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Targeting the tumor-draining lymph node with adjuvanted nanoparticles reshapes the anti-tumor immune response☆

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ABSTRACT

Accumulating evidence implicates the tumor-draining lymph node (TDLN) in tumor-induced immune escape, as it drains regulatory molecules and leukocytes from the tumor microenvironment. We asked whether targeted delivery of adjuvant to the TDLN, presumably already bathed in tumor antigens, could promote anti-tumor immunity and hinder tumor growth. To this end, we used 30 nm polymeric nanoparticles (NPs) that effectively target dendritic cells (DCs, $CD11c^+$) within the lymph node (LN) after intradermal administration. These NPs accumulated within the TDLN when administered in the limb ipsilateral (i.l.) to the tumor or in the non-TDLN when administered in the contralateral (c.l.) limb. Incorporating the adjuvants CpG or paclitaxel into the NPs (CpG-NP and PXL-NP) induced DC maturation *in vitro*. When administered daily i.l. and thus targeting the TDLN of a B16–F10 melanoma, adjuvanted NPs induced DC maturation within the TDLN and reshaped the $CD4^+$ T cell distribution within the tumor towards a Th1 ($CXCR3^+$) phenotype. Importantly, this also led to an increase in the frequency of antigen-specific $CD8^+$ T cells within the tumor. This correlated with slowed tumor growth, in contrast to unhindered tumor growth after c.l. delivery of adjuvanted NPs (targeting a non-TDLN) or i.l. delivery of free adjuvant. CpG-NP treatment in the i.l. limb also was associated with an increase in $CD8^+/CD4^+$ T cell ratios and frequencies of activated ($CD25^+$) $CD8^+$ T cells within the TDLN whereas PXL-NP treatment reduced the frequency of regulatory T ($FoxP3^+$ $CD4^+$) cells in the TDLN. Together, these data implicate the TDLN as a delivery target for adjuvant therapy of solid tumors.

1. Introduction

Cancer immunotherapy aims to activate or enhance the patient's adaptive immune system to kill tumor cells with antigen specificity [1]. A number of strategies have been described, including delivering vaccines comprised of particular tumor antigens (or tumor lysate) together with a strong dendritic cell (DC) adjuvant such as CpG oligonucleotide or PolyI:C [2,3] or adoptive T cell therapy using the patient's own T cells that are transfected to express a chimeric antigen receptor against a tumor antigen [4]. However, tumors progress in part by exploiting a variety of immune evasion and suppression mechanisms, including attracting a highly suppressive cell and cytokine repertoire in the tumor stroma [5] and inducing anergy, exhaustion or deletion of tumor antigen-specific T cells [6–8], even when anti-tumor effector T cells are circulating systemically. For example, in patients with melanoma, DC maturation and activation within the sentinel or tumor-draining lymph node (TDLN) is inhibited [9], leading to less effective presentation of tumor antigens to prime anti-tumor cytotoxic T and T helper cells [10], even in the presence of highly immunogenic melanoma antigens [11–14]. Immunotherapies that either boost anti-tumor immunity or reverse tumor-induced immune suppression could be useful in managing the disease to compliment surgical debulking of the primary tumor.

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Treatment with immune cell-activating adjuvant without co-administered antigen is emerging as an alternative approach to promote adaptive immune responses against endogenously produced tumor antigens that might simultaneously boost global immune cell activation status and dampen immune regulation [15,16]. However, such immunotherapy with adjuvants has been largely explored via systemic administration schemes, or those targeting non-associated LNs, and has demonstrated some success in improving tumor outcome [1,15–18]. Efficacy may be limited partly because of the immune suppressed state of the TDLN, which can locally dampen anti-tumor effector T cells [16]. Thus, adjuvant immunotherapy might be most efficacious in alleviating tumor burden when provided to antigen presenting cells in close proximity to tumor antigen [16,19] or when targeted to tissues of particular immunological significance in tumor immunity and progression.

As an alternative to systemic immune activation, secondary lymphoid tissues such as the lymph nodes (LNs) have been proposed as intriguing sites for targeted immunotherapy [20]. Targeted delivery of antigen and adjuvant to LNs is increasingly being explored in vaccination [21–25] as well as transplantation-associated [26] immunotherapy, given the role of the LN in supporting adaptive immune cell priming responses [27]. DCs are present in high numbers in LNs relative to peripheral tissues such as the skin, suggesting that delivery of antigen and adjuvant to the LN might enhance vaccine efficiency. However, in addition to being a primary site for initiation of effector immune responses, the LN can be an important site for induction of immune tolerance, because regulatory T (T_{reg}) cells require the LN for activation [28–31]. Moreover, lymphatic transport of antigen from the periphery to the draining LN has been implicated in tolerance induction against peripherally encountered antigens [7], such as tissue-specific self-antigens being regionally drained to and through the TDLNs. As such, the LN is an intriguing therapeutic target for not only vaccination to induce prophylactic effector immunity as previously shown [21–24,32], but also for potentially modulating endogenous immune responses, in attempting to redirect tolerogenic pro-tumor immune responses.

In the context of solid tumors, the TDLN is thought to participate in disease progression on multiple levels. First, tumor lymphangiogenesis is associated with tumor progression [33,34], and LN metastasis is one of the primary clinical indicators of tumor progression at the time of tumor resection. Second, we recently reported that the TDLN and tumor-associated lymphangiogenesis play critical roles in promoting tumor immune escape [6,7]. Tumor-derived antigen and cytokine drainage might therefore prime not only the pre-metastatic niche but also an immune suppressive environment to promote tumor immune escape.

We hypothesized that given the presumption that the TDLNs are bathed in tumor-derived antigens, delivery of adjuvant alone may be adequate to help redirect pro-tumor responses, counting on tumor drainage to provide the antigen from endogenous sources. As such, adjuvant therapy to the TDLN could exploit the unique immunological crosstalk taking place between the TDLN and tumor, potentially reshaping the local suppressive cytokine and chemokine milieu towards an inflammatory environment while simultaneously harnessing draining tumor antigen to prime anti-tumor immunity or blunt pro-tumor immune escape pathways.

To test this hypothesis, we incorporated Toll-like receptor (TLR) ligands CpG oligonucleotide (a TLR-9 agonist [35]) or paclitaxel (PXL, a TLR-4 agonist [36]) in Pluronic-stabilized poly(propylene) sulfide (PPS)-core 30 nm nanoparticles (referred to as NPs, using this abbreviation to refer specifically to these nanoparticles) developed by our laboratory, which target immune cells resident within the draining LNs [21,37,38]. We explore how local adjuvant therapy can induce inflammation in the TDLN and reshape immune regulation in the tumor.

2. Materials and methods

2.1. Reagents

All reagents were obtained from Sigma–Aldrich (Büchs, Switzerland) unless stated otherwise. Cell culture grade media, serum, and antibiotics were from Life Technologies (Basel, Switzerland) unless otherwise noted.

2.2. Animals

C57BL/6 mice were purchased from Harlan. 6–8 wk old mice weighing 20 g were used for this study. All protocols were approved by the Veterinary Authority of the Canton Vaud according to Swiss law. Isofluorane was used as anesthesia. Mice were euthanized by CO_2 asphyxiation or cervical dislocation. Mice were injected intradermally with a bolus of 30–50 μL in the forelimb or intratumorally with 10 μL rhodamine-dextran. 0.5×10^6 cells were intradermally allografted in the left dorsal skin of the animal on day 0 and treatments were administered daily from day 4–9. Naïve or control (PBS) treated mice were used in each set of experiments. Tumor volume was calculated as the product of the width, length and height. Tumor volumes of naïve or control treated mice were used to normalize tumor volumes from different experiments. Three or more independent experiments were performed.

2.3. Cell culture

B16–F10 melanoma cells were cultured in Dubelcco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin (PS). Bone marrow-derived dendritic cells DCs were harvested from C57BL/6 mice as described [32] and used at day 8 after isolation. Briefly, femurs and tibiae were removed, the surrounding muscle tissue was detached and the bones were kept in RPMI 1640 media. Marrow was flushed out with RPMI 1640 using a syringe and passed through a 70 μm nylon cell strainer (BD Biosciences) to remove debris. Cells suspensions were centrifuged and resuspended in complete RPMI 1640 medium supplemented with penicillin-streptomycin, 50 mM beta-mercaptoethanol, FBS and sodium pyruvate, which was filtered through a vacuum driven disposable filtration system (MILLIPORE Stericup Express PLUS 0.22 μm, Millipore corporations, Massachusetts, USA). Cells were seeded in 100 mm diameter bacteriological petri dishes (BD Biosciences, San Jose, CA, US) at 5×10^6 cells per dish in 10 ml of complete RPMI medium containing 100 μl of recombinant mouse granulocyte-macrophage colony-stimulating factor (rmGM-CSF). At day 3 after isolation an additional 10 ml of complete medium containing rmGM-CSF were added to the plates. At day 6 after isolation, half of the culture supernatant was collected and centrifuged, and the cell pellet was resuspended in 10 ml of fresh complete medium containing rmGM-CSF and transferred back to the original plate. DCs on day 8 after isolation were transferred in 50 ml tubes, centrifuged and resuspended in fresh complete medium. Cells were plated in 96-well plates (BD Biosciences) at a density of $0.3–0.4 \times 10^6$ cells per well in 100 μl. 80 μl of fresh complete medium were added per well and plates were incubated at 37 °C until the treatments were prepared. Treatments were added at a volume of 20 μl.

2.4. In vivo fluid clearance and migration assay

10 μL 10,000 or 70,000 Da rhodamine-dextran (Sigma–Aldrich) was injected intratumorally. 30 min later, LNs i.l. and c.l. to the tumor were excised and homogenized in 500 l T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL, USA) using Lysing Matrix D (MP Biomedicals, Illkirch, France) on a FastPrep-24 Automated Homogenizer (MP Biomedicals). Fluorescence was measured using a Safire2 TECAN plate reader (Tecan Group Ltd, Männedorf, Switzerland).

2.5. Immunofluorescence

30 min after rhodamine-dextran or AF488-NP injection, frozen LNs were cryosectioned (40 μm), counterstained with DAPI (Vector Laboratories, Burlingame, CA) and imaged using a LSM 510 confocal microscope (Carl Zeiss, Feldbach, Switzerland).

2.6. Nanoparticle synthesis and characterization

Pluronic-stabilized poly(propylene sulfide) (PPS) NPs with average diameters of 30 nm were synthesized by inverse emulsion polymerization as described previously [21,22,32,39,40]. Pluronic F-127 (a block copolymer of polyethylene glycol and polypropylene glycol terminated by hydroxyl groups) was used alone or in combination with carboxyl-terminated Pluronic derivatized as previously described [22,39,40]. Polymerization in the hydrophobic core results in PPS chains with a terminal thiolate, which can lead to stabilization of the core by intermolecular disulfide crosslinking [39]. However, since crosslinking cannot reach completion, remaining free sulfide groups on the NP surface were irreversibly capped by reaction with the alkylating reagent iodoacetamide. Alternatively, NP core thiols were reacted with a Dy488-meleimide 24 h at RT. Free dye was removed by dialysis against MilliQ water using 100,000 Da MWCO membranes. An absence of free dye was confirmed by high performance liquid chromatography (Waters, Montreux-