articles

molecular pharmaceutics

Development of a Poly(D,L-lactic-*co*-glycolic acid) Nanoparticle Formulation of STAT3 Inhibitor JSI-124: Implication for Cancer Immunotherapy

Ommoleila Molavi,[†,‡] Abdullah Mahmud,[†] Samar Hamdy,[†] Ryan W. Hung,[†,§] Raymond Lai,[‡] John Samuel,[†] and Afsaneh Lavasanifar*[,†,||]

Faculty of Pharmacy & Pharmaceutical Sciences, University of Alberta, Edmonton, T6G 2N8, Canada, Department of Laboratory Medicine & Pathology, Faculty of Medicine & Dentistry, University of Alberta, Edmonton, T6G 1Z2, Canada, Department of Radiology and Diagnostic Imaging, Faculty of Medicine & Dentistry, University of Alberta, Edmonton, T6G 2R7, Canada, and Department of Chemical and Material Engineering, University of Alberta, Edmonton, T6G 2G6, Canada

Received June 4, 2009; Revised Manuscript Received December 11, 2009; Accepted December 23, 2009

Abstract: Constitutively activated signal transducer and activator of transcription-3 (STAT3) in tumor and dendritic cells (DCs) plays a critical role in tumor-induced immunosuppression. This is considered a major challenge in effective immunotherapy of cancer. Herein we describe the development of a polymeric nanocarrier for the delivery of JSI-124 (a small molecule inhibitor of STAT3) to tumor and immunosuppressed DCs using poly(D,L-lactic-*co*-glycolic acid) nanoparticles (PLGA NPs). For this purpose, JSI-124 was chemically conjugated to PLGA and the PLGA−JSI-124 conjugate was formulated into nanoparticles using the emulsification solvent evaporation method. The attachment of JSI-124 to PLGA was confirmed by a combination of thin layer chromatography and ^{1}H NMR. The level of JSI-124 in NPs, determined by liquid chromatography−mass spectrometry, was found to be 1.7 ± 0.3 μg per mg of PLGA. The PLGA−JSI-124 NPs demonstrated a controlled drug release profile over a 1-month period and exhibited potent anticancer and STAT3 inhibitory activity comparable to the soluble JSI-124 after 24 h incubation with B16 melanoma cells, *in vitro*. Moreover, PLGA−JSI-124 NPs efficiently suppressed the level of p-STAT3 in p-STAT3^{high} DCs, generated from mouse bone marrow cells in the presence of conditioned media of B16 cells (B16CM-DCs), and improved their function as assessed by mixed lymphocyte reaction (MLR). Specifically cotreatment of B16CM-DCs with PLGA−JSI-124 NPs and PLGA NPs containing the DC adjuvant CpG resulted in higher levels of T cell proliferation in the MLR assay compared with B16CM-DCs untreated or treated with either CpG NPs or JSI-124 NPs alone. Our results indicate that PLGA NPs containing conjugated JSI-124 can potentially provide a useful platform for sustained JSI-124 release in tumor and its targeted delivery to DCs leading to the modulation of anticancer response by JSI-124 in tumor cells and immunosuppressed DCs, *in vitro*.

Keywords: Cancer immunotherapy; STAT3; JSI-124; nanoparticles; PLGA

Introduction

Over the past few decades, several vaccines for cancer immunotherapy have been developed and advanced to clinical trials. Although most of the developed vaccination strategies have shown great potential for the induction of antitumor immune responses and breaking of tolerance to

* Corresponding author. Mailing address: Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta T6G 2N8, Canada. Phone: 780-492-2742. Fax: 780-492-1217. E-mail: alavasanifar@pharmacy.ualberta.ca.

† Faculty of Pharmacy & Pharmaceutical Sciences.

‡ Department of Laboratory Medicine & Pathology, Faculty of Medicine & Dentistry.

§ Department of Radiology and Diagnostic Imaging, Faculty of Medicine & Dentistry.

|| Department of Chemical and Material Engineering.

10.1021/mp900145g
Published on Web 12/23/2009

cancer antigens in animal tumor models and cancer patients, their therapeutic efficacy in clinical trials has been poor.[1−3] Recent studies suggest that the immunosuppressive environment within tumor is a major factor responsible for the poor therapeutic outcome of cancer vaccines. The immunosuppressive tumor environment can suppress the effector phase of anticancer immune responses through inhibition of immune effector cell infiltration and function in the tumor.[4,5] Therefore, reversal of the "immunosuppressive milieu" of the tumor microenvironment may be the key for successful translation of vaccine based cancer immunotherapy strategies to clinical cancer treatment.

Constitutive activation of signal transducer and activator of transcription-3 (STAT3) in tumor and dendritic cells (DCs) contributes to the induction of immunosuppression in the tumor environment.[4−9] STAT3 is found constitutively activated at high frequency in many types of human malignancies and contributes to tumor progression through the modulation of genes involved in tumor cell growth, proliferation, angiogenesis, metastasis, and cancer immune evasion.[10,11] The critical role of STAT3 in tumor-induced immunosuppression has been highlighted in several studies that showed that constitutive activation of STAT3 in tumor resulted in diminished production of pro-inflammatory mediators and increased expression of immunosuppressive factors. These in turn led to the activation of STAT3 in diverse subsets of immune cells rendering them either dysfunctional or immunosuppressive.[7,12,13] Among the affected immune cells, immunosuppressed DCs (so-called tolerogenic DCs) play a crucial role in the establishment of the immunosuppressive network in the tumor environment.[5,7,13] DCs are professional antigen presenting cells which link the innate immunity to adaptive immune responses and play a key role in the induction of tolerance versus immunity.[14−17] Immunosuppression of DCs by cancer leads to the generation of tolerogenic DCs which not only are incapable of inducing anticancer immune responses but also are shown to activate suppressor cells such as regulatory T cells. Tolerogenic DCs and other activated immune suppressor cells produce additional immunosuppressive factors and induce activation of more immune suppressor cells, resulting in a vicious cycle of immunosuppression in the tumor environment. Because of the important role of DCs in the induction of immunosuppression in the tumor environment, restoring their function is considered as an important strategy for overcoming this immunosuppression.[5,7]

Since constitutively activated STAT3 in tumor and consequently in DCs is an important mediator in the induction of immunosuppressed DCs, blocking STAT3 in tumor and DCs represents a promising strategy for restoring the function of DCs and breaking the vicious cycle of immunosuppression in tumor environment.[18] Studies have shown that JSI-124 (cucurbitacin I), an anticancer inhibitor of STAT3,[19] reduces the level of p-STAT3 in dysfunctional p-STAT3^{high} DCs generated by culture in the presence of conditional medium

(1) Slingluff, C. L.; Speiser, D. E. Progress and controversies in developing cancer vaccines. *J. Transl. Med.* **2005**, *3* (1), 18–27.

(2) Morse, M. A.; Chui, S.; Hobeika, A.; Lyerly, H. K.; Clay, T. Recent developments in therapeutic cancer vaccines. *Nat. Clin. Pract. Oncol.* **2005**, *2* (2), 108–13.

(3) Rosenberg, S. A.; Yang, J. C.; Restifo, N. P. Cancer immunotherapy: moving beyond current vaccines. *Nat. Med.* **2004**, *10* (9), 909–15.

(4) Chen, Q.; Wang, W. C.; Evans, S. S. Tumor microvasculature as a barrier to antitumor immunity. *Cancer Immunol. Immunother.* **2003**, *52* (11), 670–9.

(5) Zou, W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat. Rev. Cancer* **2005**, *5* (4), 263–74.

(6) Stewart, C. A.; Trinchieri, G. Reinforcing suppression using regulators: a new link between STAT3, IL-23, and Tregs in tumor immunosuppression. *Cancer Cell* **2009**, *15* (2), 81–3.

(7) Yu, H.; Kortylewski, M.; Pardoll, D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. *Nat. Rev. Immunol.* **2007**, *7* (1), 41–51.

(8) Burdelya, L.; Kujawski, M.; Niu, G.; Zhong, B.; Wang, T.; Zhang, S.; Kortylewski, M.; Shain, K.; Kay, H.; Djeu, J.; Dalton, W.; Pardoll, D.; Wei, S.; Yu, H. Stat3 activity in melanoma cells affects migration of immune effector cells and nitric oxide-mediated antitumor effects. *J. Immunol.* **2005**, *174* (7), 3925–31.

(9) Kortylewski, M.; Xin, H.; Kujawski, M.; Lee, H.; Liu, Y.; Harris, T.; Drake, C.; Pardoll, D.; Yu, H. Regulation of the IL-23 and IL-12 balance by Stat3 signaling in the tumor microenvironment. *Cancer Cell* **2009**, *15* (2), 114–23.

(10) Yu, H.; Jove, R. The STATs of cancer--new molecular targets come of age. *Nat. Rev. Cancer* **2004**, *4* (2), 97–105.

(11) Al Zaid Siddiquee, K.; Turkson, J. STAT3 as a target for inducing apoptosis in solid and hematological tumors. *Cell Res.* **2008**, *18* (2), 254–67.

(12) Wang, T.; Niu, G.; Kortylewski, M.; Burdelya, L.; Shain, K.; Zhang, S.; Bhattacharya, R.; Gabrilovich, D.; Heller, R.; Coppola, D.; Dalton, W.; Jove, R.; Pardoll, D.; Yu, H. Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. *Nat. Med.* **2004**, *10* (1), 48–54.

(13) Kortylewski, M.; Kujawski, M.; Wang, T.; Wei, S.; Zhang, S.; Pilon-Thomas, S.; Niu, G.; Kay, H.; Mule, J.; Kerr, W. G.; Jove, R.; Pardoll, D.; Yu, H. Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. *Nat. Med.* **2005**, *11* (12), 1314–21.

(14) Banchereau, J.; Steinman, R. M. Dendritic cells and the control of immunity. *Nature* **1998**, *392* (6673), 245–52.

(15) Novak, N.; Bieber, T. 2. Dendritic cells as regulators of immunity and tolerance. *J. Allergy Clin. Immunol.* **2008**, *121* (2 Suppl), S370–4, quiz S413.

(16) Dhodapkar, M. V.; Dhodapkar, K. M.; Palucka, A. K. Interactions of tumor cells with dendritic cells: balancing immunity and tolerance. *Cell Death Differ.* **2008**, *15* (1), 39–50.

(17) Cools, N.; Ponsaerts, P.; Van Tendeloo, V. F.; Berneman, Z. N. Balancing between immunity and tolerance: an interplay between dendritic cells, regulatory T cells, and effector T cells. *J. Leukocyte Biol.* **2007**, *82* (6), 1365–74.

(18) Nefedova, Y.; Gabrilovich, D. I. Targeting of Jak/STAT pathway in antigen presenting cells in cancer. *Curr. Cancer Drug Targets* **2007**, *7* (1), 71–7.

(19) Blaskovich, M. A.; Sun, J.; Cantor, A.; Turkson, J.; Jove, R.; Sebti, S. M. Discovery of JSI-124 (cucurbitacin I), a selective Janus kinase/signal transducer and activator of transcription 3 signaling pathway inhibitor with potent antitumor activity against human and murine cancer cells in mice. *Cancer Res.* **2003**, *63* (6), 1270–9.

from p-STAT3 hyperactive tumors, and restore their function *in vitro*.[18,20,21] Furthermore, treatment of tumor-bearing mice with JSI-124 has been shown to modulate immunosuppression in the tumor environment leading to increased infiltration of immune effector cells.[18,22−24] We have recently shown that simultaneous inhibition of STAT3 by JSI-124 and activation of DCs by CpG (a Toll-like receptor 9 (TLR9) ligand) modulate tumor-induced immunosuppression and generate synergistic antitumor effects compared to CpG or JSI-124 alone in a B16 mouse melanoma model.[24] Kortylewski et al. have reported a similar effect while studying the anticancer effects of a CpG-based cancer immunotherapy approach in combination with STAT3 inhibition induced by a small molecule inhibitor of STAT3 (CPA7) in a B16 tumor model.[25]

Despite potent STAT3 inhibitory effects of JSI-124 and its potential for overcoming tumor-induced immunosuppression, the clinical application of JSI-124 has been hampered due to its poor water solubility and nonspecific toxicity. Application of polymeric nanocarriers as drug delivery systems for JSI-124 is a promising approach for overcoming both limitations and enhancing the benefit of this important therapeutic agent. Development of poly(D,L -lactic-*co*-glycolic acid) (PLGA) nanoparticles containing chemically conjugated JSI-124 has been pursued in the present study as a strategy for efficient delivery of JSI-124 to tumor and DCs.

PLGA is a biocompatible and biodegradable polymer approved by the FDA for human use. PLGA nanoparticles (NPs) are naturally targeted to DCs by phagocytosis because their size is similar to that of pathogens. They have been extensively studied for delivery of antigens, adjuvants and several small therapeutic molecules to DCs.[26−31] PLGA NPs have also been used for sustained delivery of several anticancer agents to tumor.[32−38] Therefore, PLGA nanoparticulate formulations of JSI-124 are expected not only to provide sustained delivery of JSI-124 to cancer cells but also to target JSI-124 to immunosuppressed DCs. Finally, PLGA NPs can provide a platform for codelivery of cancer antigens, adjuvants and STAT3-inhibitor JSI-124 to DCs paving the way toward development of efficient cancer therapeutic vaccines.

Materials and Methods

Materials. Polyvinyl alcohol (PVA, MW 31−50 kDa), carboxylic acid and ester terminated PLGA polymers,

(20) Nefedova, Y.; Cheng, P.; Gilkes, D.; Blaskovich, M.; Beg, A. A.; Sebti, S. M.; Gabrilovich, D. I. Activation of Dendritic Cells via Inhibition of Jak2/STAT3 Signaling. *J. Immunol.* **2005**, *175* (7), 4338–46.

(21) Nefedova, Y.; Nagaraj, S.; Rosenbauer, A.; Muro-Cacho, C.; Sebti, S. M.; Gabrilovich, D. I. Regulation of dendritic cell differentiation and antitumor immune response in cancer by pharmacologic-selective inhibition of the janus-activated kinase 2/signal transducers and activators of transcription 3 pathway. *Cancer Res.* **2005**, *65* (20), 9525–35.

(22) Fujita, M.; Zhu, X.; Sasaki, K.; Ueda, R.; Low, K. L.; Pollack, I. F.; Okada, H. Inhibition of STAT3 promotes the efficacy of adoptive transfer therapy using type-1 CTLs by modulation of the immunological microenvironment in a murine intracranial glioma. *J. Immunol.* **2008**, *180* (4), 2089–98.

(23) Molavi, O.; Ma, Z.; Hamdy, S.; Lavasanifar, A.; Samuel, J. Immunomodulatory and Anticancer Effects of Intra-Tumoral Co-Delivery of Synthetic Lipid A Adjuvant and STAT3 Inhibitor, JSI-124. *Immunopharmacol. Immunotoxicol.* **2008**, 1–14.

(24) Molavi, O.; Ma, Z.; Hamdy, S.; Lai, R.; Lavasanifar, A.; Samuel, J. Synergistic antitumor effects of CpG oligodeoxynucleotide and STAT3 inhibitory agent JSI-124 in a mouse melanoma tumor model. *Immunol. Cell Biol.* **2008**, *86* (6), 506–14.

(25) Kortylewski, M.; Kujawski, M.; Herrmann, A.; Yang, C.; Wang, L.; Liu, Y.; Salcedo, R.; Yu, H. Toll-like receptor 9 activation of signal transducer and activator of transcription 3 constrains its agonist-based immunotherapy. *Cancer Res.* **2009**, *69* (6), 2497–505.

(26) Waeckerle-Men, Y.; Gander, B.; Groettrup, M. Delivery of tumor antigens to dendritic cells using biodegradable microspheres. *Methods Mol. Med.* **2005**, *109*, 35–46.

(27) Elamanchili, P.; Lutsiak, C. M.; Hamdy, S.; Diwan, M.; Samuel, J. Pathogen-mimicking" nanoparticles for vaccine delivery to dendritic cells. *J. Immunother.* **2007**, *30* (4), 378–95.

(28) Schlosser, E.; Mueller, M.; Fischer, S.; Basta, S.; Busch, D. H.; Gander, B.; Groettrup, M. TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses. *Vaccine* **2008**, *26* (13), 1626–37.

(29) Jiang, W.; Gupta, R. K.; Deshpande, M. C.; Schwendeman, S. P. Biodegradable poly(lactic-co-glycolic acid) microparticles for injectable delivery of vaccine antigens. *Adv. Drug Delivery Rev.* **2005**, *57* (3), 391–410.

(30) Waeckerle-Men, Y.; Groettrup, M. PLGA microspheres for improved antigen delivery to dendritic cells as cellular vaccines. *Adv. Drug Delivery Rev.* **2005**, *57* (3), 475–82.

(31) Foged, C.; Sundblad, A.; Hovgaard, L. Targeting vaccines to dendritic cells. *Pharm. Res.* **2002**, *19* (3), 229–38.

(32) Kalaria, D. R.; Sharma, G.; Beniwal, V.; Ravi Kumar, M. N. Design of Biodegradable Nanoparticles for Oral Delivery of Doxorubicin: In vivo Pharmacokinetics and Toxicity Studies in Rats. *Pharm. Res.* **2009**, *26* (3), 492–501.

(33) Bala, I.; Hariharan, S.; Kumar, M. N. PLGA nanoparticles in drug delivery: the state of the art. *Crit. Rev. Ther. Drug Carrier Syst.* **2004**, *21* (5), 387–422.

(34) Tong, W.; Wang, L.; D'Souza, M. J. Evaluation of PLGA microspheres as delivery system for antitumor agent-camptothecin. *Drug Dev. Ind. Pharm.* **2003**, *29* (7), 745–56.

(35) Hussain, M.; Beale, G.; Hughes, M.; Akhtar, S. Co-delivery of an antisense oligonucleotide and 5-fluorouracil using sustained release poly (lactide-co-glycolide) microsphere formulations for potential combination therapy in cancer. *Int. J. Pharm.* **2002**, *234* (1−2), 129–38.

(36) Wang, Y. M.; Sato, H.; Adachi, I.; Horikoshi, I. Preparation and characterization of poly(lactic-co-glycolic acid) microspheres for targeted delivery of a novel anticancer agent, taxol. *Chem. Pharm. Bull. (Tokyo)* **1996**, *44* (10), 1935–40.

(37) Danhier, F.; Lecouturier, N.; Vroman, B.; Jerome, C.; Marchand-Brynaert, J.; Feron, O.; Preat, V. Paclitaxel-loaded PEGylated PLGA-based nanoparticles: in vitro and in vivo evaluation. *J. Controlled Release* **2009**, *133* (1), 11–7.

(38) Yoo, H. S.; Lee, K. H.; Oh, J. E.; Park, T. G. In vitro and in vivo anti-tumor activities of nanoparticles based on doxorubicin-PLGA conjugates. *J. Controlled Release* **2000**, *68* (3), 419–31.

monomer ratio 50:50, MW 4−7 kDa, were purchased from Absorbable Polymers International (Pelham, AL). *N*,*N*-Dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP) were purchased from Sigma (St. Louis, MO). Recombinant murine granulocyte monocyte colony stimulating factor (GM-CSF) was purchased from Peprotech (Rocky Hill, NJ). RPMI-1640, L-glutamine, and gentamicin were purchased from Invitrogen Canada, Inc. (Burlington, ON, Canada). Fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT). JSI-124 was purchased from Indofine Chemicals, Inc. (Hillsborough, NJ). Anti-p-STAT3 antibody and its respective isotype control were purchased from Santa Cruz Biotechnology. CpG (unmethylated CpG dinucleotides, ODN#1826) was obtained from InvivoGen (San Diego, CA).

Chemical Conjugation of JSI-124 to PLGA. For the conjugation of JSI-124 to COOH-terminated PLGA polymer, DMAP (52.41 mg, 0.429 mM) and DCC (88.5 mg, 0.429 mM) were added to a stirring solution of PLGA (300 mg, 0.0429 mM) in anhydrous tetrahydrofuran (THF, 25 mL) under argon gas. The reaction mixture was stirred for 1 h at room temperature. A solution of JSI-124 (22 mg, 0.0429 mM) in anhydrous THF (10 mL) was then added to the reaction mixture, and the reaction was continued for an additional 96 h. Evaporation of the reaction mixture gave a residue, which was dissolved in a 1:5 ratio of HPLC grade THF and methanol (10 mL). The solution of PLGA−JSI-124 polymer was then added to 20 mL of water in a dropwise manner while stirring. The resulting solution was dialyzed against water for 48 h to remove the unreacted JSI-124 and any other byproduct. PLGA−JSI-124 conjugate was lyophilized to a bright yellow solid form for further use. Thin layer chromatography of the conjugate compared to free drug using ethyl acetate as the mobile phase and vanillin/phosphoric acid as a JSI-124 indicator confirmed the conjugation of JSI-124 to PLGA and the absence of free JSI-124 in the purified product.[39] To further confirm the absence of free drug in the drug−polymer conjugate, PLGA−JSI-124 conjugate was dissolved in 1:4 methanol:chloroform and analyzed by liquid chromatography/mass spectrometry (LC−MS) for the presence of free JSI-124. A Waters Micromass ZQ 4000 spectrometer, coupled to a Waters 2795 separations module with an autosampler (Milford, MA) was used for LC−MS analysis. No peak corresponding to the molecular ion of JSI-124 was observed in the SIR chromatogram of PLGA−JSI-124, indicating the absence of free drug in the drug−polymer conjugate. Prepared drug−polymer conjugate was characterized by ^{1}H NMR (Bruker Unity-300 spectrometer) using deuterated dimethyl sulfoxide (DMSO-d_6) as the solvent and tetramethylsilane as an internal reference.

Preparation and Characterization of PLGA NPs Containing JSI-124 or CpG. PLGA−JSI-124 conjugate was formulated into NPs by the single emulsion solvent evaporation method. Briefly, 60 mg of PLGA−JSI-124 polymer was dissolved in 450 μL of chloroform and the resulting solution was emulsified in 2 mL of PVA solution (9%, w/v PVA in PBS) by sonication for 45 s at level 4, using a microtip sonicator (Heat systems Inc., Farmingdale, NY). The emulsion was added into 8 mL of stirring PVA solution in a dropwise manner. The final emulsion was further stirred for 3 h and then collected by centrifugation of the emulsion at 40000*g* for 10 min at 4 °C. The NPs were washed twice with cold deionized water and lyophilized. The volume mean diameter and polydispersity index of the nanoparticles were determined by the dynamic light scattering (DLS) technique using a Zetasizer 3000 (Malvern, U.K.). PLGA−JSI-124 conjugate was further characterized for the level of conjugated JSI-124 by LC−MS. To measure the degree of JSI-124 conjugation, NPs were suspended in a formic acid solution at pH = 2 containing % 20 ethanol (at a concentration of 0.5 mg of NPs per mL) and then incubated in the water bath at 37 °C for 4 h. After 4 h incubation, the NPs solution was centrifuged but no precipitation of NPs was observed. The level of JSI-124 in the solution of NPs was measured by the LC−MS method as described previously.[40] To assess the release profile of JSI-124 from the formulation, NPs (3 mg) were suspended in 15 mL of distilled water (ddH_2O) and then aliquoted into 15 samples of 1 mL in microcentrifuge tubes. The samples were shaken in a water bath at 37 °C. At predetermined time intervals, the supernatant of one sample was collected by centrifugation (12000*g* for 10 min) and analyzed for the level of released JSI-124 by LC−MS.

Physical encapsulation of CpG in PLGA NPs was achieved by the double emulsion solvent evaporation method. Briefly CpG ODN (200 μg in 60 μL of TE buffer at pH 8) was emulsified with ester-terminated PLGA solubilized in chloroform (600 μL, 30% w/v) for 20 s using the microtip sonicator. The resulting primary (W/O) emulsion was then combined with PVA solution (4 mL, 7.5% w/v in TE buffer) and sonicated for 40 s at level 4 to form a secondary (W/O/W) emulsion. This secondary emulsion was then added in a dropwise manner to a beaker containing PVA solution (16 mL, 7.5% w/v in TE buffer) under constant stirring. The final emulsion was stirred for 3 h at room temperature and collected by centrifugation at 40000*g* for 10 min at 4 °C. The NPs were washed twice with cold deionized water and lyophilized. The level of encapsulated CPG was determined as described in a previous publication.[41]

Cell Viability Assay. Antiproliferative activity of free and PLGA NP-conjugated JSI-124 was assessed in B16.F10, a melanoma cell of C57BL/6 origin (American Type Culture

(39) Afifi, M. S.; Ross, S. A.; El-Sohly, M. A.; Naeem, Z. E.; Halaweish, F. T. Cucurbitacins of Cucumis prophetarum and Cucumis prophetarum. *J. Chem. Ecol.* **1999**, *25*, 847–859.

(40) Molavi, O.; Shayeganpour, A.; Somayaji, V.; Hamdy, S.; Brocks, D. R.; Lavasanifar, A.; Kwon, G. S.; Samuel, J. Development of a sensitive and specific liquid chromatography/mass spectrometry method for the quantification of cucurbitacin I (JSI-124) in rat plasma. *J. Pharm. Pharm. Sci.* **2006**, *9* (2), 158–64.

(41) Diwan, M.; Elamanchili, P.; Cao, M.; Samuel, J. Dose sparing of CpG oligodeoxynucleotide vaccine adjuvants by nanoparticle delivery. *Curr. Drug Delivery* **2004**, *1* (4), 405–12.

Collection, ATCC). B16 cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 IU/mL penicillin/streptomycin at 37 °C in 5% CO_2 atmosphere. Cell viability was monitored using the 3-(4,5-dimethylthiazol-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) assay.[42] To evaluate cytotoxicity, B16 cells were obtained from exponentially growing 90−95% confluent cultures and seeded at a density of 2000 cells/well in 96-well plates. After two days incubation the cells were washed twice in serum-free medium and treated with control or four different concentrations of JSI-124 either dissolved in methanol or conjugated to PLGA NPs. After 24 h incubation at 37 °C the medium was removed and cells were washed three times with the media followed by the addition of 20 μL of a 2 mg/mL solution of tetrazolium MTT dye. The plates were returned to the incubator for a period of 4 h. The residual MTT solution was removed from the wells, then 0.2 mL of DMSO was added to each well and the absorbances were read at 570 nm using a plate reader (PowerWave 340, Bio-Tek instruments Inc.).

Generation of Tumor-Induced Immunosuppressed DCs. DC primary cultures were generated from murine bone marrow precursors of C57BL/6 mice in complete media in the presence of GM-CSF as described previously.[43] Briefly, femurs were removed and purified from the surrounding muscle tissue. Then intact bones were left in 70% ethanol for 2 min for disinfection, washed with PBS, then cut on both ends with scissors, and the bone marrow flushed with PBS using a syringe with a 0.45 mm diameter needle. After one wash in PBS, about $1-1.5 \times 10^7$ leukocytes were obtained per femur. Cell culture medium was RPMI-1640 supplemented with gentamicin (80 μg/mL), L-glutamine (2 mM), and 10% heat-inactivated FCS. At day 0, bone marrow leukocytes were seeded at 2×10^6 per 100 mm dish in 10 mL of complete medium containing 20 ng/mL GM-CSF. At day 3, another 10 mL of complete medium containing 20 ng/mL GM-CSF was added to the plates. At day 6, half of the culture supernatant was collected and replaced with 10 mL of fresh medium containing 20 ng/mL GM-CSF. By day 7 the cells were ready for use. The purity of the DC population on day 7 was found to be between 70 and 75% based on flow cytometric analysis of the expression of CD11c on the semiadherent and nonadherent cell populations. To generate immunosuppressed p-STAT3^{high} DCs, conditioned media (CM) from B16 cells was added to the culture of bone marrow originated DCs on day 7 and then DCs were incubated for 8−12 h before treatment with different formulations. To make B16CM, B16 cells were kept in a reduced (2%) FBS concentration for 48 h and then the supernatants were collected, filtered and used in the experiments.

Analysis of p-STAT3 Level by Flow Cytometry. Intracellular staining of p-STAT3 (phosphorylated STAT3) was done using PE labeled-anti-pSTAT3 antibody or isotype control according to the manufacturer's instructions. Briefly DCs or B16 cells were collected, washed twice with PBS and then fixed with PBS/paraformaldehyde fixation solution (Santa Cruz Biotechnology) at 4 °C for 30 min. After two washes, the cells were permeabilized by a saponin solution (Santa Cruz Biotechnology) at room temperature for 15 min, and then stained with PE labeled-anti-pSTAT3 antibody or isotype control (2 μg/10^6 cells) for 60 min at room temperature. After three washes with FCM wash buffer (Santa Cruz Biotechnology), samples were acquired on a Becton-Dickinson FACSort and analyzed with Cell-Quest software.

Assessment of the Functional Characteristics of Tumor-Induced Immunosuppressed DCs by Mixed Lymphocyte Reaction (MLR). DCs derived from C57BL/6 mice (on day 7 of their culture) were incubated with CM from B16 cells, for 8−12 h, and then treated with different formulations. After 18 h incubation with the formulations, cells were harvested, washed, and irradiated (2000 rads). T cells were isolated from the spleen of Balb/c mice using the EasySep mouse T cell enrichment kit (Stem Cell Technologies). The irradiated DCs were cocultured with T cells (1:10 ratio) for 60 h, and the proliferation of T cells was determined by incorporation of ^{3}H-thymidine for the last 18 h of the culture.

Statistical Analysis. The significance of differences among groups was analyzed by one-way analysis of variance (ANOVA) for parametric data, followed by the Student−Newman−Keuls posthoc test for multiple comparisons, or by the Kruskal−Wallis one-way ANOVA for nonparametric data. A p-value of ≤ 0.05 was set for the significance of difference among groups. The statistical analysis was performed with SigmaStat software (Systat Software Inc. San Jose, CA).

Results

Synthesis and Characterization of PLGA−JSI-124. PLGA−JSI-124 conjugate was synthesized by forming an ester bond between a hydroxyl group from JSI-124 and the terminal carboxyl group of PLGA polymer using DMAP and DCC as the coupling agent and catalyst (Figure 1).

The conjugation of the JSI-124 molecule to PLGA polymer was confirmed by the ^{1}H NMR spectrum of PLGA−JSI-124 (Figure 2a), free JSI-124 (Figure 2b), and PLGA polymer (Figure 2c). The ^{1}H NMR spectrum of PLGA−JSI-124 in DMSO-d_6 (Figure 2a) demonstrated characteristic JSI-124 peaks at 1.75, 3.6, and 5.69 ppm. Similar peaks were also present in the ^{1}H NMR spectrum of the free JSI-124 (Figure 2b). Further evidence for the conjugation of JSI-124 to PLGA polymer was provided by TLC and LC−MS analysis. TLC analysis of PLGA−JSI-124 conjugate showed the absence of the free form of JSI-124 in PLGA−JSI-124 conjugate (Figure 2d). Consistent with TLC results, PLGA−JSI-124 conjugate dissolved in 1:4

(42) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65* (1−2), 55–63.

(43) Lutz, M. B.; Kukutsch, N.; Ogilvie, A. L.; Rossner, S.; Koch, F.; Romani, N.; Schuler, G. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* **1999**, *223* (1), 77–92.

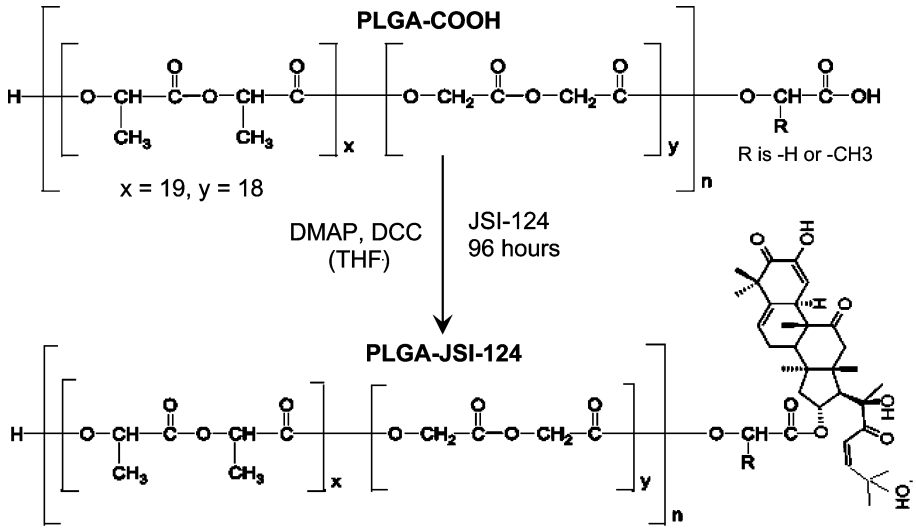

Figure 1. Synthetic scheme for the preparation of PLGA−JSI-124 conjugate.

methanol:chloroform did not show the peak corresponding to the molecular ion of JSI-124 by LC−MS analysis (Figure 2e) indicating the absence of free drug in PLGA−JSI-124 conjugate. On the other hand, NPs of PLGA−JSI-124 conjugates showed the presence of a molecular ion peak at 559 *m*/*z* corresponding to MW of [JSI-124 + formic acid − H] when they were incubated in formic acid solution at pH = 2 for 4 h and analyzed by LC−MS (Figure 2f).

Characterization of PLGA−JSI-124 NPs. The PLGA−JSI-124 NPs were 329 ± 44 nm in diameter with a polydispersity less than 0.1 as determined by DLS (Table 1). The level of conjugated JSI-124 was found to be 1.7 ± 0.3 μg per mg of PLGA polymer as assessed by the LC−MS method of quantification. This corresponds to 0.023 mol of JSI-124/mol of PLGA.

In Vitro **Release Profile of JSI-124 from NPs.** PLGA−JSI-124 NPs showed a burst release (30%) of JSI-124 within the first 2 days followed by the establishment of a plateau phase in the next 21 days. PLGA NPs released 58% of conjugated JSI-124 to the receiving media within 21 days (Figure 3). The cumulative release of JSI-124 from the PLGA NPs over a period of 34 days was 86%. The triphasic release of JSI-124 from the PLGA NPs suggests a bulk erosion and biodegradation dependent release profile for this drug from PLGA NPs (Figure 3).

Anticancer and STAT3 Inhibitory Activity of PLGA−JSI-124 NPs in B16 Cells *in Vitro*. Figure 4a depicts the anticancer activity of JSI-124 either free or conjugated to PLGA NPs in B16−F10 melanoma cells lines after 24 h incubation. Treatment of the murine B16 melanoma cells with increasing concentrations of free or PLGA-conjugated JSI-124 resulted in a significant loss of cell viability (ANOVA, $p < 0.001$). Treatment of B16 cells with PLGA−JSI-124 NPs at concentrations of 1.25, 2.5, 5, and 10 μM for 24 h reduced the cell viability to 37, 25, 11 and 4%, which were not significantly different from the viability of the cells treated with free drug at identical concentrations ($P > 0.05$, ANOVA). As shown in Figure 4a, JSI-124 either fee or conjugated to PLGA NPs inhibited B16 cell growth in a dose-dependent manner. PLGA−JSI-124 NPs were able to efficiently suppress the level of p-STAT3 in B16 melanoma cell line at a concentration of 2 μM after 24 h incubation (Figure 4b).

STAT3 Inhibitory Effects of PLGA−JSI-124 NPs on DCs. To evaluate the STAT3 inhibitory effects of the NPs on DCs, p-STAT3$^+$ DCs were generated from C57BL/6 mouse bone marrow cells in the presence of B16CM. As shown in Figure 5a, treatment of DCs with B16CM results in a higher level of p-STAT3 in treated DCs as compared to untreated DCs. B16CM-treated DCs characterized by higher level of p-STAT3 are referred to as B16CM-DCs. Treatment of B16CM-DCs with JSI-124 conjugated to PLGA NPs at concentrations of 100, 200, and 400 nM for 24 h reduced the percentage of p-STAT3$^+$ DCs to 18, 7, and 5%, which were significantly different from the percentage of p-STAT3$^+$ DCs in untreated group or the cells treated with empty NPs (Figure 5b, $p < 0.01$ ANOVA). The percentage of p-STAT3$^+$ DCs treated with free JSI-124 at identical concentrations for the same period of time was not significantly different from what was observed with PLGA−JSI-124 NPs (ANOVA, $p > 0.05$).

Immunomodulatory Effects of PLGA NPs Delivering JSI-124 and CpG on DCs. DCs treated with CM from B16 cells (B16CM-DCs) expressed higher level of p-STAT3 (Figure 5a) and were found to be dysfunctional in stimulating T cells in an MLR reaction (Figure 6). Treatment of B16CM-DCs with CpG NPs resulted in a significant, albeit modest, increase in the level of T cell proliferation induced by treated B16CM-DCs, but it could not completely restore their function. This was indicated by significantly lower level of T cell proliferation in CpG treated group compared to control untreated DCs ($p < 0.05$). However, when B16CM-DCs were treated with 200 nM JSI-124 either free or conjugated to the PLGA NPS for 24 h, the impaired DCs regained their ability to induce T cell proliferation in an MLR assay. The level of T cell proliferation induced by B16CM-DCs treated with either soluble or PLGA−JSI-124 NPs for 24 h was significantly higher than that induced with untreated B16CM-

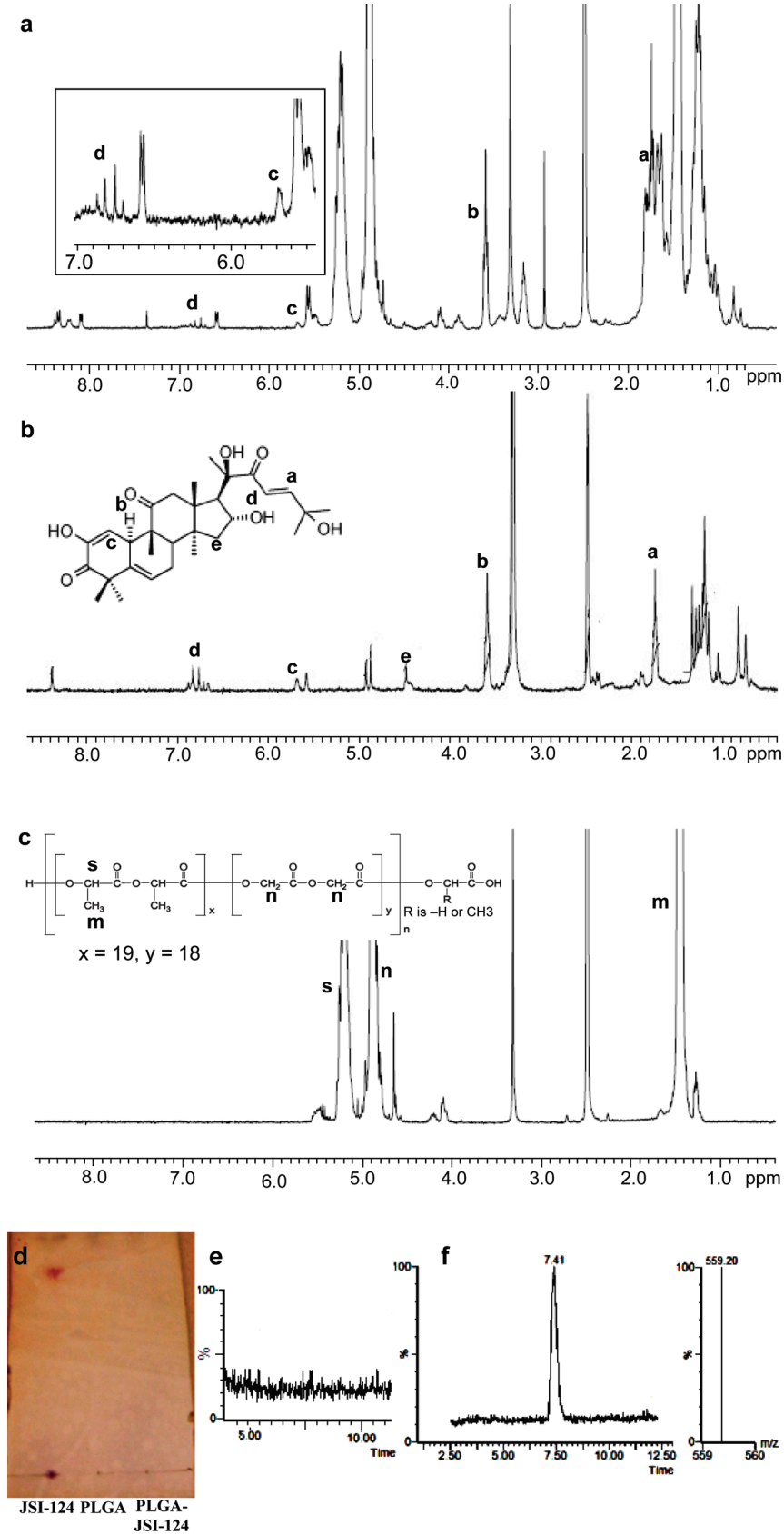

Figure 2. Characterization of PLGA–JSI-124 conjugate. ^{1}H NMR spectrum of (a) PLGA–JSI-124 conjugate in DMSO-d_6, (b) free JSI-124 in DMSO-d_6, (c) PLGA polymer in DMSO-d_6; (d) TLC analysis of PLGA–JSI-124, PLGA and free JSI-124; (e, f) SIR chromatogram and mass spectra of (e) PLGA–JSI-124 conjugate in 1:4 methanol:chloroform and (f) PLGA–JSI-124 NPs after incubation in formic acid (pH = 2) for 4 h, and monitored at 559 *m*/*z*.

Table 1. Characteristics of PLGA−JSI-124 NPs

size (nm)	329 ± 44
polydispersity index	0.09 ± 0.05
JSI-124 conjugated (μg per mg of PLGA)	1.7 ± 0.3

DCs or empty NP or CpG NP B16CM-DCs ($p < 0.001$, ANOVA). There was no statistically significant difference in the level of T cell proliferation between the groups of B16CM-DCs treated with soluble and nanoparticulate formulation of JSO-124 (Figure 6, $p > 0.05$ ANOVA). Cotreatment of B16CM-DCs with CpG NPs and JSI-124, either free or nanoparticulate, resulted in higher level of T cell proliferation compared with the DCs treated with either CpG NPs or JSI-124 formulations alone (Figure 6, $p < 0.01$ ANOVA). The level of T cell proliferation in the group of DCs treated with CpG + JSI-124 free was not significantly different from that in DCs treated with CpG + JSI-124 PLGA NPs.

Discussion

Activation of STAT3 is shown to play a major role in tumor growth, survival and invasion in several types of cancer. In addition, STAT3 activation in tumor is also known to provoke STAT3 activation in several tumor infiltrating immune cells, leading to the formation of an immunosuppressive network in the tumor milieu characterized by the accumulation of tolerogenic DCs and regulatory T cells as well as reduced level of mature DCs and Th1 cytokines.[7,9,12,13] Moreover, recent observations document an important role for STAT3 activation in restraining the immunostimulatory effect of toll-like receptor (TLR) ligands that are regularly used as adjuvants to boost the effect of prophylactic or therapeutic cancer vaccines.[25] Therefore, inhibition of STAT3 in the tumor microenvironment and in tolerogenic DCs is an attractive approach not only to reduce tumor proliferation and metastasis but also to break the vicious cycle of tumor-induced immunosuppression and increase the therapeutic efficacy of cancer immunotherapy strategies.

JSI-124, a natural product from the cucurbitacin family, is a potent small molecule inhibitor of STAT3 that has been extensively studied for its STAT3 and anticancer activity in several human and murine cancers.[19,23,24,44−48] In addition, the immunomodulatory effects of JSI-124 mediated through inhibition of STAT3 have been illustrated in numerous *in vitro* and *in vivo* models.[20−24] Our group has reported on the development of polymeric micellar formulations that can increase the water solubility of JSI-124, sustain its release rate *in vitro*, and maintain the cytotoxic and immunomoulatory activity of JSI-124 while limiting the exposure of drug

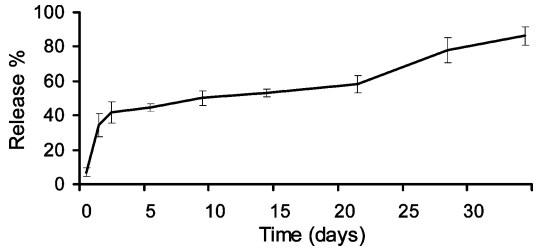

Figure 3. JSI-124 release profile from PLGA NPs *in vitro*. Each point represents mean ± SD ($n = 3$).

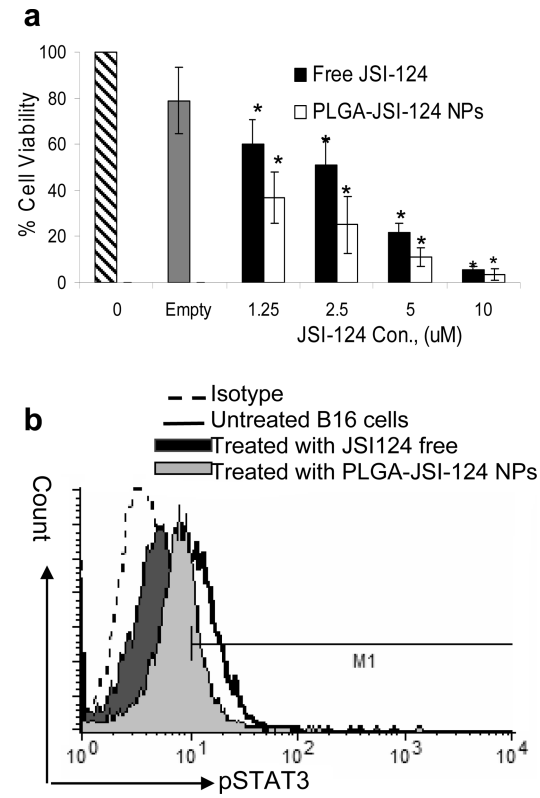

Figure 4. Assessment of the anticancer and STAT3 inhibitory activity of PLGA−JSI-124 NPs against B16 cell line. (a) Anticancer activity of JSI-124 free and chemically conjugated to PLGA NPs, against B16 melanoma cell line after 24 h incubation, *in vitro*. B16 cells were treated with four different concentrations of JSI-124 either free or conjugated to PLGA NPs. After 24 h incubation, cell viability was estimated by MTT assay and expressed as percentage of untreated controls. The data represent the mean ± SD of three independent experiments; *significantly different from untreated B16 cells ($P < 0.05$); (b) p-STAT3 inhibitory activity of JSI-124 and JSI-124-PLGA NPs in B16 cells after 24 h incubation. The data represent one out of three independent experiments which showed similar results.

(44) Jayaprakasam, B.; Seeram, N. P.; Nair, M. G. Anticancer and antiinflammatory activities of cucurbitacins from Cucurbita andreana. *Cancer Lett.* **2003**, *189* (1), 11–6.

(45) Molavi, O.; Ma, Z.; Mahmud, A.; Alshamsan, A.; Samuel, J.; Lai, R.; Kwon, G. S.; Lavasanifar, A. Polymeric micelles for the solubilization and delivery of STAT3 inhibitor cucurbitacins in solid tumors. *Int. J. Pharm.* **2008**, *347* (1−2), 118–27.

(46) Shi, X.; Franko, B.; Frantz, C.; Amin, H. M.; Lai, R. JSI-124 (cucurbitacin I) inhibits Janus kinase-3/signal transducer and activator of transcription-3 signalling, downregulates nucleophosmin-anaplastic lymphoma kinase (ALK), and induces apoptosis in ALK-positive anaplastic large cell lymphoma cells. *Br. J. Haematol.* **2006**, *135* (1), 26–32.

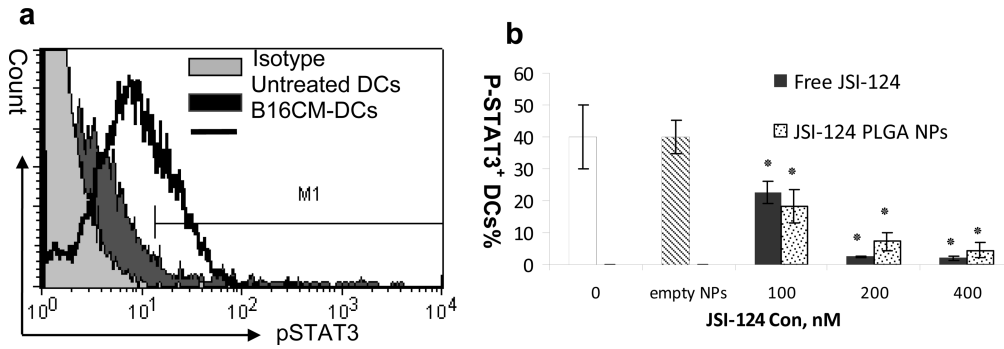

Figure 5. Assessment of the STAT3 inhibitory activity of PLGA−JSI-124 NPs in DCs. (a) p-STAT3 expression in DCs treated with CM of B16 cell (B16CM-DCs). The data represent one out of three independent experiments which showed the similar results. (b) p-STAT3 inhibitory activity of JSI-124 and PLGA−JSI-124 NPs in B16CM-DCs after 24 h incubation. The data represent the mean ± SD of three independent experiments; *significantly different from untreated B16CM-DCs or empty NP treated B16CM-DCs ($P < 0.01$).

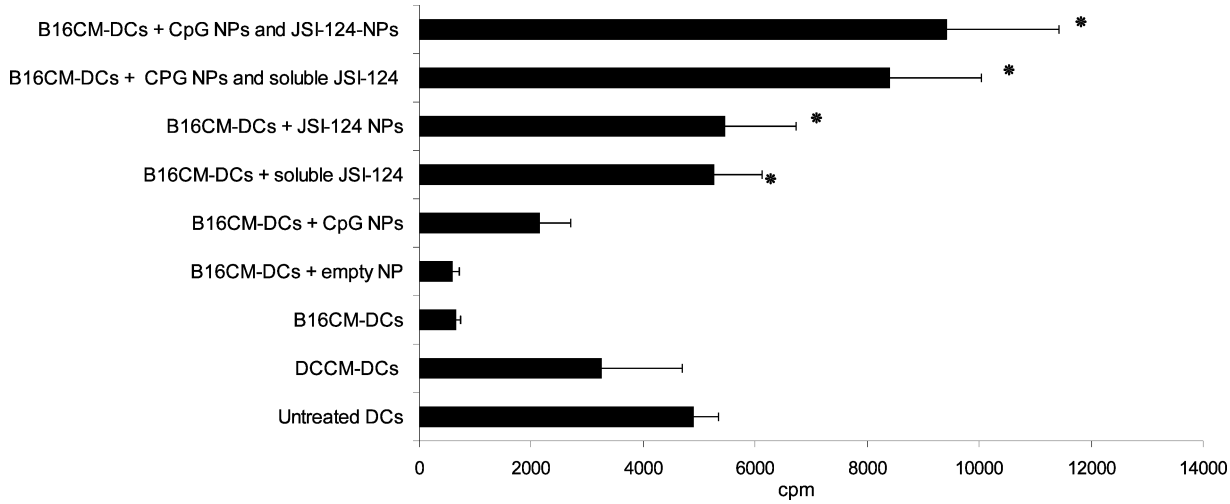

Figure 6. The effects of PLGA-NP delivery of JSI-124 on the function of DCs. DCs treated with B16CM (B16CM-DCs) were incubated with different formulations for 24 h then cocultured with T cells isolated from the spleen of Balb/c mice for a MLR reaction. Proliferation of T cells was determined by incorporation of ^{3}H-thymidine for the last 24 h of the culture. *Significantly higher from B16CM-DC group ($P < 0.01$).

to nontarget tissues after intratumoral administration in a murine B16 melanoma model.[45,49] Although polymeric micellar formulations are promising nanodelivery systems for sustained JSI-124 delivery to the tumor microenvironment, their small size (<100 nm) and the dense hydrophilic coating of poly(ethylene glycol) may limit their interaction with DCs. The purpose of this study was to achieve an optimal nanocarrier for selective delivery of JSI-124 to immunosuppressed DCs. Unlike polymeric micelles, PLGA-NPs are naturally targeted to DCs through phagocytosis, as their size is comparable to that of pathogens[31] and their surface is not protected by a hydrophilic palisade. The successful delivery of several antigens, adjuvants and small molecules to DCs by PLGA NPs has been well documented by our group and others.[27,50−53] Besides, PLGA NPs may provide a promising platform for codelivery of cancer vaccines and STAT3 inhibitors to the same population of DCs. In addition to DCs, cancer cells have also been shown to be able to take up PLGA NPs.[54]

In this study PLGA NPs containing chemically conjugated JSI-124 was generated for local (intratumoral) delivery of JSI-124 to cancer cells and DCs. Development of the PLGA NP formulations of JSI-124 was accomplished through chemical conjugation of JSI-124 to COOH-terminated PLGA and further assembly of polymer conjugates to NPs as the physical encapsulation efficiency of JSI-124 in PLGA-NPs was found to be very low (unpublished data). Chemical conjugation of JSI-124 to PLGA NPs resulted in the generation of nanocarriers which are able not only to carry

(47) Van Kester, M. S.; Out-Luiting, J. J.; von dem Borne, P. A.; Willemze, R.; Tensen, C. P.; Vermeer, M. H. Cucurbitacin I Inhibits Stat3 and Induces Apoptosis in Sezary Cells. *J. Invest. Dermatol.* **2008**, *128* (7), 1691–5.

(48) Chen, J. C.; Chiu, M. H.; Nie, R. L.; Cordell, G. A.; Qiu, S. X. Cucurbitacins and cucurbitane glycosides: structures and biological activities. *Nat. Prod. Rep.* **2005**, *22* (3), 386–99.

(49) Mahmud, A.; Patel, S.; Molavi, O.; Choi, P.; Samuel, J.; Lavasanifar, A. Self-Associating Poly(ethylene oxide)-*b*-poly(α-cholesteryl carboxylate-ε-caprolactone) Block Copolymer for the Solubilization of STAT-3 Inhibitor Cucurbitacin I. *Biomacromolecules* **2009**, *10*, 471–8.

a fairly high load of drug (0.023 drug/polymer on molar basis) but also to control the release of JSI-124 from NPs over a 1-month period, *in vitro* (Figure 3). The level of JSI-124 conjugated to PLGA NPs (1.7 μg per 1 mg of PLGA) is believed to be therapeutically relevant since, based our present finding, JSI-124 either free or conjugated to PLGA NPs is able to suppress the level of p-STAT3 at concentration as low as 200 nM *in vitro*. The PLGA−JSI-124 NPs showed a burst release within the first 2 days probably due to the release of drug from PLGA−JSI-124 during the NP preparation process and/or the accelerated degradation and/or release of drug from low molecular weight PLGAs or those located on the surface of NPs. The triphasic release suggested a degradation-dependent mode of drug release, which is expected to favor the release of JSI-124 following endocytosis in the acidic environment of endosomes, where hydrolysis of the ester linkage between JSI-124 and PLGA would be catalyzed, and bulk erosion of the PLGA NPs would be accelerated.[55] At the same time, the degradation-dependent delivery of free JSI-124 is expected to limit the release of conjugated JSI-124 at physiological pH before NP uptake by DCs or tumor cells restricting drug distribution into nontarget tissues after *in vivo* administration. The uptake of PLGA NPs by DCs has been assessed and reported in previous publications from our lab.[50,56−58] Furthermore PLGA NPs have been shown to be quickly taken up by B16 cancer cells.[34] Since the bond between JSI-124 and PLGA is an ester bond, it is expected to be broken in the acidic pH of endosome after NPs are taken up by the cell leading to the release of free functional drug mostly inside the cells. Our *in vitro* studies found comparable cytotoxicity in B16 melanoma cells (Figure 4), as well as a similar STAT3 inhibitory effects in B16 cells and DCs for PLGA−JSI-124 NPs as compared to free JSI-124 (Figure 4 and 5). While the identification of the pathway through which functional drug becomes available to cancer cells and DCs needs more investigation, we presume that functional JSI-124 is delivered to the cells either by uptake of NPs and release of drug in endosome or by diffusion of free drug released prior to the uptake of NPs. STAT3 is constitutively active in B16 melanoma cells and plays a major role in their growth, survival, and resistance to apoptosis. JSI-124 inhibit B16 tumor growth through the inhibition STAT3 pathway on which B16 cell are very dependent for their growth and survival.[19,59]

It has been shown that p-STAT3^{high} DCs generated in the presence of CM from STAT3 hyperactive tumor cells are dysfunctional and unable to efficiently stimulate T cell proliferation *in vitro*.[21] Consistent with these results, DCs treated with CM from B16 cells (B16CM-DCs) generated here were unable of stimulating T cell proliferation (Figure 6). We have previously reported successful development and characterization of PLGA NP formulations of CpG, a potent adjuvant which activates untreated DCs through TLR9 and induces Th1 immune responses.[41,60] The results of previously published work from our lab show that the delivery of CpG in PLGA NPs is a more efficient approach for DC activation and induction of T cell responses than the use of CpG in solution. Based on our findings PLGA delivery of CpG to DCs strongly stimulates DC activation and induces Th1 type immune responses.[41,56,60] However, PLGA NP delivery of CpG to DCs pretreated with B16CM was found to be ineffective in restoring the function of impaired DCs in terms of stimulating T cell proliferation in the MLR reaction. This is consistent with other studies showing that CpG on its own cannot activate impaired DCs in tumor.[61] On the other hand, treatment of B16CM-DCs with JSI-124 (either soluble or conjugated to PLGA NPs) significantly improved their T cell mitogenic activity when compared with untreated B16CM-DCs or B16CM-DCs treated with empty NPs. This is consistent with previous reports where inhibition of STAT3 by JSI-124 in CM-DCs has been shown to improve DC function, *in vitro*.[20] Consistent with our observation on the

(50) Elamanchili, P.; Diwan, M.; Cao, M.; Samuel, J. Characterization of poly(D,L-lactic-co-glycolic acid) based nanoparticulate system for enhanced delivery of antigens to dendritic cells. *Vaccine* **2004**, *22* (19), 2406–12.

(51) Samuel, J.; Kwon, G. K. *Polymeric Nanoparticle Delivery of Cancer Vaccines*; Informa Healthcare: 2005.

(52) Hamdy, S.; Elamanchili, P.; Alshamsan, A.; Molavi, O.; Satou, T.; Samuel, J. Enhanced antigen-specific primary CD4+ and CD8+ responses by codelivery of ovalbumin and toll-like receptor ligand monophosphoryl lipid A in poly(D,L-lactic-co-glycolic acid) nanoparticles. *J. Biomed. Mater. Res. A* **2007**, *81* (3), 652–62.

(53) Gerelchuluun, T.; Lee, Y. H.; Lee, Y. R.; Im, S. A.; Song, S.; Park, J. S.; Han, K.; Kim, K.; Lee, C. K. Dendritic cells process antigens encapsulated in a biodegradable polymer, poly(D,L-lactide-co-glycolide), via an alternate class I MHC processing pathway. *Arch. Pharm. Res.* **2007**, *30* (11), 1440–6.

(54) Feng, S. S.; Mu, L.; Win, K. Y.; Huang, G. Nanoparticles of biodegradable polymers for clinical administration of paclitaxel. *Curr. Med. Chem.* **2004**, *11* (4), 413–24.

(55) Yoo, J. Y.; Kim, J. M.; Seo, K. S.; Jeong, Y. K.; Lee, H. B.; Khang, G. Characterization of degradation behavior for PLGA in various pH condition by simple liquid chromatography method. *Biomed. Mater. Eng.* **2005**, *15* (4), 279–88.

(56) Diwan, M.; Elamanchili, P.; Lane, H.; Gainer, A.; Samuel, J. Biodegradable nanoparticle mediated antigen delivery to human cord blood derived dendritic cells for induction of primary T cell responses. *J. Drug Targeting* **2003**, *11* (8−10), 495–507.

(57) Newman, K. D.; Elamanchili, P.; Kwon, G. S.; Samuel, J. Uptake of poly(D, L-lactic-co-glycolic acid) microspheres by antigen-presenting cells in vivo. *J. Biomed. Mater. Res.* **2002**, *60* (3), 480–6.

(58) Lutsiak, M. E.; Robinson, D. R.; Coester, C.; Kwon, G. S.; Samuel, J. Analysis of poly(D,L-lactic-co-glycolic acid) nanosphere uptake by human dendritic cells and macrophages in vitro. *Pharm. Res.* **2002**, *19* (10), 1480–7.

(59) Niu, G.; Heller, R.; Catlett-Falcone, R.; Coppola, D.; Jaroszeski, M.; Dalton, W.; Jove, R.; Yu, H. Gene therapy with dominant-negative Stat3 suppresses growth of the murine melanoma B16 tumor in vivo. *Cancer Res.* **1999**, *59* (20), 5059–63.

(60) Diwan, M.; Tafaghodi, M.; Samuel, J. Enhancement of immune responses by co-delivery of a CpG oligodeoxynucleotide and tetanus toxoid in biodegradable nanospheres. *J. Controlled Release* **2002**, *85* (1−3), 247–62.

similar effect of free and conjugated JSI-124 on STAT3 activation, restoration of DC activity in terms of T cell proliferation by free and conjugated JSI-124 was comparable.

We also found that PLGA NP delivery of CpG along with JSI-124 (soluble or conjugated) to B16CM-DCs results in an increased level of T cell proliferation as compared with B16CM-DCs treated with either JSI-124 or CpG alone. This is consistent with the result of our previously published data showing that intratumoral coadministration of JSI-124 and CpG in B16 tumor-bearing mice resulted in an increased percentage of activated DCs in tumor-draining lymph nodes and in the tumor itself.[24]

It has been recently shown that activation of STAT3 in DCs can limit the efficacy of CpG for the induction of DC maturation and activation which is a key process in the induction of antitumor immunity.[25] This study suggests a mechanism by which codelivery of JSI-124 and CpG to immunosuppressed B16CM-DCs improves the function of B16CM-DCs and results in a significant increase in T cell proliferation as compared with the groups of B16CM-DCs treated with CpG alone. In other words, STAT3 activation in DCs might act like a "brake" on TLR9 induced activation of DCs, while JSI-124 can release this "brake" and restore efficient activation of DCs by CpG. The results of this study showed that JSI-124 either free or conjugated to PLGA NPs enhanced the stimulatory effects of CpG NPs on immunosuppressed p-STAT3^{high} DCs. The statistically insignificant difference in the level of T cell proliferation between the group stimulated with CpG NP + free JSI-124 and with CpG NP + PLGA−JSI-124 NPs indicates that functional JSI-124 is efficiently delivered by these newly developed nanocarriers to DCs.

In summary, chemical conjugation of JSI-124 to PLGA NPs generates an efficient drug delivery system for JSI-124 with degradation-controlled release properties. The developed PLGA−JSI-124 NPs were found to provide comparable anticancer and STAT3 inhibitory activity against B16 melanoma cells with free JSI-124, *in vitro*. Furthermore, JSI-124 conjugated to PLGA NPs was able to suppress the level of p-STAT3 in B16CM-DCs and to significantly improve their function in stimulating T cell proliferation in an MLR reaction. These findings show the potential of PLGA−JSI-124 NPs for the delivery of functional JSI-124 to tumor and DCs. The results also demonstrate the ability of PLGA NP formulation of JSI-124 to restore the function of tumor suppressed DCs. Our present *in vitro* findings also points to the potential of PLGA NPs as efficient carriers for codelivery of a small molecule inhibitor of STAT3 (e.g., JSI-124) and a TLR ligand (CpG) to DCs, leading to improved responses in cancer immunotherapy.

Acknowledgment. This work was supported by a research grants from Canadian Institute of Health Research (CIHR, Grant MOP 42407 and MOP 82884) and the National Science and Engineering Research Council (NSERC, Grants G121210926 and G121220086). O.M. has been supported by Rx & D Health Research Foundation/Canadian Institute of Health Research graduate studentship and a scholarship from the Iranian Ministry of Health and Medical Education.

MP900145G

(61) Vicari, A. P.; Chiodoni, C.; Vaure, C.; Ait-Yahia, S.; Dercamp, C.; Matsos, F.; Reynard, O.; Taverne, C.; Merle, P.; Colombo, M. P.; O'Garra, A.; Trinchieri, G.; Caux, C. Reversal of tumor-induced dendritic cell paralysis by CpG immunostimulatory oligonucleotide and anti-interleukin 10 receptor antibody. *J. Exp. Med.* **2002**, *196* (4), 541–9.