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A multi-trimeric fusion of CD40L and gp100 tumor antigen activates dendritic cells and enhances survival in a B16-F10 melanoma DNA vaccine model

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ABSTRACT

Vaccination with tumor-associated antigens can induce cancer-specific CD8+ T cells. A recent improvement has been the targeting of antigen to dendritic cells (DC) using antibodies that bind DC surface molecules. This study explored the use of multi-trimers of CD40L to target the gp100 melanoma tumor antigen to DC. The spontaneously-multimerizing gene Surfactant Protein D (SPD) was used to fuse gp100 tumor antigen and CD40L, creating the recombinant protein SPD-gp100-CD40L. This "third generation" DC-targeting vaccine was designed to both target antigen to DC and optimally activate dendritic cells by aggregating CD40 trimers on the DC membrane surface. SPD-gp100-CD40L expressed as a 110 kDa protein. Analytical light scattering analysis gave elution data corresponding to 4-trimer and multi-trimer SPD-gp100-CD40L oligomers. The protein was biologically active on dendritic cells and induced CD40-mediated NF-κB signaling. DNA vaccination with SPD-gp100-CD40L plasmid, together with plasmids encoding IL-12p70 and GM-CSF, significantly enhanced survival and inhibited tumor growth in a B16-F10 melanoma model. Expression of gp100 and SPD-CD40L as separate molecules did not enhance survival, highlighting the requirement to encode gp100 within SPD-CD40L for optimal vaccine activity. These data support a model where DNA vaccination with SPD-gp100-CD40L targets gp100 to DC in situ, induces activation of these DC, and generates a protective anti-tumor response when given in combination with IL-12p70 and GM-CSF plasmids.

1. Introduction

Q3 Cancer vaccination has attracted renewed attention as a therapy for the treatment of tumor growth and metastasis. The use of Tumor Associated Antigens (TAA) is particularly promising. Therapeutic effects specific to cancer cells can be generated through the careful selection of TAA preferentially expressed on tumor cells [1,2]. In particular, it has been reported that DNA vaccination using an exogenous plasmid encoding TAA can induce cancer-specific cytotoxic T lymphocytes (CTL) with antitumor activity [3,4]. However, optimal CTL activity requires that the antigen be selectively and efficiently presented by antigen presenting cells (APC). APC, including dendritic cells (DC), are critical for the initiation, programming and regulation of anti-cancer immunity [5]. One approach to increase DNA vaccine efficacy is to encode molecular adjuvants within the vaccine. Previous studies have evaluated a number of molecular adjuvants including cytokines/chemokines as well as members of the TNF superfamily of ligands [6,7]. Of particular interest is the TNFSFL molecule CD40L, the cognate ligand for CD40, which is involved in DC activation.

Abbreviations: ALS, analytical light scattering; APC, antigen presenting cell; BMDC, bone marrow derived dendritic cells; CD40L, CD40 ligand; CTL, cytotoxic T lymphocytes; DC, dendritic cell; DLS, dynamic light scattering; GM-CSF, granulocyte macrophage colony-stimulating factor; GVAX, B16-F10 cells expressing GM-CSF; IRES, internal ribosome entry site; MHC, major histocompatibility complex; M_n, number-average molar mass; M_w, weight-average molar mass; R_g, radius of gyration; R_h, hydrodynamic radius; *Ro*, Raleigh ratio; SEAP, secreted alkaline phosphatase; SLS, static light scattering; SPD, surfactant protein D; TAA, tumor associated antigen; TNFSFL, TNF superfamily ligand.

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Melanoma-specific antigen gp100 is known to induce anti-gp100 immune responses and suppress melanoma tumor growth in DNA and viral vector vaccine models [8]. However, adjuvants encoded in the vaccine may further enhance the immune response to gp100. As important, targeting of tumor antigens directly to DC using the DC receptor DEC-205 has previously been shown to increase immune responses [9]. Similarly, it has also been shown that delivery of antigens to DC via CD40 can enhance cross-presentation of antigen to CD8+ T cells via MHC I [10,11].

CD40L stimulation induces the production of IL-12p70 and other cytokines by DC [12] and enhances the differentiation of effector T cells [13]. Based on previously published data [14,15] a 4-trimer secreted soluble form of CD40L has been shown to be particularly effective as a DNA vaccine adjuvant. Surfactant Protein D (SPD) was used as a scaffold to generate the 4-trimer CD40L complex. SPD is a member of the collectin family that spontaneously trimerizes and forms disulfide bonds to generate a four-trimer oligomer [16].

In addition to CD40L, other adjuvants previously tested in cancer vaccine models include GM-CSF and IL-12p70. Systemic co-administration of IL-12p70 [17] or GM-CSF [18] has been shown to induce antitumor immunity. Studies have also evaluated these cytokines as DNA-encoded adjuvants for DNA vaccines, where they have shown modest efficacy [19,20].

In the present study, a fusion protein (SPD-gp100-CD40L) was generated encoding the murine CD40L extracellular domain fused to the collagen-like domain of murine SPD, with gp100 antigen inserted within the SPD coding region. We reasoned that these soluble CD40L multi-trimers would deliver gp100 to DC while simultaneously activating the DC, thereby inducing an enhanced anti-tumor CD8+T cell response. As we report, SPD-gp100-CD40L protein was stable, formed large polymeric complexes, and was biologically active on DC, suggesting proper assembly of CD40L trimers. Co-delivery of SPD-gp100-CD40L, GM-CSF, and IL-12p70 plasmids by intramuscular injection enhanced survival of mice challenged with B16-F10 melanoma, and significantly suppressed tumor growth. This response was not observed with any other vaccine combination, and was not observed when gp100 and SPD-CD40L were delivered as separate molecules, with or without the addition of GM-CSF and IL-12p70. Overall, these data support a model whereby SPD-gp100-CD40L targets gp100 antigen to DC in situ, activates these DC via CD40 stimulation, and induces an immune response that, when augmented with GM-CSF and IL-12p70, controls tumor growth and enhances survival in a murine B16-F10 tumor model.

2. Materials and methods

2.1. Construction and preparation of DNA plasmids

Plasmid encoding human glycoprotein 100 (pgp100) was a gift of Dr. Patrick Hwu [21]. Plasmid encoding murine SPD-CD40L was generated as detailed [15]. To generate pSPD-gp100-CD40L, DNA encoding amino acids 25 to 596 (sequence KVPRNQD to EAGLGQV) of human gp100, incorporating the full extracellular domain or gp100, was inserted between amino acids 105 and 106 of mouse SPD within construct SPD-CD40L. The coding sequence was inserted between amino acid sequences GERGLSG and PPGLPGI of murine SPD. Murine IL-12p70 plasmid pIL-12 was purchased from Invivogen and encodes a single chain dimer of IL-12 p35 and p40 (InvivoGen). Murine GM-CSF plasmid was constructed using a codon-optimized gene encoding murine GM-CSF inserted into plasmid pcDNA3.1. Clone pgp100-IRES-SPD-CD40L was generated by placing an IRES sequence between human gp100 (amino acids 1-594) and murine SPD-CD40L [15]. All plasmids were propagated in *Escherichia coli* strain TOP10. DNA was initially prepared using the Giga Endofree plasmid kit (Qiagen, Inc.), followed by additional endotoxin removal with further rounds of Triton-X114 extraction [14]. All plasmid were confirmed by LAL endotoxin assay (Lonza Inc.) to be endotoxin free (<0.2 EU/ml) prior to use.

2.2. Western blot analysis

293T cells were transfected with constructs using Genjet Plus transfection reagent (Signagen Laboratories). Supernatant was collected, centrifuged and filtered 48-h later. Samples were then loaded on a 10% polyacrylamide-sodium-dodecyl sulfate gel (Bio-Rad) in the presence of DTT. Following electrophoresis, protein was blotted onto PVDF membrane (Pierce) by electrophoretic transfer, blocked, and probed with goat anti-mouse CD40L antibody (R&D Systems). For analytical light scattering analysis, supernatant following transfection of 293T cells with pSPD-gp100-CD40L was collected and concentrated 10-fold using an Amicon centrifugal filtration system with 100 kDa cutoff (Millipore).

2.3. Analytical light scattering to study hydrodynamic characterization of SPD-gp100-CD40L protein

SPD-gp100-CD40L was analyzed by Analytical Light Scattering (ALS) as described previously [22]. Briefly, a Hiload Superdex 200 size-exclusion column was used, controlled by an Akta FPLC system (GE), maintained at 10 °C in a chromatography refrigerator. Analysis was performed using a miniDAWN TREOS triple-angle static light scattering detector and QELS dynamic light scattering detector (Wyatt), coupled to an Optilab rEX differential refractive index detector (Wyatt). 293T cell supernatant of SPD-gp100-CD40L was loaded on the Superdex 200 column at 10–50 μM starting concentration. The flow rate was maintained at 1 ml/min. Data was collected using ASTRA software. Wyatt miniDAWN TREOS detector equipped with three scattering detectors positioned at 42°, 90° and 138° was employed in the flow mode, allowing resolution of the angular and concentration-dependence of static light scattering (SLS) intensity for SPD-gp100-CD40L. The QELS detector was positioned at 90° relative to the incident laser light for the resolution of time- and concentration-dependence of dynamic light scattering (DLS) intensity fluctuation. Hydrodynamic parameters associated with solution behavior of SPD-gp100-CD40L, including weighted-average molar mass (M_w), number-average molar mass (M_n), weighted-average hydrodynamic radius (R_h) and weighted-average radius of gyration (R_g) were determined using SLS data based on the Zimm model [23] and by non-linear least-squares fit of DLS data to an autocorrelation function [24]. In both the SLS and DLS measurements, protein concentration (c) along the elution profile of each protein species was determined in the ASTRA software from the change in refractive index (Δn) with respect to the solvent as measured by the Wyatt Optilab rEX detector using the following relationship: $c = (\Delta n)/(dn/dc)$ where dn/dc is the refractive index increment of the protein in solution.

2.4. CD40 SEAP assay

The ability of CD40L recombinant protein to stimulate CD40-mediated signaling was measured using a CD40-293-SEAP cell line. This reporter cell line was generated from HEK293 cells stably transfected with a plasmid expressing human CD40 and a second plasmid expressing secreted alkaline phosphatase (SEAP) under the control of NF-κB [25]. Reporter cells were plated on a 96-well plate (80,000 cells per well) and cultured with 100 μl of 293T supernatant (generated by transfection of 293T cells with CD40L recombinant expression plasmid or pcDNA3.1 control). Supernatant was added

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