomodulation to induce an effective immunologic response while minimizing systemic toxicities.

These observations lead us to hypothesize that targeted manipulation of cytokine expression, specifically in the TME, could be more relevant and beneficial for cancer immunotherapy.

Therefore, in the current study, we developed a novel targeted therapeutic approach to specifically manipulate the immune TME in vivo. To this end, we engineered legumain-targeted liposome NPs loaded with a CDDO-Im payload capable of direct STAT-3 inhibition in the TME. We showed that TME-specific inhibition of STAT-3 altered the expression of an array of cytokines and growth factors in primary tumors. Importantly, this change was shown by a shift from a protumor Th2 to an antitumor Th1 immune cytokine microenvironment, characterized by increased protein expressions...
of IFN-γ, IL-12, IL-15, GM-CSF, and activated STAT-1 and decreased expression of IL-6, IL-10, and TGF-β. Together, these factors regulate inflammatory and antitumor functions of immune cells including CD8+ T cells and macrophages (24).

Immune responses derived from DNA vaccines depend primarily on functions of CD8+ T cells (27). Cytokines that can activate CD8+ T cells include IFN-γ, which promotes CD8+ T-cell expansion (28). IFN-γ also increases tumor cells expression of MHC class I antigen, making them better targets for tumor-specific CD8+ T cells (29). Importantly, IFN-γ was shown to prevent formation of lymphomas and squamous cell carcinomas initiated by soluble carcinogens in mice (30–31). Similarly, IL-12 was reported to protect against 7,12-dimethylbenz(a)anthracene/12-O-tetradecanoylphorbol-13-acetate (DMBA/TPA)-induced tumors whereas mice lacking IL-12 showed increased papilloma development, compared with wild-type mice (32). Significantly, both IL-12 and IFN-γ were found to induce Th1 polarization of CD4+ T cells (28) and increase IFN-γ production, thereby promoting the expansion of cytotoxic CD8+ T cells (28). These findings relate to our study because mice treated with Leg-NP-CDDO significantly increased protein expressions of both IFN-γ and IL-12 and correlated with increased percentages of activated CD8+ T cells in the TME.

Increased CD8+ T cells in tumors of Leg-NP-CDDO–treated mice also correlated with marked increases in IL-15 expression, a potent chemotactrant for T cells (33). Importantly, IL-15 stimulates Th1 T-cell differentiation and proliferation of naïve human and memory CD8+ T cells in vitro (34). Significantly, these findings are consistent with our observations correlating increased IL-15 expression in the TME with improved CD8+ T-cell function as a result of STAT-3 inhibition with Leg-NP-CDDO.

Tumor-associated macrophages (TAM) are among the most common immune cells in solid tumors (24). TAMs mediate protumor inflammation by cytokine release prompting further recruitment of inflammatory cells (24). Concordantly, we found here a decrease in protein expressions of IL-10 and TGF-β in primary tumors, both reported to induce the cancer-promoting M2 phenotype of TAMs (28). In contrast, macrophages that are activated by IFN-γ possess a phenotype associated with tumor destruction (28). These M1 macrophages are characterized, in part, by expression of Nos2 (35, 36). Intriguingly, we observed an increased infiltration of Nos2+ macrophages in primary tumors of mice treated with Leg-NP-CDDO, which corresponded with an increased expression of GM-CSF in primary tumors. Importantly, GM-CSF was shown to induce recruitment of enhanced professional antigen-presenting cells including DCs and macrophages (22).

Finally, we showed that targeted manipulation of the immune TME with Leg-NP-CDDO combined with an HER-2 DNA vaccine (pNeuTm) essentially prevented breast cancer recurrence in our mouse tumor model. Combination therapy also significantly improved antitumor CTL responses of CD8+ T cells, when compared with mice receiving single therapy alone. Furthermore, mice treated with the combination therapy showed enhanced CTL responses specifically against primary tumor cells, but not HER-2low endothelial cells, thus showing a tumor antigen–specific immune response. Importantly, our combination therapy delayed tumor growth after rechallenging with HER-2+ primary tumor cells and protected against recurrence in 40% of mice. These results clearly show that therapeutic manipulation of the immune TME can improve the efficacy of cancer immunotherapy.

Taken together, the results of our study align with findings of several phase I/II clinical trials, showing limited effects by single cytokine therapies, which strongly emphasized the need for combination therapies and specific targeting of multiple cytokines (24). Significantly, our findings here represent a novel targeted therapeutic approach to manipulate a major repertoire of immune cytokines and growth factors in the TME. Importantly, by targeting immune manipulations for Th1/Th2 transitions specifically in the TME, we begin to circumvent the serious systemic toxicities of many immune-stimulating cytokines while utilizing their immune-promoting effects. By improving the antitumor effects of cancer vaccine therapy and preventing cancer recurrence, Leg-NP-CDDO represents a potentially useful therapeutic compound that can ultimately improve the efficacy of cancer immunotherapy to increase life span and health of cancer patients.

Disclosure of Potential Conflicts of Interest

The content of this study is solely the responsibility of the authors and does not necessarily represent the official views of the National Heart, Lung, and Blood Institute or the NIH. No potential conflicts of interest were disclosed.

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