Targeted therapeutic remodeling of the tumor microenvironment improves a HER-2 DNA vaccine and prevents recurrence in a murine breast cancer model.

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ABSTRACT

The tumor microenvironment (TME) mediates immune suppression resulting in tumor cell escape from immune surveillance and cancer vaccine failure. Immune suppression is mediated by the STAT-3 transcription factor, which potentiates signaling in tumor and immune cells. Since immune suppression continues to be a major inhibitor of cancer vaccine efficacy, we examined in this study whether therapeutically targeted delivery of a synthetic STAT-3 inhibitor to the TME, combined with a HER-2 DNA vaccine can improve immune surveillance against 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 demonstrated an effective therapeutic inhibition of STAT-3 activation in primary tumors. Furthermore, we showed that treatment with these NPs resulted in priming of the immune TME, characterized by increased IFN-γ, pSTAT-1, GM-CSF, IL-2, IL-15 and IL-12b and reduced TGF-β, IL-6 and IL-10 protein expression. Additionally, we found significantly increased tumor infiltration by activated CD8⁺ T cells, M1 macrophages, and dendritic cells. These changes correlated with delayed growth of orthotopic 4TO7 breast tumors and, when combined with a HER-2 DNA vaccine, prevented HER-2⁺ primary tumor recurrence in immune competent mice. Furthermore, anti-tumor T cell responses were enhanced in splenocytes isolated from mice treated with this combination therapy. Together, these data demonstrate 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 (1). Additionally, through cytokine production and release, these cells create an immune suppressive TME that facilitates tumor cell escape from clearance by the immune system, thus mediating tumor maintenance (2). The role of immunity in cancer was demonstrated by studies showing that chemically-induced carcinomas in mice require an inflammatory response (3). For example, RAG-1-/- mice, lacking NK, B and T cells, developed sarcomas faster and more frequently following methylcholanthrene injection than wild-type mice with intact immune systems (2). Similar results were reported in mice with functional ablation of natural killer cells, γδ T cells, αβ T cells, IFN-γ, or IL-12 (2). In humans, immunosuppression of transplant patients and disease-associated immunodeficiencies correlated with significantly higher risk for cancer development (2). Furthermore, clinical studies have associated chronic inflammation with increased risk of colon and breast cancers (4, 5). Cumulatively, these studies indicate that immunity plays a major role in cancer, in both mice and humans.

The Signal Transducer and Activators of Transcription 3 (STAT-3) 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 anti-tumor immune responses (6). STAT-3 inhibits dendritic cell (DC) maturation and macrophage function by suppressing their expression of antigen presentation and co-stimulatory molecules (7). Furthermore, STAT-3 signaling promotes proliferation and survival of regulatory T cells
that inhibit CD8$^+$ T cell 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 nM 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 demonstrated that Legumain-targeting dramatically improved NP drug delivery to solid tumors, while preventing non-specific accumulation in the reticuloendothelial system (RES) (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 demonstrate that this novel combination therapy improved the anti-tumor 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 (La Jolla, CA, USA), FVB/NJ mice from Charles River Laboratories (Wilmington, MA, USA) and housed in our AAALAC accredited facility. Animal protocols, approved by TSRI Animal Care Committee, were performed according to NIH Guides for the Care and Use of Laboratory animals.

Authenticated 4TO7/4T1 murine breast carcinoma cells were provided by Suzanne Ostrand-Rosenberg (University of Maryland, College Park, MD) and maintained in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS, 1% HEPES, 1% sodium bicarbonate and 1% sodium pyruvate. Cell lines are authenticated by in vivo growth/metastasis in Balb/c mice, by expressions of IL-6 and S100A8/A9, and resistance to 6-thioguanine. Cells were tested negative for mycoplasma using MycoALERT (2008, Lonza, Basel, Switzerland). MMTV-Neu primary tumor was provided by Michael Karin (University of California, San Diego, CA, USA) 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, Lakewood, NJ, USA) in RPMI-1640 medium supplemented with 2.5% FBS and 10mM Hepes. 1x10⁶ cells 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 500mm³.
Nanoparticle Formulation. Synthesis of the Legumain specific inhibitor RR-11a was previously described (15). Phospholipids (Avanti Polar Lipids, Alabaster, AL, USA) 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 1.1:6.7:6.7:2.2:1 as previously described (16). CDDO-Imadazolide (CDDO-Im), provided by Michael Sporn (Dartmouth Medical School, Hanover, NH, USA), was added to the lipids prior to roto-evaporation. Unencapsulated CDDO-Im was removed by ultrafiltration using 100nm pore size polycarbonate filters. Size distributions and zeta potentials were determined by dynamic light scattering on a Zetasizer Nano (Malvern, Worcestershire, UK) and TEM performed as previously described (17). Loaded concentrations of CDDO-Im were determined by NP sonication in 2% Tween and UV spectrometry analysis.

Western blot. Protein extracts were prepared as previously described (18). Western blots were probed with the following antibodies: rabbit anti-phospho-STAT-3 (Cell Signaling, Danvers, MA, USA), 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, GM-CSF and IFN-γ, and mouse-anti-IL-15 (all Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-ERBB2 (Abcam, Cambridge, MA, USA). Protein band intensities were quantified using ImageJ software and normalized to β-actin.

In vivo tumor studies. 4TO7 (5x10³) cells or MMTV-Neu (1x10⁴) primary tumor cells were injected orthotopically into female BALB/c or FVB/NJ mice, respectively. NPs in
200μl of PBS (~1.36x10^{13} particles) were administered i.v. and tumor dimensions measured using digital microcalipers. Tumor volume was calculated using the formula 
\[ \frac{a^2 \times b}{2} \], where 'a' is the larger of two perpendicular diameters. For recurrence studies, primary tumors were surgically removed and mice re-challenged orthotopically in the contralateral mammary fat pad. Mice were vaccinated 3 times orally at 1 week intervals by gavage with attenuated salmonella typhimurium (1x10^8 CFU per mouse) transduced with either pNeuTm (provided by Wei-Zen Wei, Karmanos Cancer Center, Detroit, MI, USA) or empty vector, as previously described (19).

**Flow cytometry.** Splenocytes and tumor infiltrating lymphocytes were isolated as previously described (19) and incubated (1x10^6 cells per tube) with fluorescein-conjugated antibodies (0.25μg antibody per 10^6 cells in 100μl volume) against mouse CD8, CD25, CD14, CD11c, CD11b, CD80, CD45, F4/80 (Biolegend, San Diego, CA USA) and/or Granzyme B (0.125μg antibody per 10^6 cells in 100μl volume) (eBioscience, San Diego, CA, USA). Data were collected on a digital LSR-II (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed with FlowJo software (Tree Star, Inc., Ashland, OR, USA).

**Immunohistochemistry.** Tumor sections fixed in acetone were stained with the following primary antibodies: rat anti-mouse F4/80 (1:50 dilution, AbD Serotec, Raleigh, NC, USA) and rabbit anti-mouse Nos2 (1:50 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), 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, Carlsbad, CA, USA), respectively. For staining controls, tissue...
sections were incubated with secondary antibodies only. Cell nuclei were stained with DAPI-dilactate (Sigma, St. Louis, MO, USA).

**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, La Jolla, CA, USA).

**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 45μM CDDO-Im (data not shown), which is approximately 450-fold more concentrated than the dose of 100nM required for effective STAT-3 inhibition. Analysis of NPs by dynamic light scattering and TEM showed an optimal average NP diameter of 100nm and a zeta potential close to zero (Fig. 1 A-D), indicating uniform composition.

**CDDO-Im inhibits STAT-3 activation in murine breast cancer cells.**
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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 100nM-1μM concentrations (Fig. 2A). We next confirmed the ability of encapsulated CDDO-Im to inhibit STAT-3 activation by incubating IL-6 stimulated 4TO7 tumor cells with empty targeted NPs (Leg-NP), non-targeted (NP-CDDO) or targeted (Leg-NP-CDDO) NPs loaded with CDDO-Im, or free CDDO-Im. Western blot analysis showed that encapsulated CDDO-Im blocked STAT-3 phosphorylation as well as free CDDO-Im (Fig. 2B). Importantly, cells treated with Leg-NP did not show inhibition of STAT-3 phosphorylation, thus demonstrating that inhibition was due solely to CDDO-Im and not by any non-specific effect of NPs (Fig. 2B).

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 i.v. 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 one day after the last injection showed that Leg-NP-CDDO effectively inhibited STAT-3 phosphorylation in primary tumors (Fig. 2C). Collectively, these data demonstrate that CDDO-Im inhibits STAT-3 phosphorylation in murine breast cancer cells. Additionally, we demonstrated 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 5x10^3 4TO7 tumor cells and 4 days later, treated them with 8 i.v. 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 non-targeted particles was markedly less effective at suppressing tumor growth when compared with Leg-NP-CDDO. Additionally 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 i.v. 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 to tumors derived from 4TO7 cell lines.

Leg-NP-CDDO modulates cytokine and growth factor expression in primary tumors. STAT-3 signaling mediates tumor-associated immune suppression in vivo by modulating cytokine and growth factor expression by tumor cells and other cells in the
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TME, including macrophages (6). Therefore, to determine the effects of Leg-NP-CDDO on expression levels of these factors, whole cell extracts were derived from primary tumors of mice treated with Leg-NP-CDDO, Leg-NP or PBS. Western blot analysis showed markedly upregulated protein expressions of pSTAT-1 (715-fold), IL-15 (37-fold), IL-12b (9-fold), IFN-γ (24-fold) and GM-CSF (6-fold) in mice treated with Leg-NP-CDDO as compared with controls (Fig. 5A). Conversely, protein expressions of IL-6, IL-10 and TGF-β showed a 2 to 5-fold decrease in primary tumors of Leg-NP-CDDO treated mice (Fig. 5B). Leg-NP-CDDO treatment also downregulated expressions of anti-apoptotic proteins Bcl-XL (8-fold) and Bcl-2 (1.4-fold) (Fig. 5C). Intriguingly, these results indicate a Th1 cytokine polarization of the TME as a result of Leg-NP-CDDO therapy.

Increase in antigen presenting cells and CD8+ T cells in primary tumors of Leg-NP-CDDO treated mice. Immune cells recruited by tumors secrete different cytokines and growth factors depending upon whether they receive Th1 or Th2 polarizing signals from the TME (22). Therefore, the Th1 shift we observed in cytokine expression suggested that changes in the immune cell milieu in tumors might also be evident. To support this contention, live single cell suspensions of primary tumors were derived from mice treated with either Leg-NP-CDDO, Leg-NP or PBS and analyzed by flow cytometry to detect activated CD8+ T cells, DCs and macrophages (Figs. 6 A-C and Supplementary Figs. 1-3). Mice treated with NP-Leg-CDDO showed a 4.6-fold increase in CD8+/CD25+ T cells compared with PBS controls (Fig. 6A). Additionally, mice treated
with Leg-NP-CDDO revealed a 5.6 and 2-fold increase in macrophages (CD45+/F4/80+) (Fig. 6B) and DCs (CD14+/CD11c+ and CD80+/CD11b+) (Fig. 6C), respectively.

Macrophages have very different effects on immune function and tumor growth depending on their mode of activation and polarization. ‘Classically activated’ M1 macrophages typically show high expression of NOS2 in association with anti-tumor immune responses (23). In contrast, ‘alternatively activated’ M2 macrophages do not express NOS2 and are typically associated with immune suppression and pro-tumor responses (23). Therefore, we determined if macrophages in primary tumors of Leg-NP-CDDO treated mice corresponded to either M1 or M2. To this end, immunohistochemistry and fluorescence microscopy analysis of tumors revealed a marked increase in F4/80+/Nos2+ positive cells in tumors derived from Leg-NP-CDDO treated mice (Fig. 6D), whereas control tumors showed robust F4/80+ staining that was predominantly NOS2- (Fig. 6D). These findings suggest that M1 polarization of tumor infiltrating macrophages is a result of Leg-NP-CDDO treatment.

**Combination therapy improves the anti-tumor effects of a Her-2 DNA vaccine.**

Thus far, our findings suggested that treatment with Leg-NP-CDDO blocks TME-mediated immune suppression. Furthermore, based on cytokine expression profiles and immune effector cell infiltration, the immune TME appeared sufficiently primed for an anti-tumor 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 1x10⁴ MMTV-Neu primary tumor cells and treated with a combination
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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 500mm³, and after 4 weeks of recovery, mice were re-challenged with 1x10⁴ 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% (2/5) 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 anti-tumor 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 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⁺/Granzyme B⁺ splenocytes compared with controls (Fig. 7B). Additionally, to determine whether this boost in CTL responses was tumor cell specific, we compared the CTL response of splenocytes from Leg-NP-CDDO/pNeuTm treated mice cultured with either HER-2[^high] MMTV-Neu tumors cells versus HER-2[^low] HEVc mouse endothelial cells (Fig. 7C). Flow cytometry analysis of these splenocytes revealed a 4-fold increase in percentage of CD8⁺/Granzyme B⁺ cells in response to HER-2[^high] cells versus HER-2[^low] cells (Fig. 7C), thus demonstrating that
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the immune response of mice treated with the combination therapy was indeed tumor antigen specific.

**DISCUSSION**

Inflammation and immunity perform unequivocal roles in cancer, as demonstrated 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 demonstrating the important relationship between immunity and cancer (2).

The complexity of the TME and the difficulty of manipulating the fine balance between anti-tumor and autoimmunity *in vivo*, without prohibitive toxicities, are evidenced by the relatively low clinical success rates of cancer immunotherapies for solid tumors. Since 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 immune modulation to induce an effective immunological 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 demonstrated by a shift from a pro-tumor Th2 to an anti-tumor Th1 immune cytokine microenvironment, characterized by increased protein expressions of IFN-γ, IL-12, IL-15, GM-CSF, activated STAT-1, and decreased expression of IL-6, IL-10 and TGF-β. Together, these factors regulate inflammatory and anti-tumor 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 induced by soluble carcinogens in mice (30, 31). Similarly, IL-12 was reported to protect against 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 since 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 chemoattractant 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 (TAMs) are among the most common immune cells in solid tumors (24). TAMs mediate pro-tumor 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 NOS-2 (35, 36). Intriguingly, we observed an increased infiltration of NOS-2⁺ 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 demonstrated that targeted manipulation of the immune TME with Leg-NP-CDDO combined with a HER-2 DNA vaccine (pNeuTm) essentially prevented breast cancer recurrence in our mouse tumor model. Combination therapy also
significantly improved anti-tumor 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-2$^-$ endothelial cells, thus demonstrating a tumor antigen specific immune response. Importantly, our combination therapy delayed tumor growth after re-challenging with HER-2$^+$ primary tumor cells and protected against recurrence in 40% of mice. These results clearly demonstrate 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 anti-tumor 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 lifespan and health of cancer patients.

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FIGURE LEGENDS

Figure 1. Physicochemical characterization of RR-11a-coupled NPs. Legumain-targeted NPs loaded with CDDO-Im (A), without CDDO-Im (B), or non-targeted NPs loaded with CDDO-Im (C) or without CDDO-Im (D), were analyzed by dynamic light scattering and TEM (inset) to determine particle size distribution (diameter, nm) and zeta potential (mV). Scale bar=100nm.

Figure 2. Encapsulated CDDO-Im inhibits STAT-3 phosphorylation in murine breast cancer cells and primary tumors by Western Blot analysis. (A) 4T1 murine breast cancer cells were treated with IL-6 (10ng/mL) and CDDO-Im at varying concentrations. (B) 4TO7 murine breast cancer cells were treated with IL-6 (10ng/mL) in combination with free CDDO-Im (Free CDDO), empty targeted NPs (Leg-NP), non-targeted NP-encapsulated CDDO-Im (NP-CDDO) or targeted NP-encapsulated CDDO-Im (Leg-NP-CDDO). (C) MMTV-Neu primary tumor extracts were prepared from mice treated with 8 i.v. injections of PBS (Lane 1), Leg-NP (Lane 2) or Leg-NP-CDDO (Lane 3).

Figure 3. Therapeutic treatment with Leg-NP-CDDO inhibits growth of 4TO7 tumors. (A) Treatment schematic of mice challenged with 5x10^3 4TO7 tumor cells and treated with Leg-NP-CDDO or controls (PBS, free CDDO, NP-CDDO or Leg-NP) (n=8 mice/group). (B) Tumors were palpated every 2 days and tumor size calculated. Data represent means±s.e.m. (C) Tumor weights were measured on day 19 and compared to
body weights to determine percent tumor burden. Data represent means±s.e.m. *p<0.05.

Figure 4. Therapeutic treatment of MMTV-Neu primary tumors with Leg-NP-CDDO delays tumor growth. (A) Treatment schematic of mice challenged with 1x10^4 MMTV-Neu primary tumor cells and treated with Leg-NP-CDDO or controls (PBS or Leg-NP) (n=8 mice/group). (B) Tumors were palpated every 3 days and tumor size calculated. Data represent means±s.e.m. (C) Tumor weights were measured on day 46 and used to calculate percent tumor burden. Data represent means±s.e.m, *p<0.05.

Figure 5. Leg-NP-CDDO modulates tumor cytokine and growth factor expression profiles in vivo. Whole tissue extracts were prepared from MMTV-Neu primary tumors isolated from mice treated as described in Fig. 4A. Western blot analysis (left panels) was performed and quantified relative to Actin (right graphs) to determine protein expression of Th1 (A) and Th2 (B) associated growth factors and cytokines. Additionally, expression of anti-apoptotic proteins was also determined (C). Data represent means±s.e.m. from 3 independent experiments. *p<0.05 and **p<0.005.

Figure 6. Therapeutic treatment with Leg-NP-CDDO modulates infiltration of immune cells into primary tumors. Mice were treated as depicted in Figure 4A. (A-C) 46 days after tumor cell challenge, live primary tumor single cell suspensions were analyzed by flow cytometry to detect activated CD8^+ T cells (A), macrophages (B) or dendritic cells (C). Data represent means±s.e.m. (D) M1 macrophages were identified in
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Frozen tumor sections by immunohistochemistry using antibodies against F4/80 (red staining) and NOS2 (green staining). Cell nuclei were stained with DAPI (blue staining).

Scale bar = 100μm.

Figure 7. Leg-NP-CDDO and pNeuTm combination therapy enhances anti-tumor immune surveillance and prevents breast cancer recurrence. (A) Schematic of treatment schedule for tumor recurrence study. Mice were challenged orthotopically with MMTV-Neu primary tumor cells (Day 0, black dashed arrow), treated with Leg-NP-CDDO or control NPs (gray solid arrows), and vaccinated with pNeuTm or pVector (gray dashed arrows). Primary tumors were surgically removed after reaching a size of ~500mm³ (black solid arrow). Four weeks later, mice were re-challenged in the contralateral mammary fat pad with MMTV-Neu primary tumor cells (Day 53, black dashed arrow). Tumor dimensions were measured and used to calculate tumor size (n=5 mice/group). Data represent means±s.e.m. (B) Mice were sacrificed when secondary tumors reached a volume of 500mm³. Tumor free mice were sacrificed 128 days after initial tumor cell challenge. Splenocytes from pNeuTm vaccinated mice treated with either PBS, Leg-NP or Leg-NP-CDDO were cultured with irradiated MMTV-Neu primary tumor cells and analyzed by flow cytometry. Data represent means±s.e.m. *p<0.05. (C) Splenocytes from Leg-NP-CDDO/pNeuTM treated mice were cultured with irradiated HEVc or MMTV-Neu primary tumor cells and analyzed by flow cytometry. HER-2 protein expression was confirmed by Western blotting. Data represent mean±s.e.m. ***p<0.0005.
Figure 1.
Figure 2.

A

+IL-6

0 100 300 500 1000 CDDO-Im (nM)

86 kDa - p-STAT3 (Y705)
86 kDa - Total STAT3
43 kDa - β-actin

B

+ IL-6

Unreated Free CDDO Lsg-NP CDDO Lsg-NP-CDDO

86 kDa - p-STAT3 (Y705)
86 kDa - Total STAT3
43 kDa - β-actin

C

1 2 3

86 kDa - p-STAT3 (Y705)
86 kDa - Total STAT3
43 kDa - β-actin
Figure 3.

A Tumor cell Challenge

Leg-NP-CDDO or Controls

Isolate Tumors

Day 0 4 6 8 10 12 14 16 18 19

B

Tumor Size (nm³)

0 500 1000 1500 2000 2500

Days After Tumor Cell Challenge

PBS  CDDO  NP-CDDO  Leg-NP  Leg-NP-CDDO

C

% Tumor Burden

PBS  CDDO  NP-CDDO  Leg-NP  Leg-NP-CDDO

*
Figure 4.

A) Tumor cell challenge

B) Tumor size (mm$^3$) over days

C) % Tumor Burden
Figure 5.

A

25 kDa - IFN-γ
40 kDa - IL-12b
91 kDa - p-STAT1
15 kDa - IL-2
14 kDa - IL-15
14 kDa - GM-CSF
43 kDa - β-actin

B

18 kDa - IL-6
20 kDa - IL-10
25 kDa - TGF-β
43 kDa - β-actin

C

30 kDa - BCL-Xₐ
29 kDa - BCL-2
43 kDa - β-actin
Figure 6.

A

%CD8+CD25+ cells

PBS
Leg-NP
Leg-NP-CDDO

B

%CD4+FoxP3+ cells

PBS
Leg-NP
Leg-NP-CDDO

C

%CD11c+CD11c+ cells

PBS
Leg-NP
Leg-NP-CDDO

D

PBS
Leg-NP
Leg-NP-CDDO

Legend:

- PBS
- Leg-NP
- Leg-NP-CDDO

*(p < 0.05) compared to PBS control.
Figure 7.

A

![Graph showing tumor size over days for different conditions](image)

B

![Bar graph showing %CD8+GranzymeB+ Cells](image)

C

![Western blot showing HER-2 and ACTIN](image)

Legend:
- Log-NP-CDDO + pNouTM
- Log-NP + pNouTM
- Log-NP-CDDO + pVector
- Log-NP + pVector
- PBS
Targeted therapeutic remodeling of the tumor microenvironment improves a Her-2 DNA vaccine and prevents recurrence in a murine breast cancer model.

Debbie Liao, Ze Liu, Wolfgang J. Wrasidlo, et al.

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