Cross-presentation of gp100 melanoma antigen

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Abstract

In this study we examine the utility of using CD8+ T cell hybridomas to measure the ability of bone marrow dendritic cells (BMDCs) to internalize cancer proteins and display them to cytotoxic T cells, a process termed cross-presentation. We test the ability of a newly generated T cell hybridoma called BUSA14 to detect cross-presentation of the melanoma antigen gp100. BUSA14 produces a dose-dependent response to human and mouse gp100 peptides. However, cross-presentation of gp100 by BMDCs using SK-MEL-28 human melanoma cell lysates or direct MHC class I-restricted presentation by B16 murine melanoma cells was not detected. Both SK-MEL-28 and B16 cells express gp100 protein by immunoblot, and gp100 as a membrane bound protein may be concentrated by cell fractionation techniques. We validated our cross-presentation assay with another T cell hybridoma B3Z to detect cross-presentation of the model antigen ovalbumin. Lastly, we determined that although BUSA14 expresses the co-receptor CD8, BUSA14 lacks CD3 expression, which likely impairs the ability of this hybridoma to respond to engagement of the T cell receptor and contributes to the inability to detect presentation of native gp100 protein. To resolve these issues, we plan to use primary gp100-specific T cells from pmel mice expressing the same T cell receptor as the BUSA14 hybridoma to detect presentation of gp100 protein. Ultimately, we plan to evaluate the requirements for cross-presentation of gp100, including a role for gamma-interferon-inducible lysosomal thiol reductase (GILT), a disulfide bond reducing enzyme.

Introduction

Although traditional cancer therapies have had limited success in the treatment of melanoma, improving the immune system's ability to detect and destroy cancer cells results in improved overall survival of metastatic melanoma patients. Cytotoxic CD8+ T cells are a major mechanism of immune-mediated tumor destruction. Dendritic cells are the primary cell type to present cancer-associated antigens and activate CD8+ T cells. This process termed cross-presentation is not fully understood. An improved understanding of the mechanisms regulating cross-presentation will likely be translated into improving T cell-mediated tumor therapies. T cell hybridomas are a cost-effective and efficient method to detect cross-presentation. The BUSA14 hybridoma was recently created by Dr. Lea Eisenbach's laboratory and expresses a T cell receptor recognizing a peptide derived from gp100 and presented by MHC class I. This hybridoma expresses LacZ under the IL-2 promoter, providing a rapid readout of signaling through the T cell receptor. We tested the ability of this hybridoma to detect cross-presentation of gp100 protein with the ultimate goal of evaluating requirements for efficient cross-presentation of melanoma antigens, including a role for GILT.

Materials and Methods

Cell lines: T cell hybridoma BUSA14 recognizes $gp100_{(25\text{-}32)}$ peptide presented by the MHC class I molecule H-$2D^b$, and B3Z recognizes OVA peptide (SIINFEKL) presented by the MHC class I molecule H-$2K^b$. Both T cell hybridomas have a LacZ reporter expressed under the IL-2 promoter to detect downstream T cell receptor signaling. Human melanoma cell lines (SK-Mel-28, WM-2664, A375 and SK-Mel-3) and murine melanoma cell line B16.F10 were tested for gp100 expression and used as a source of native gp100 protein. Murine keratinocyte line PDV and human epithelial line HEK-293T do not express gp100 protein and served as negative controls. Cell lines were cultured in RPMI or DMEM media fortified with 10% FBS and antibiotics.

Antigen presentation assay: BMDCs were generated from wild-type and GILT-/- mice femurs after 5 d of culture with media containing 20 ng/ml GM-CSF. Antigen was incubated with immature BMDCs for 2 h, then BMDCs were matured with 10 ng/ml LPS for 8 h and co-cultured with the T cell hybridoma for 18 h. Freeze thaw lysates of SK-Mel-28 and HEK293T cells were used as a source of native protein antigen. Human (KVPRNQDWL) and mouse gp100 peptide (EGSRNQDWL) and OVA peptide served as controls (1-10,000 ng/ml). In direct antigen presentation assays, BUSA14 T cell hybridoma was cultured with B16F10 melanoma or PDV cells (negative control) for 18 h. β-galactosidase (LacZ) activity was measured by lysing cells, exposing to chlorophenol red-β-D-galactopyranoside (CPRG) substrate, and measuring OD_{570}/OD_{650}.

Immunoblotting: Cells were lysed with 1% Triton X-100 in TBS and resolved with SDS-PAGE. Cell fractionation was achieved by hypotonic lysis in 10mM Tris pH 7.5 and a Dounce homogenizer. The post-nuclear supernatant was centrifuged for 45,000 rpm for 45 min to generate the membrane pellet and cytosolic fraction. After transferring to Immobilon-P membrane, gp100 was detected with anti-gp100 (EP4863(2), 120 ng/ml). GAPDH (polyclonal, 80 ng/ml) served a as loading control.

Protein assay: Flow cytometry: Cells were stained in PBS containing 0.1% bovine serum albumin and 0.1% sodium azide with CD3-PE, CD8-APC, H-$2D^b$-PE, and CD11c-APC. Fluorescence was measured on a BD LSRII flow cytometer.

CD3/CD28 activation assay: BUSA 14 and B3Z hybridoma cells were stimulated with plate-bound anti-CD3ε (145-2C11;10 μg/ml) and soluble anti-CD28 (37.51; 2 μg/ml). CPRG assay was performed after 6 h.

Results

1.) BUSA14 hybridoma displays a dose-dependent response to human and murine gp100 peptides. The BUSA14 T cell hybridoma recognizes human gp100 peptide with approximately 10-fold greater efficiency than mouse gp100 peptide.

Figure 1

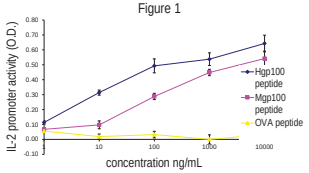

Figure 1: BUSA14 T cell hybridoma cells were cultured with wild type BMDCs that were loaded with mouse gp100 peptide, human gp100 peptide or OVA peptide (negative control) at varying concentrations.

2.) Gp100 is expressed in murine melanoma B16.F10 and human melanoma cell line SK-Mel-28. Gp100 is a membrane bound protein and can be enriched through membrane fractionation.

Figure 2

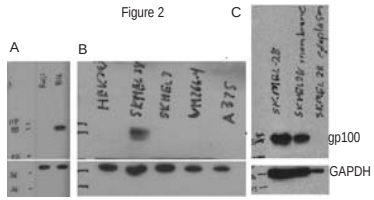

Figure 2: A) Gp100 expression in murine melanoma cell line B16-F10. B) Gp100 expression in human melanoma cell line SKMEL28; gp100 was not detected in human melanoma cell lines SK-Mel-3, WM266-4 and A375 (1 x 10^5 cell equivalents/lane). C) Detection of gp100 in SK-Mel-28 membrane fraction, but not in the cytoplasmic fraction. GAPDH served as a loading control.

3.) BUSA14 hybridoma did not detect cross-presentation of native human gp100 from melanoma lysates by BMDCs or direct presentation of gp100 from B16 murine melanoma cells. Wild-type and GILT-/- BMDCs express equivalent MHC class I (H-$2D^b$).

Figure 3

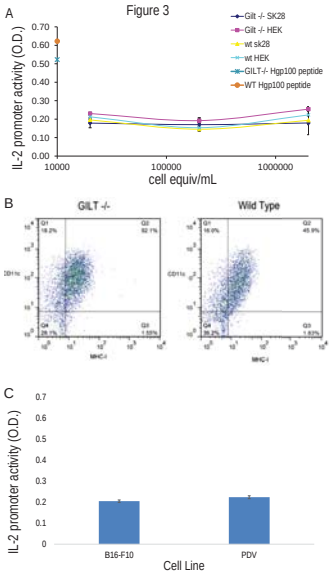

Figure 3: A) BUSA14 hybridoma cells were co-cultured with BMDCs cultured with various concentrations of SK-Mel-28 lysates. Human gp100 peptide at 10000ng/mL served as positive control and HEK293T lysates served as a negative control. B) FACS analysis demonstrating similar DC purity (CD11c+) and MHC class I expression in BMDCs generated from GILT-/- and wild type mice. C) BUSA14 hybridoma cells were co-cultured with B16.F10 tumor cells or PDV cells (negative control).

4.) Validation of the cross-presentation assay using the B3Z hybridoma recognizing a ovalbumin peptide presented by MHC class I. A dose-dependent response in cross-presentation was observed with increasing ovalbumin protein concentration.

Figure 4

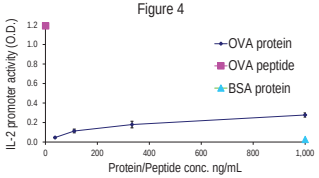

Figure 4: B3Z T cell hybridoma cells were cultured with BMDCs that were loaded with full-length ovalbumin (OVA) protein, OVA peptide (positive control), or full-length bovine serum albumin (BSA, negative control).

5.) BUSA14 T cell hybridoma expresses the CD8 co-receptor, but lacks expression of CD3. The CD3 protein complex associates with the T cell receptor and mediates signaling. The BUSA14 T cell hybridoma cannot be stimulated with antibodies to CD3 and CD28. Results are compared with B3Z as a positive control. The lack of CD3 on BUSA14 likely contributes to the inability of BUSA14 to detect cross-presentation of gp100 by BMDCs and direct presentation of gp100 by B16 melanoma cells.

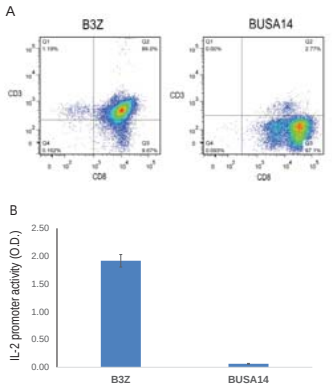

Figure 5: BUSA 14 and B3Z comparison A) Cells were stained with anti-CD3-PE and anti-CD8-APC antibodies and analyzed using FACS. B) Cells were cultured in a 96 well plate with immobilized CD8 and soluble CD28 antibodies.

Discussion and Conclusions

The BUSA14 was highly specific and sensitive for detecting $gp100_{(25\text{-}32)}$ peptides presented by BMDCs but was unable to produce a measurable signal when using cell lysates shown to contain gp100 protein on immunoblot. After several failed attempts to optimize our cross-presentation protocol to produce detection of gp100 by BUSA14, we validated our protocol using a different T cell hybridoma B3Z specific for an ovalbumin peptide and detected cross-presentation of ovalbumin using BMDCs fed purified ovalbumin protein. We compared the BUSA14 hybridoma with the B3Z hybridoma and discovered an absence of the CD3 receptor on the BUSA14 hybridoma. We hypothesize that the absence of CD3 decreases BUSA14's ability to detect presentation of gp100 protein. It is also possible that the quantity of gp100 present in the cell lysates is insufficient to produce a measurable response. Future experiments involve testing the BUSA14 hybridoma with purified, recombinant gp100 protein. Additionally, we will test cross-presentation protocol using primary gp100-specific T cells obtained from pmel mice in order to verify the ability of BMDC to cross-present gp100.

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