

**THE ROLE OF GILT IN THE CROSS PRESENTATION OF THE MELANOMA ANTIGEN GP100**

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## **Abstract**

In this study we examine the utility of using CD8<sup>+</sup> 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.

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## **Introduction/Significance**

Many of the current therapies to cancer involve cytotoxic agents that have significant side effects usually related to the unintended consequence of destroying healthy cells. By targeting the approach to destroying only malignant cells immunotherapy promises better efficacy with significantly less side effects. Much like the response to cancer, viral infections also require that the cellular arm of the immune system be activated to target affected cells. By understanding the pathways involved in developing an immune response to intracellular pathogens, better immunotherapy can be created to help the immune system identify and eliminate these threats.

To help the immune system identify potential threats, proteins from the extracellular or intracellular environment are processed into short peptide fragments and attached to receptors located on the outside of cells. The receptors are called major histocompatibility complexes (MHCs) and there are two important classes of these receptors named MHC class I and MHC class II.

Although it is not always the case, the MHC class I receptors are generally involved with activating the cellular response of the immune system and the MHC class II activates the humoral response. The humoral response involves the release of antibodies that are able to attach to pathogens or their products thereby inactivating or aiding the elimination of these threats. The humoral response may not always be able to eliminate intracellular threats or tumor cells. MHC class II can also be involved with cellular immunity in the case of macrophages presenting to CD4+ helper cells for permission to destroy an organism that they have phagocytosed.

The cellular immune response of an organism is primarily charged with the destruction of infected cells and tumor cells. If an infected cell has the ability to destroy intracellular pathogens, as is done in a macrophage, a signal is provided by a CD4+ helper cell to kill the organism. Tumor cells and infected cells incapable of destroying intracellular organisms must be dealt with by a different mechanism. These cells are eliminated by CD8+ T cells that identify MHC class I bound cytoplasmic-derived pathological processes. All cells express MHC class I receptors on their outer cell membranes and these receptors are loaded with short peptide

fragments derived from proteins in their cytoplasm. Using the MHC class I receptor, self-antigens as well as intracellular pathogenic antigens are displayed to CD8+ T cells. To prevent the destruction of normal cells expressing self-antigens, CD8+ T cells must first be activated to identify and kill MHC class I bound peptides derived from only pathogens or tumor cells. This important activation step is termed cross-priming or cross-presentation.<sup>1</sup>

Cross presentation begins when a dendritic or antigen presenting cell endocytosis an abnormal protein that may have entered the extracellular space from the lysis of a tumor cell or an infected cell. Once inside the dendritic cell, the protein is processed into small peptide fragments that are bound to MHC class I receptors and transported to the cell membrane of the dendritic cell. This mechanism is termed cross presentation because instead of ending up on MHC class II receptors that normally present extracellular antigens to CD4+ T cells, the antigen is crossed over into the MHC class I pathway that normally handles intracellular antigens. This is an important pathway because it allows CD8+ T cells to be activated to target proteins on MHC class I receptors.<sup>6</sup>

The tumor cells of melanoma cancer express some unique proteins that are potential targets for the immune system. By helping the immune system identify and kill cells that contain these proteins we may improve an organism's ability to eliminate melanoma tumor cells. One potential protein target that has been identified from the melanoma cancer line is called gp100. Because gp100 is made inside tumor cells it will normally be presented on MHC class I receptors located on the outside of the tumor cells. The appropriate response to tumor cells expressing gp100 peptides on MHC class I receptors is elimination by CD8+ T cells. As mentioned earlier these CD8+ T cells must first be activated by dendritic cells cross presenting gp100 proteins obtained from tumor lysates in the extracellular environment.<sup>2</sup>

The mechanism of cross presentation has not been fully elucidated and it is likely that several pathways exist. One such pathway utilizes the enzyme gamma interferon-inducible lysosomal thio reductase (GILT). Previous experiments on the role of GILT in both MHC class I and class II pathways have shown that some proteins require GILT and some do not. Further it has been shown that some proteins are presented better without GILT. One proposal for the different effects that GILT has on antigen presentation is that it may be involved with breaking disulfide

bonds that form the tertiary structure of some proteins. Because gp100 contains two disulfide bonds we hypothesize that it may be a GILT dependent protein. Understanding the mechanisms and enzymes involved in the cross presentation of gp100 is an important first step in developing treatment for individuals suffering from melanoma cancer.<sup>5,6,7,8</sup>

It can be difficult to accurately measure the cross presentation of any antigen and gp100 is no exception. We are excited, however, to try a new method of measuring the cross presentation of gp100 using a specialized hybridoma provided to us by Dr. Eisenbach and her team at The Weizmann Institute of Science, Rehovot, Israel. This hybridoma has the ability to detect gp100 peptides presented on MHC class I peptide and activate the LacZ reporter gene. This hybridomas will be used to quantify the amount of cross presentation of gp100 and how cross presentation changes in GILT knockout mice. <sup>3</sup>

## Research Material and Methods

### *Media and Reagents*

Mouse cell lines were cultured in cRPMI (RPMI 1640, 1% L-glutamine, 1% penicillin/streptomycin, 50  $\mu$ M 2-mercaptoethanol, 10% heat inactivated FBS GIBCO). Human cell lines were cultured in cDMEM (Dulbecco's Modification of Eagles Medium, 4.5 g/L glucose, 1% L-glutamine, 1% sodium pyruvate, 1% penicillin/streptomycin, 10% heat inactivated FBS GIBCO). BMDC were cultured in DCgrow (cRPMI, 20ng/mL GMCSF). CPRG lysis buffer (PBS with 9 mM  $MgCl_2$ , 0.125% NP40, 0.3 mM Chlorophenol Red- $\beta$ -D-galactopyranoside) was used to detect activity of the BUSA 14 IL-2/LacZ reporter. FACS staining buffer: PBS containing 0.1% (m/v) bovine serum albumin, 0.1% Na<sub>3</sub>N.

### *Synthetic Peptides and Antibodies*

Human peptide gp100<sub>25-32</sub> (KVPRNQDWL) Sigma-Aldrich, Mouse Peptide gp100<sub>25-32</sub> ((EGSRNQDWL) Sigma-Aldrich, Anti-melanoma gp100 antibody [ab137078] Abcam. GAPDH loading control antibody (GA1R) Thermofisher. PE-MHC class I (H-2Db) and APC-CD11c eBioscience,

### *Cell lines*

Human melanoma cell lines SkMel28, SkMel3, A375 and WM266-4 were generously provided by Dr. Sekulic, Mayo Clinic Scottsdale AZ. HEK293t (human embryonic kidney, neg. control). The BUSA14 cell line is a LacZ inducible T cell hybridoma specific for human and mouse gp100 peptide consisting of residues 25-33 (gp100<sub>25-33</sub>) and was kindly provided by Dr. Lea Eisenbach [Cafri G, Sharbi-Yunger A, Tzehoval E, Eisenbach L (2013) Production of LacZ Inducible T Cell Hybridoma Specific for Human and Mouse gp100<sub>25-33</sub>

Peptides. PLoS ONE 8(2): e55583. doi:10.1371/journal.pone.0055583]



## *Mice*

C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). GILT knock out mice were obtained from Dr. Cresswell, Yale University. [M Maric et al. Science 294 (5545), 1361-1365. 2001 Nov 09.]

## *Preparing bone marrow dendritic cells*

Wild type C57BL/6 mice and GILT KO mice were sacrificed. The hind legs were removed and cleaned using scissors and clean Kim Wipes. Then the femur and tibias were placed in a 12.5 mm petri dish containing fresh RPMI media. After soaking for a few minutes the remaining tissue on the bones was removed. The bones were then placed in 70% ethanol for 1 min and then quickly washed with PBS three times to remove the ethanol. After washing the bones were placed immediately into a 12.5 petri dish containing fresh 25 mL RPMI. The ends of the bones were carefully cut off using sterile scissors while in the media. A 22-gauge needle was used to draw up the media in the dish and push out the bone marrow into the dish for each long bone. The entire mixture of media, marrow and bones were then filtered in to new 50mL centrifuge tube. The cells were centrifuged at 150g for 5 min and the media was aspirated off. Cells were resuspended in 5mL of ACK lysis buffer and the tube was slowly rotated by hand for 3 min to lyse the RBCs. Immediately 45mL of RPMI was added and the tube centrifuged again at 150g for 5 min. The media was aspirated off and the cells were resuspended in 20mL of DCgrow media and counted. Additional DCgrow was added to make the concentration  $10^7$  cells per mL. To a 24 well plate 1 mL of cell mixture was added to each well. At day 2, 1 mL of DCgrow was added to each well. At day 4, 1 mL of media was carefully aspirated off each well and replenished with 1mL fresh DCgrow. On day 6 the cells were ready for use in either the peptide assay or lysate assay

### *Immunoblotting*

Cell pellets of the 4 melanoma cell lines and HEK293t (neg control) were made by centrifuging  $10^7$  cells, aspirating off media and washing in PBS twice. Cells were then lysed with 1% Triton X-100 in TBS for 30 min on ice. A total of  $10^5$  cell equivalents of post-nuclear supernatants was resolved by SDS-PAGE (10% w/v acrylamide) and electrophoretically transferred to Immobilon-P membrane (Millipore, Bedford, MA). The membrane was blocked in PBS with 0.2% Tween 20 and 5% dehydrated milk, and then incubated with gp100 primary Ab (1:3000) and GAPDH antibody (1:5000) as loading control. The membranes were then washed, incubated with HRP-conjugated goat anti-rabbit or mouse IgG (1:4000; Jackson ImmunoResearch Laboratories) and enhanced chemiluminescent substrate (SuperSignal West Pico; Pierce, Rockford, IL), and exposed to film.

### *CPRG assay*

After the BUSA14 hybridoma and BMDC were co-cultured, activation of the BUSA 14 hybridoma was assessed by measuring LacZ quantity produced by the IL-2/LacZ reporter. Cells were washed with PBS two times and resuspended in CPRG lysis buffer for 1 min. Using a multi-tip pipet, the CPRG lysis buffer and lysed cells were then moved to a clear flat bottom 96 well plate. LacZ activity on the CPRG was measured using a Tecan Safire<sup>2</sup> microplate reader and  $\Delta$ OD was recorded with a 570nm measure and 630nm reference.

### *BUSA 14 response to mouse gp100<sub>25-32</sub> and human gp100<sub>25-32</sub> peptides.*

Frozen BMDC prepared from a prior experiment were thawed and cultured for 2 days. 60k of these BMDC in 50  $\mu$ l cRPMI and 60k BUSA 14 cells in 50  $\mu$ l in cRPMI were co-cultured in a 96 well plate in triplicate with 100  $\mu$ l of peptides: human gp100 @ 100  $\mu$ g/mL, mouse gp100 @ 100  $\mu$ g/mL or TRP-1 @ 200  $\mu$ g/mL After a co-culture of 8 hours the plate was centrifuged and the media was aspirated off. The CPRG assay was then performed.

### *Peptide dose response assay*

Fresh BMDC were harvested on day 6 by pipetting loosely adherent cells into a centrifuge tube and spinning for 5 min at 250g. The BMDC were then resuspended to a concentration of  $1.2 \times 10^6$  cells per mL in DCgrow. The BUSA 14 hybridoma was counted, spun and resuspended to  $1.2 \times 10^6$  cells per mL in similar manner. Serial peptide dilutions were made using the hp100 peptide in cRPMI media in ranges of 6-6000 ng/mL and 20-20000 ng/mL. In a 96 well round bottom culture plate 60k hybridoma cells in 50 $\mu$ L and 60k BMDC cells in 50 $\mu$ L were added to 100 $\mu$ L of peptide dilutions for a final peptide dilution of 3-3000 ng/mL and 10-10000 ng/mL per well in triplicate. After a co-culture of 8 hours the plate was centrifuged and the media was aspirated off. The CPRG assay was then performed.

### *Freeze thaw cell lysate*

SKmel28 cells and HEK293t cells at 80-90% confluence were counted and resuspended in RPMI without serum in a 15 mL conical to a concentration of  $10^8$  cell equivalents per mL. The cells were then frozen to -80C and allowed to thaw at room temperature. This was repeated 5 times. The freeze thaw lysate was then centrifuged and the supernatant was collected. Serial lysate dilutions were then made from the supernatant by diluting the lysate 1:10 in serum free RPMI.

### *Flow cytometry*

From the BMDC culture used in the cross-presentation assay immature wild type and GILT KO cells were collected before exposure to lysate. The cells were counted and  $10^6$  cells were placed in FACS tubes and spun down for 5 min at 250g and the aspirate was removed. The cells were then resuspended in 50  $\mu$ L of FACS Staining buffer with 1  $\mu$ g of Fc Block (BD) for 5 min on ice. Next 50  $\mu$ L of FACS staining buffer containing 0.5  $\mu$ g PE-MHC I and 0.5 $\mu$ g of APC-CD11c was added to each tube and incubated on ice for 30 min. The cells were washed with 1 mL of FACS and resuspended on 500 $\mu$ L of 1% paraformaldehyde. Cell-associated fluorescence was

measured using an LSRII flow cytometer (BD Biosciences), analysis was performed using FlowJo software (Tree Star, Ashland, OR).

#### *Cross-presentation assay*

On day 6 of BMDC culture for both wild type and GILT KO, the media was carefully removed and replaced with 900  $\mu$ L of SKmel28 or HEK293t (neg control) freeze thaw lysate in 3 different dilutions. The BMDC were allowed to culture in the lysate for 5 hours at which time the lysate was gently removed and replaced with 1 mL of DC grow. The lysate loaded BMDC were harvested by pipetting the loosely adherent cells from the 24 well plate into a centrifuge tube and spinning for 5 min at 250g. Loaded BMDC were then resuspended to a concentration of  $1.2 \times 10^6$  cells per mL in DC grow. In a 96 well round bottom culture plate 60k hybridoma cells in 50 $\mu$ L and 60k BMDC cells in 50 $\mu$ L were added in triplicate for each lysate dilution. LPS was added at a concentration of 10ng/mL to further BMDC maturation. After a co-culture of 18 hours the plate was centrifuged and the media was aspirated off. The CPRG assay was then performed.

#### *Direct-presentation assay*

180k BUSA14 cells were co-cultured with 60k B16 melanoma cells or 60k PDV cells (negative control) in a 96 well flat bottom plate in 200  $\mu$ l of cRPMI. After 12 hours of co-culture the plate was centrifuged and the media was aspirated off. The CPRG assay was then performed and  $\Delta$  OD was read by Tecan safire2 scanner.

#### *CD3/CD28 activation assay*

BUSA 14 and B3Z hybridoma cells were stimulated with plate-bound anti-CD3 $\epsilon$  (145-2C11; 10  $\mu$ g/ml) and soluble anti-CD28 (37.51; 2  $\mu$ g/ml). CPRG assay was performed after 6 h.

## Results

### **BUSA14 hybridoma displays a dose-dependent response to human and murine gp100 peptides.**

We show that the BUSA 14 hybridoma responds to the bone marrow dendritic cells that were loaded with either human or murine gp100 peptides but did not respond to the ovalbumin peptide, a negative control. The hybridoma is not only specific for the gp100 peptide but also demonstrates a logarithmic dose response. It was also observed that the hybridoma responds greater to the human version of the gp100 than the murine version. The human and murine versions of the gp100 peptide differ by three amino acids which may account for this observation.

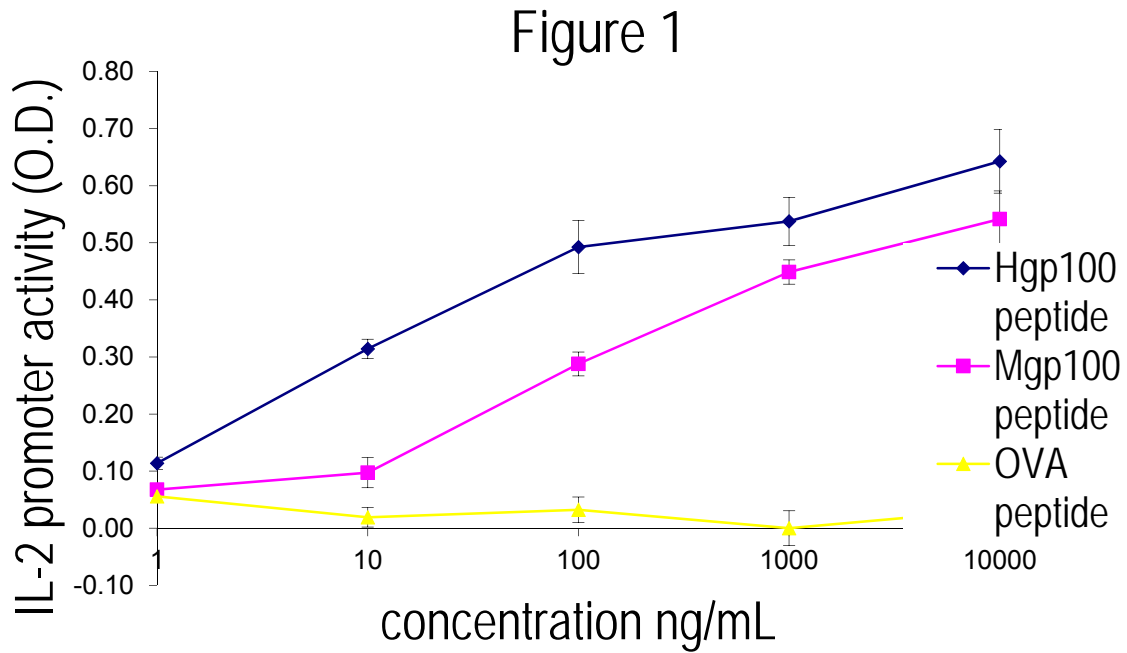


Figure 1: A) BUSA14 T cell hybridoma cells were cultured with wild type and GILT<sup>-/-</sup> BMDC that were loaded with mouse gp100 peptide, human gp100 peptide or OVA peptide (negative control) at varying concentrations.

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

To identify a source of native gp100 protein we performed western analysis of 4 human melanoma cell lines and identified gp100 present in the Sk-Mel-28 cell line. Cell fractionation was performed on the Sk-Mel-18 cell line and it was observed that the gp100 protein was located in the cell membrane fractionation. This suggests that gp100 protein concentration in the cell lysate may be increased through cell fractionation techniques. To identify a murine cell line that could autonomously display gp100 peptide fragments on its MHC-I receptor we performed western analysis on a murine cell line B16.F10 and verified the presence of gp100 protein.

Figure 2

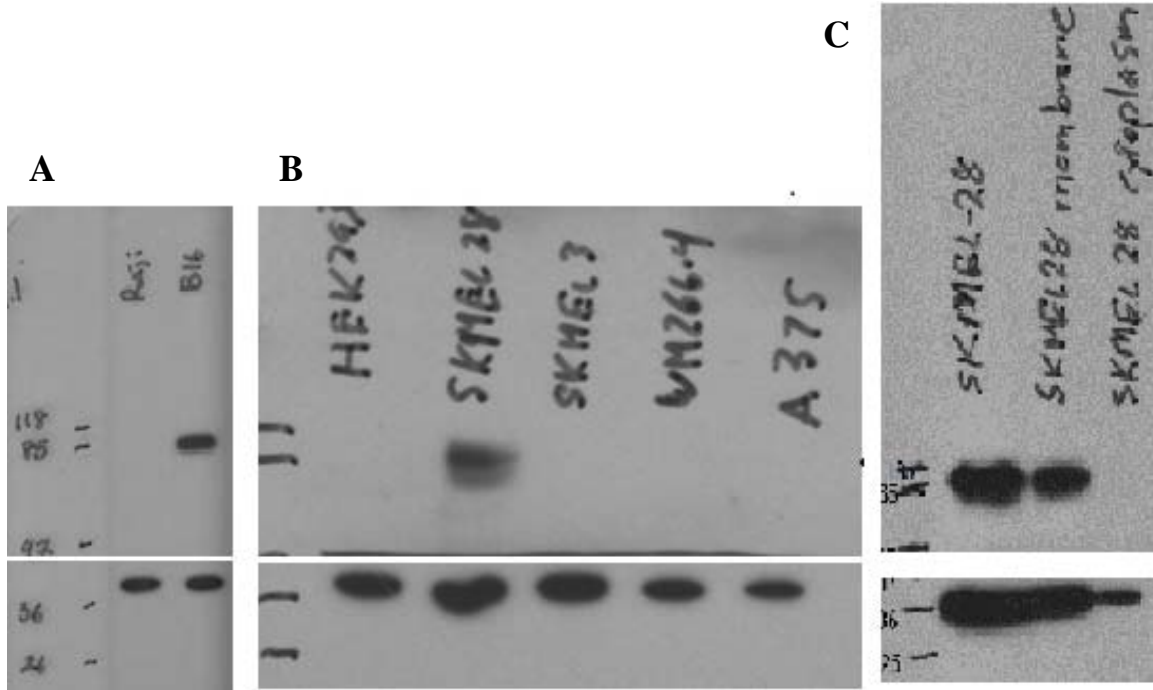


Figure 2: A) gp100 expression in human melanoma cell lines. 100k cell equivalents were loaded for four human melanoma cell lines (SK-MEL-28, SK-Mel-3, WM266-4 and A375), HEK293T as negative. B) gp100 expression in murine melanoma cell line B16-F10 controls. C) detection of gp100 in SK-Mel-28 membrane fraction. GAPDH served as a loading control.



**BUSA14 hybridoma did not detect cross-presentation of human gp100 from melanoma lysates by BMDCs or direct presentation of gp100 from B16 murine melanoma cells. Wild-type and GILT<sup>-/-</sup> BMDCs express equivalent MHC class I (H-2K<sup>b</sup>).**

We did not observe a dose specific response to bone marrow dendritic cells obtained from wild type and GILT knockout mice, pulsed with Sk-Mel-28 cell lysate previously shown to contain gp100. FACS analysis verified that the two populations of bone marrow dendritic cells contained similar percentages of MHC –I presentation. This was done to account for possible differences in quantity of dendritic cells capable of presenting the gp100 peptide.

Because we did not observe a measurable response to Sk-Mel-28 cell lysate, we further tested the hybridoma's ability to detect direct presentation of gp100 by B16 murine cells previously shown to contain gp100. Since all cells present internal proteins on MHC –I receptors for surveillance of the immune system we hypothesized that BUSA 14 would produce a response to B16 cells. We did not observe a measurable response to the B16 cells versus PDV cells our negative control. This lead us to consider problems with either our methods or in the hybridoma itself.

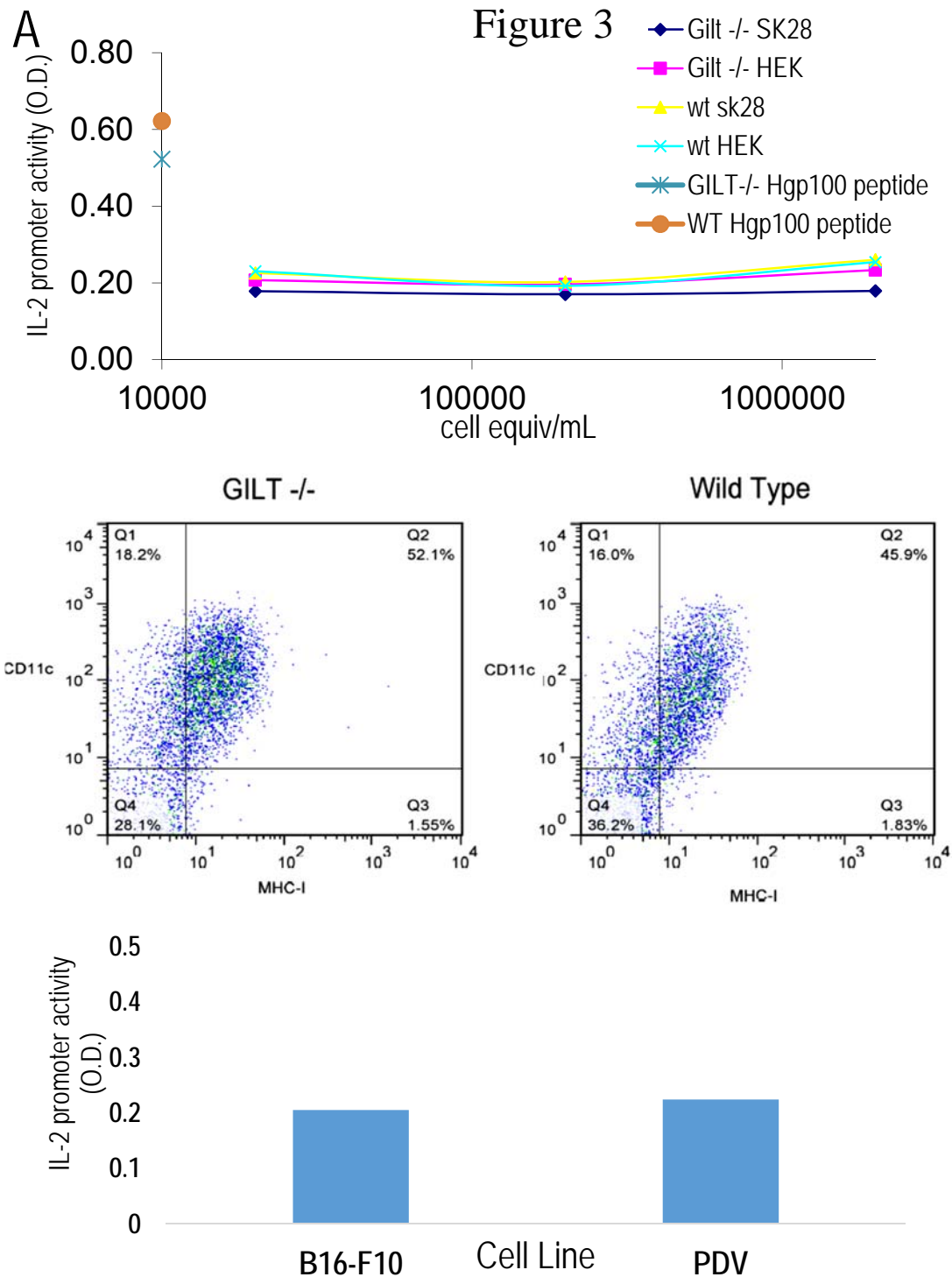


Figure 3: A) BUSA14 hybridoma cells were co-cultured with BMDCs cultured with various concentrations of SK-Mel-28 lysates and HEK293T lysates served as a negative control. B) FACS comparing concentration of MHC-I+ BMDC harvested from GILT-/- and wildtype mice. C) 100k BMDCs were cultured with 100k B16.F10 tumor cells or 100k PDV cells.

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

We then decided to obtain another hybridoma cell line, B3Z (shown to detect a protein called ovalbumin) to test our experimental design. We did not however use cell lysate as a source of ovalbumin but instead used pure ovalbumin protein. The B3Z hybridoma produced a measureable response to ovalbumin indicating that our experimental design was adequate. This led us to further explore the differences between the two hybridomas.

Figure 4

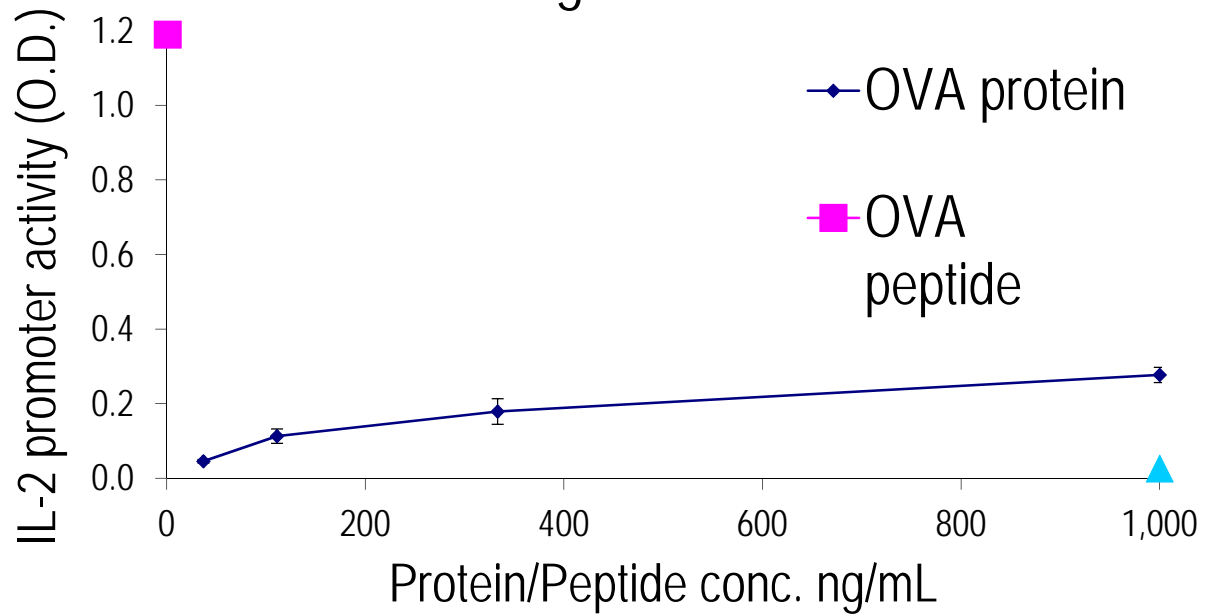


Figure 4: B3Z T cell hybridoma cells were cultured with BMDCs that were loaded with full-length ovalbumin (OVA) protein, OVA peptide (pos. control), or full-length bovine serum albumin (BSA, neg. control).

**BUSA14 T cell hybridoma expresses the CD8 co-receptor, but lacks expression of CD3. The CD3 protein complex associated with the T cell receptor and mediates signaling.**

We performed FACS analysis of the two hybridomas B3Z and BUSA14. It was observed that the BUSA14 hybridoma had very little expression of CD3, an essential part of the T cell receptor complex. To further test the BUSA 14 hybridoma we performed a CD3 activation assay that utilized plate bound CD3. CD28 was added to aid in activation. 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

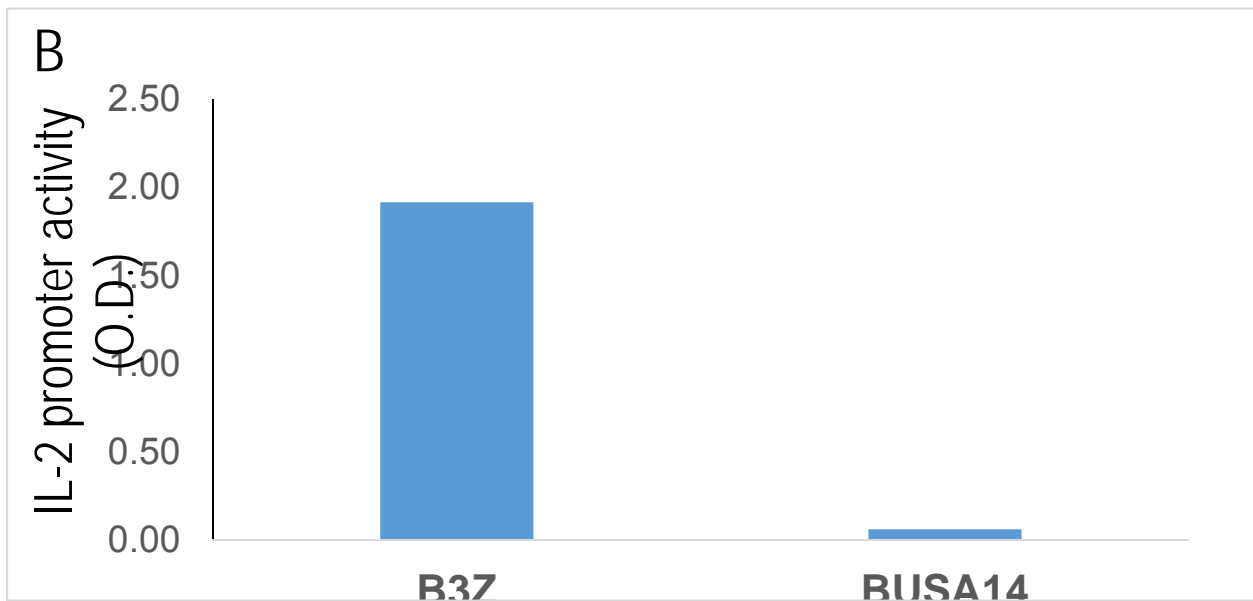
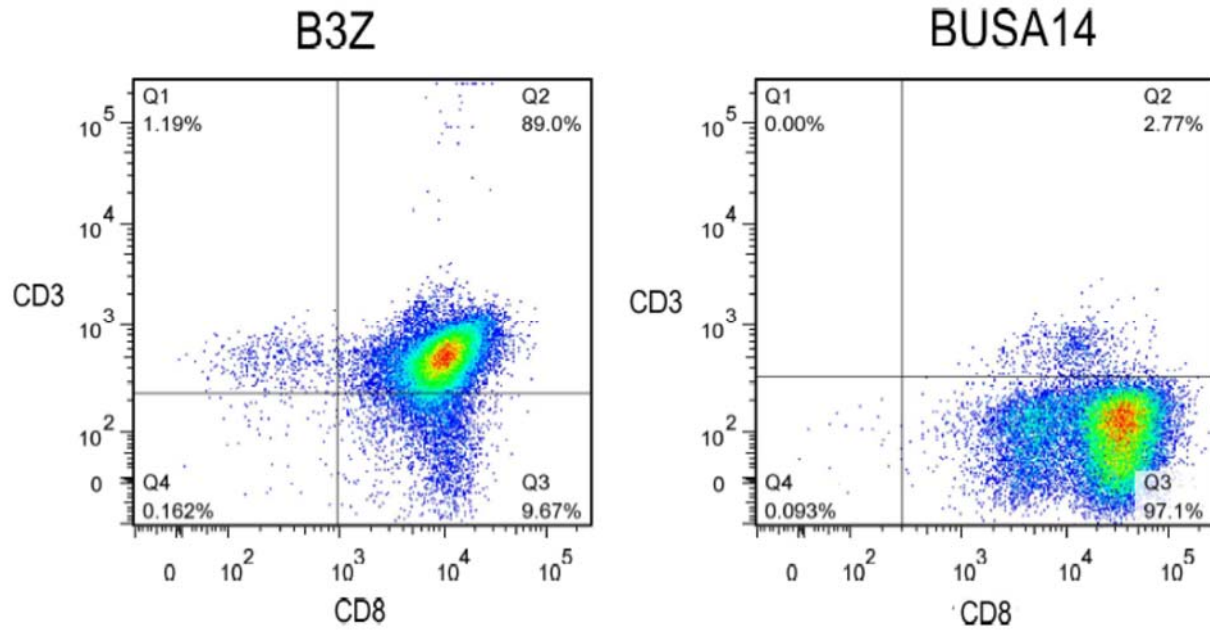


Figure 5: BUSA 14 and B3Z comparison A) Cells were stained with anti-CD3-PE and anti-CD8-APC antibodies and analyzed using FACS. B) Cells were cultured in a 96 well plate with immobilized CD8 and soluble CD28 antibodies.

## Discussion

The BUSA14 was highly specific and sensitive for detecting gp100<sub>(25-32)</sub> 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.

## **Conclusions**

This experiment originally sought to utilize a T cell hybridoma to quickly measure cross-presentation and see how GILT might be involved in cross-presentation. Scientists actively studying cross-presentation can use hybridomas to speed up the process of testing methods of changing cross-presentation. We discovered, however that hybridomas may be lacking critical cell components. The CD3 is known by some scientists who frequently use hybridomas but is not well documented in the literature. Since I was using hybridomas for the first time and did not have knowledge of the problems that hybridomas can have I spent considerable time trouble shooting my experimental methods. This was complicated by the fact that the hybridoma continued to have a dose specific response to the peptide. I hope that documenting this experiment and the problems that we encountered with BUSA 14 hybridoma may aid other researchers who are beginning to use hybridomas.



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