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Synapse-directed delivery of immunomodulators using T-cell-conjugated nanoparticles

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ABSTRACT

Regulating molecular interactions in the T-cell synapse to prevent autoimmunity or, conversely, to boost anti-tumor immunity has long been a goal in immunotherapy. However, delivering therapeutically meaningful doses of immune-modulating compounds into the synapse represents a major challenge. Here, we report that covalent coupling of maleimide-functionlized nanoparticles (NPs) to free thiol groups on T-cell membrane proteins enables efficient delivery of compounds into the T-cell synapse. We demonstrate that surface-linked NPs are rapidly polarized toward the nascent immunological synapse (IS) at the T-cell/APC contact zone during antigen recognition. To translate these findings into a therapeutic application we tested the NP delivery of NSC-87877, a dual inhibitor of Shp1 and Shp2, key phosphatases that downregulate T-cell receptor activation in the synapse, in the context of adoptive T cell therapy of cancer. Conjugating NSC-87877-loaded NPs to the surface of tumor-specific T cells just prior to adoptive transfer into mice with advanced prostate cancer promoted a much greater T-cell expansion at the tumor site, relative to co-infusing the same drug dose systemically, leading to enhanced survival of treated animals. In summary, our studies support the application of T-cell-linked synthetic NPs as efficient drug delivery vehicles into the IS, as well as the broad applicability of this new paradigm for therapeutically modulating signaling events at the T-cell/APC interface.

1. Introduction

Immune cells communicate via the formation of a receptor-containing adhesive contact zone termed an immunological synapse [1]. Upon encounter with antigen-presenting cells (APCs), T lymphocytes spatially rearrange membrane receptors, accessory molecules and downstream signaling molecules toward the T cell-APC junction to facilitate the accurate interaction of stimulatory and inhibitory ligands with their respective receptors, to modulate T-cell expansion and, ultimately, to determine T-cell fate [2]. Therefore, regulation of these molecular interactions arises as an important therapeutic strategy to prevent pathological self-reactivity (autoimmunity) or, conversely, to boost immunity against infections or tumor cells [3]. To this end, antibodies targeting stimulatory receptors (CD28, OX-40, 4-1BB) or inhibitory molecules (e.g., PD-1) in the immunological synapse have entered advanced clinical testing [4], and an antibody blocking the inhibitory receptor CTLA-4 expressed by activated T-cells has recently received FDA approval for cancer therapy [5]. A challenge with targeting proteins involved in signaling at the IS is that these receptors may be sterically shielded from antibody or recombinant ligand therapeutics by virtue of the tight contact formed between T-cells and antigen-presenting cells or target cells [6–8]. For example, CTLA-4 is sequestered in intracellular stores in T-cells and on T-cell receptor triggering, the receptor is delivered to the cell surface directly within the synapse [7,9]. In addition, potent ligands that promote T-cell function by engaging stimulatory receptors or blocking inhibitory receptors have the potential for serious autoimmune consequences when administered systemically in a manner that allows the entire lymphocyte compartment to be stimulated [10,11]. Thus, strategies to focus these T-cell-amplifying signals on antigen-specific cells attacking a disease target are desired to simultaneously increase the efficacy and saftey of these agents.

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In parallel to efforts targeting cell surface receptors involved in T-cell stimulation, membrane-permeable small molecule compounds that can suppress or activate T-cell receptor (TCR) proximal intracellular signaling pathways have undergone preclinical and early-stage clinical evaluation [12–15]. Critical kinases, phosphatases, and adapters involved in TCR signaling associate with receptors or lipid rafts trafficked into the IS, leading these critical intracellular signaling components to also be enriched in the synapse at the cytoplasmic face of the T-cell membrane [13,16]. Clinical success of such synapse signaling-targeting compounds will hinge on delivering therapeutically meaningful doses of these drugs to the IS where these pathways are active. In addition, like reagents targeting receptors involved in the regulation of T-cell priming, these small-molecule drugs have potential for serious off-target as well as on-target autoimmune consequences, and their safety and efficacy will likely require a means to focus their action on disease-specific lymphocytes.

An attractive clinically-relevant setting for targeted delivery of immunomodulatory drugs to T-cells is in adoptive cell therapy (ACT). In ACT, autologous disease-specific T-cells are isolated from patients [17,18] or generated *ex vivo* by genetic engineering [19,20], stimulated to differentiate into potent effectors, and then reinfused into the patient to treat metastatic cancer or infectious disease [21,22]. To enhance the efficacy of ACT, we recently showed that lipid or polymer particles in the 100–300 nm size range can be linked to T-cells via maleimide-thiol conjugation without compromising cell function [23]. Using this simple *ex vivo* conjugation process, we showed that immunocytokines that promote T-cell function could be slowly released from cell-bound nanoparticles (NPs) and primarily recaptured by particle-carrying cells in autocrine signaling loops. Surface-decorating tumor-specific T cells with cytokine-loaded NPs greatly increased T-cell number, anti-tumor function, and longevity after subsequent *in vivo* transfer [23,24].

In this study, we investigated whether, beyond providing infused T cells with autocrine sources of growth factors, covalent coupling of maleimide-functionlized nanocarriers to free thiol groups on T-cell membrane proteins could enable efficient delivery of therapeutic small-molecule drugs into the T-cell synapse. We assessed by time-lapse microscopy whether plasma membrane-anchored NPs alter their surface distribution in response to the dynamic behavior of their carrier T-cell and polarize toward the nascent immunological synapse at the T-cell/APC contact zone, and we identified by mass spectrometry predominant anchor proteins of T-cell-conjugated NPs. To evaluate the therapeutic potential of T-cell-linked synthetic NPs as drug delivery vehicles into the immunological synapse we tested the delivery of NSC-87877 [25], a small molecule dual inhibitor of Shp1 and Shp2, key phosphatases which downregulate T-cell receptor activation in the synapse [26], in the context of adoptive T-cell therapy of cancer.

2. Methods

2.1. Preparation of lipid nanoparticles

To synthesize stable maleimide-functionalized liposomes, we hydrated a dry lipid film (1.2 mg total lipids) composed of the maleimide-headgroup lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidomethyl) cyclohexane-carboxamide] (MCC-PE), hydrogenated soybean phosphatidylcholine (HSPC), and cholesterol (all Avanti Polar Lipids) in a molar ratio of 40:30:30, respectively, with 50 mM HEPES/150 mM NaCl-buffer pH 6.5 (500 μl) at 62 °C for 2 h to form large multilamellar vesicles (MLVs). The resulting vesicles were sized by repeated extrusion at 62 °C through a polycarbonate membrane (Nucleopore, Whatman, NJ) of 200 nm pore size. After extrusion, liposomes were washed with 12 ml phosphate buffered saline pH 7.4 (PBS) and collected via ultracentrifugation at 30,000 rpm for 6 h. The liposome pellet was resuspended in 1 ml PBS and used immediately. For the preparation of fluorescent liposomes, 0.11 mg 1,1′-dioctacdecyl-3,3,3′,3-tetramethylindodicarbocyanine (DiD, Invitrogen) was admixed to the lipids before solvent evaporation. The SHP1/2 PTPase inhibitor NSC-87877 (EMD chemicals) was encapsulated in the aqueous core of liposomes by dissolving 300 μg NSC-87877 in 500 μl of the 50 mM HEPES/150 mM NaCl-lipid hydration buffer. Particle sizes were determined by dynamic light scattering on a Brookhaven 90Plus particle sizer.

2.2. Preparation of 2C effector T cells for adoptive transfer and retroviral transduction with click beetle red luciferase

Animals were housed in the MIT Animal Facility and all mouse studies were performed in accordance with protocols approved by the MIT Division of Comparative Medicine following federal, state, and local guidelines. To isolate antigen-specific T-cells, spleens of 2C TCR transgenic mice were harvested, macerated over a filter, and resuspended in ACK lysing buffer (Biosource, Rockville, MD). Effector 2C $CD8^+$ T cells were prepared by incubating splenocytes (3×10^6/mL) in complete RPMI 1640 with 1 ng/mL interleukin-7 (PeproTech, Rocky Hill, NJ) and 2 μg/mL concavalin A (Calbiochem, La Jolla, CA) at 37 °C. Two days later, dead cells were removed by Ficoll gradient separation (GE Healthcare) and $CD8^+$ cells were isolated using a mouse CD8 negative isolation kit (Stemcell Technologies, Vancouver, BC).

Introduction of luciferase into T-cells for bioluminescence imaging studies was performed by retroviral transduction. Concentrated click beetle red luciferase (CBR-luc)-expressing retrovirus [27] 1 mL, kindly provided by Dr. Michel Sadelain (Memorial Sloan-Kettering Cancer Center, New York) was preloaded onto six-well non-tissue culture treated dishes coated with RetroNectin (TakiraBio) and incubated at 37 °C incubation for 1 h. An equal volume of isolated T-cells (3×10^6 cells/mL supplemented with 10 ng mIL-2/mL) was added and centrifuged at 2000 × g for 30 min). 6 hr after spinoculation, 1 mL of fresh, prewarmed RPMI, containing 10 ng mIL-2 (PeproTech, Rocky Hill, NJ) was added. T cells were used for adoptive transfer experiments 1 day after gene transfer.

2.3. Nanoparticle conjugation and in situ PEGylation

Lipid NPs were conjugated to the surface of effector T lymphocytes as previously described [23]. Briefly, 60×10^6 T cells/mL were resuspended in serum-free X-Vivo 10 medium (Cambrex, East Rutherford, NJ), mixed with an equal volume of NPs (T cell:NP ratio = 1:1000), and incubated at 37 °C for 30 min with gentle agitation every 10 min. After a PBS wash to separate cells from unbound particles, residual maleimide groups present on cell-bound particles were quenched by incubation of 3×10^6 cells/mL with 1 mg/mL thiol-terminated 2 KDa poly(ethylene glycol) (PEG, Laysan Bio, Arab, AL) at 37 °C for 30 min in complete RPMI medium, followed by two PBS washes to remove unbound PEG.

2.4. Confocal and time-lapse videomicroscopy

We stained $CD8^+$ T cells with FITC-conjugated cholera toxin B subunit (FITC-CTB, Sigma–Aldrich, St. Louis, MO) as described previously [28]. For confocal imaging, T cells were transferred onto fibronectin (Sigma–Aldrich)-coated 10 mm teflon ring glass slides (Electron Microscopy Sciences, Hatfield, PA), fixed with 2% paraformaldehyde, mounted with ProLong Gold Antifade reagent (Life Technologies, Grand Island, NY) for 24 h and imaged with a Zeiss LSM 510 laser scanning confocal microscope. For cell migration analyses using time-lapse videomicroscopy we mixed cells with a collagen precursor solution (1.8 mg/mL type I bovine collagen, Advanced BioMatrix, San Diego, CA) and incubated at 37 °C to allow polymerization of the solution into a fibrillar collagen gel. T-cell trans-endothelial migration imaging was performed in 24 mm diameter 3 μm pore size Transwell plates (Costar). 1×10^5 MS1 endothelial cells (ATCC) were seeded on gelatin (0.2%)-coated upper wells and allowed to grow for 2 days until confluence. We plated 1 $x10^5$ $CD8^+$ effector T lymphocytes, conjugated with 100 DiD^+ NPs per T-cell onto confluent MS1 monolayers which had been pre-activated for 4 h with 25 ng/mL TNF-α (Preprotech). The T-cell chemoattractant MCP-1 (10 ng/ml, Preprotech), was added to the lower wells. Cells were imaged in a 5% CO_2 humidified environmental chamber on a Zeiss Axiovert 200 inverted microscope.

2.5. Mass spectrometry

Amine-modified polystyrene nanospheres (200 nm, Life technologies) were functionalized with maleimide groups using NHS-PEG_{24}-maleimide crosslinkers according to the manufacturer's protocol (Thermo Scientific, Rockford, IL), and were surface-coupled to 2C effector $CD8^+$ T lymphocytes as described under 2.3. 30 x 10^6 NP-decorated T cells were subsequently lysed in 3 ml of 9.5 M urea, 2% (w/v) CHAPS, 0.8% (w/v) Pharmalyte pH 3-10, 1% (w/v) dithiothreitol (DTT) and 5 mM Pefabloc (all Sigma–Aldrich) with vigorous vortexing, followed by 3 × 20 s sonication (40 Watt power) on ice. To purify NPs from T-cell lysate we pelleted NPs through a 20% sucrose layer at 13.000 g for 15 min and washed the NP-pellet three times with distilled water. We adapted the "filter-aided sample preparation (FASP)" procedure [29] to isolate and identify proteins that became linked to maleimide-functionalized NPs during cell conjugation. Each sample was suspended in 200 ?L of a solution of 8 M urea and 50 mM ammonium bicarbonate, pH 8.5. Dithiothreitol (Sigma–Aldrich) was added to a concentration of 10 mM and disulfide