Impact of Unfolded Protein Response, USP14, and NEDD8 Inhibitors Upon Antigen Presentation in SaI/Ak and 4T1 Tumor Cell Lines

by
Jamila Godil

A THESIS

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AN ABSTRACT OF THE THESIS OF

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Abstract approved:_____

Brian Dolan

Antigen presentation is a mechanism used by cells to present peptides on the cell surface. Peptides, derived from both viral or self-proteins are presented on the cell surface by MHC Class I molecules to be recognized and detected by cytotoxic T cells. T cells comprise the defense system to protect cells from infection by intracellular pathogens and oncogenic transformation. T cells are constantly scanning the surface of cells for specific peptides, a signal for the T cell to kill the infected or transformed cell. The consequences of antigen presentation are important for a variety of clinical diseases. Erroneous detection by the T cell can result in labeling a healthy cell as diseased and lead to the development of an autoimmune disease. Antigen presentation can also be used in tumor immunotherapy. By manipulating a tumor cell, it can cause the presentation of own tumor peptides to be recognized and targeted by the T cell.

Three pathways that may assist in antigen presentation are the Unfolded Protein Response Pathway, USP14 Pathway and Nedd8 Pathway. Each pathway interacts with diverse aspects of antigen presentation. The Unfolded Protein Response combats unfolded proteins floating within the ER by using two sensor proteins, IRE1-alpha and PERK. USP14 pathway targets proteins for degradation by the proteasome. Nedd8 pathway binds and modified proteins called cullins, thereby, signaling cell division and protein degradation.

The goal of this research study is to analyze the total MHC Class I levels at the cell surface after treatment with inhibitor. The three mechanisms, Unfolded Protein Response, USP14 or Nedd8, are tested and analyzed by treating murine tumor cell lines, SaI/Ak and 4T1, with inhibitor. Before treatment with an inhibitor, the cells undergo an acid wash procedure to denature existing MHC Class I levels at the cell surface, allowing us to measure recovery of MHC class I levels. Following six hours of incubation with inhibitor, cells are stained with antibodies specific to MHC class I molecules and analyzed via flow cytometry.

Our results conclude that unfolded protein response inhibitors slightly decrease MHC Class I levels in SaI/Ak cells, and do not change MHC Class I Levels in 4T1 cells. Overall, USP14 inhibitors had no effect in SaI/Ak and 4T1 cell lines. MLN-4924, the inhibitor of Nedd8 conjugation, slightly decreases MHC Class I levels in SaI/Ak cell line, but not in 4T1 cell line.

Key Words: Antigen presentation, Nedd8, USP14, Unfolded Protein Response

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Jamila Godil, Author

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Introduction

The adaptive immune system is comprised of a variety of cells that are responsible for responding to specific pathogens and providing immunological memory. Cells included in this category are B cells, T cells, and professional antigen presenting cells (APC) (20). Antigens are transported from the sites of infection to the lymph node to be recognized by naïve B and T cells. After exposure to antigens, lymphocytes differentiate into effector cells, adaptive immune cells that are educated about the infectious agent, and take action to remove the infection. Cytotoxic CD8 T cells eliminate self-cells that have become infected or transformed. This is accomplished by the binding of the T cell Receptor (TCR), expressed on the T cell, to the Major Histocompatibility Complex (MHC) Class I and antigenic peptide complex.

Checks and balances are incorporated in the development of adaptive immune cells to ensure they do not react with self-peptides. When the checks and balances fail, the T or B cell can become auto-reactive which leads to the development of autoimmune diseases, such as Type-1 Diabetes (T1D). An important protein involved with autoimmunity is the autoimmune regulator (AIRE) protein (1). In order for our immune system not to react to self-peptides in the eye, brain, major organs, etc. the AIRE protein will present these peptides in the medulla of the thymus for negative selection during T cell development (1, 20). If the self-peptide is not presented, then an autoimmune disease can develop. In the case of T1D, scientist hypothesized that the insulin peptide from the pancreas was not presented to T cells during development in the medulla of the thymus. Therefore, when a T cell encountered the peptide, it was registered as foreign and killed (1).

Type-1-Diabetes (T1D) is an autoimmune disease diagnosed during ones youth. Currently, 1.25 million Americans live with T1D and about 40,000 people are diagnosed each year in the US (2). According to the Juvenile Diabetes Research Foundation (JDRF), by 2050 5 million people will be living with T1D (2). The prevalence of this disease is linked with the T cell recognition and antigen presenting system associated with these cells. In order to understand the impact of antigen presenting on T1D and other autoimmune diseases, it is important to understand how the presentation system works and study aspects that assist it.

The immune system plays a large role in tumor clearance (25). Most tumors are comprised of self-antigens, and therefore do not elicit a strong immune response (1). Tumors also secrete anti-inflammatory cytokines, which result in T-cell anergy and an easier growing environment for the tumor (1). Tumor-immunotherapy is a developing method for targeting tumors where T lymphocytes play a central role. The goal of tumor immunotherapy is to activate T cells, which recognize tumor specific antigens, so they may attack and eliminate the tumor cells. One strategy involves enhancing ones own T-cells via co-stimulation through Adoptive Cell Therapy (ACT). In the ACT process, a patients T cells are extracted and activated in-vitro by the binding event between CD28 receptors to the B7 ligand. This binding event differentiates the naïve T cell to an effector T cell (20). T-cells scan for tumor proteins and high affinity T-cells are selected and further expanded. Patients' lymphocytes are depleted to create space for transferred T-cells. Clinical trials with melanoma patients using ACT as method for treatment, has been successful (1,20, 23). Scientists further expanded the idea of using self-immune cells for cancer treatment and cultivated a new path, Chimeric Antigen Receptor T cells (CAR-T) (1, 23). CAR-T cells are custom built receptors that are placed within a vector and allow for expression within a patients T cells. The receptor is created using an antibody-binding

site that is linked to a T cell transmembrane receptor. This method allows for T cells to bind to an antigen, without MHC presentation and thereby, by passing the activation process required (1, 23).

The lack of functional T cells result in disease prevalence. For example, Acquired Immunodeficiency Syndrome (AIDS), the most severe phase of Human Immunodeficiency Virus (HIV), attacks and depletes the amount of T lymphocytes. The suppression of the immune system by AIDS has made this disease deadly. T cells are believed to play a role in many other diseases as well (20). CD8 Cytotoxic T cells, bind to MHC Class I and stimulate cell death. Recognition of the target cell by the T lymphocyte is necessary for both autoimmune disease and tumor immunotherapy. Understanding the process for simulating antigen presentation, where healthy or disease-associated peptides are presented at the cell surface via MHC, for recognition by T lymphocytes is important for effective tumor-immunotherapy and autoimmune disease prevention (20). Research on how T cells provide signals for antigen presentation is critical for the future of medicine. The ability to modulate antigen presentation may lead to better clinical outcomes for a variety of diseases.

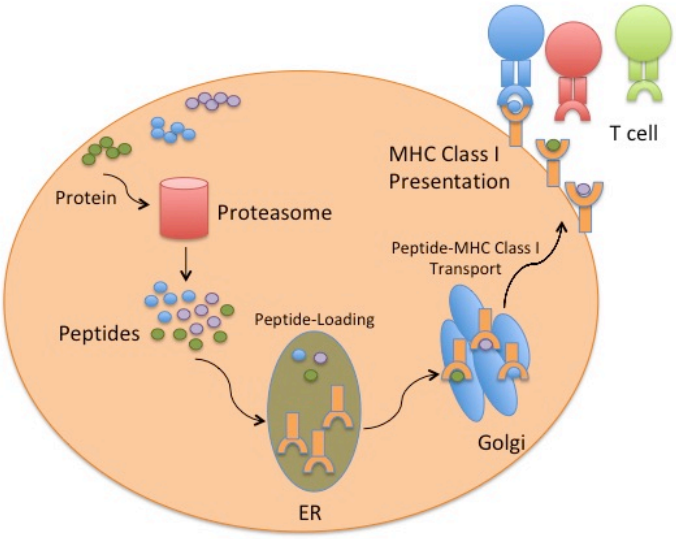

Figure 1. Antigen presentation

A process used by cells to present healthy and viral peptides at the cell surface, to be recognized by T cells. T cells have the capability to differentiate between a healthy peptide and a viral peptide.

Antigen presentation follows many steps to promote viral peptides to the surface of the cell for T cell recognition, summarized in Figure 1. Cells use MHC I to present intracellular proteins, including those derived from self-proteins and those from intracellular pathogens like a virus, for interaction with T-cells. This mechanism allows our immune system to easily recognize foreign pathogens and target them for destruction via Cytotoxic T-cells (20). MHC Class I antigen presentation occurs in all nucleated cells. This allows the adaptive immune system to detect which cells have been infected with an intracellular pathogen (2, 20).

The objective of antigen presentation is to recognize cells that contain foreign viral peptides and induce apoptosis via the adaptive immune system. The peptides generated are delivered to the endoplasmic reticulum (ER) and bound to the MHC Class I molecule. The MHC Class I and peptide complex are presented to the cell surface. CD8 Cytotoxic T-cells scan the peptides within the MHC Class I-protein complex for viral peptides. When the T lymphocyte recognizes a viral antigen located within MHC class I, the T cells induce apoptosis in target cell by injecting granules filled with toxic material into the target cell (1, 20). Any cell that is recognized by activated CD8 T cells will be eliminated (1, 20).

Many steps of the antigen presentation system are ambiguous or unknown. To further our understanding of direct MHC class I antigen presentation, we examined three cellular pathways, which are likely to impact antigen presentation (Figure 2): The Unfolded Protein Response (UPR), USP14 deubiquitinating activity, and the NEDD8-conjugation pathway.

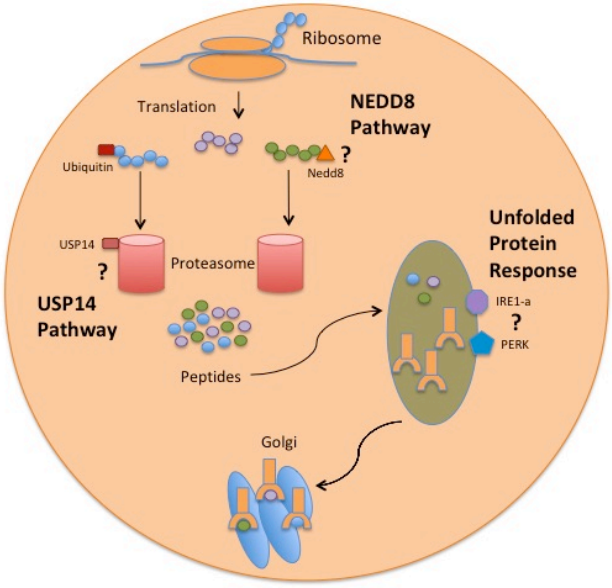

Figure 2. Three Pathways Influence Antigen Presentation

Diagram highlights the role of each pathway in the cell. USP14 removes ubiquitin chains from substrates prior to degradation by the proteasome. Nedd8 protein binds and activates cullins, which signal cell division and protein degradation. Unfolded Protein Response pathway combats unfolded proteins found in the cytoplasm.

The Unfolded Protein Response:

Protein folding is a complex and meticulous process. When the steps to protein folding break down, proteins begin to unfold and toxic levels can build within the cell. Chaperone proteins are present in the Endoplasmic Reticulum (ER) to monitor protein folding (4). Two sensory proteins, IRE1α and PERK, are present in the ER and bound to chaperone proteins. When chaperones are necessary to support the protein folding process, the chaperone proteins separate from the sensory protein, resulting in sensory proteins forming homodimers. Homodimerization transmits signals to the cell to initiate the UPR in an attempt to enhance protein folding. IRE1α signaling up-regulates synthesis of chaperone proteins to combat unfolded proteins in the ER (4). PERK signaling halts translation to decrease the amount of unfolded proteins (5). Chemical inhibitors and activators of the unfolded protein response, which selectively alter the unfolded protein response, are available. The compound known as 4μ8c blocks IRE1α signaling, therefore, preventing increased synthesis of chaperone proteins (4, 5).

Guanabenz acetate and GSK2656157 (Calbiochem) disrupt PERK signaling (6, 7). Conversely, Salubrinal is an inhibitor of eukaryotic translation initiation factor 2 subunit alpha (eIF2a) dephosphorylation (8), thereby maintaining elevated levels of phosphorylated eIF2a. As the PERK signaling pathway is further activated by p-eIF2a (22) Salubrinal acts as a mimic of the UPR.

USP14 Deubiquitinating Activity

Protein degradation into peptides is an important step in MHC class I antigen presentation. Proteins are degraded into peptides by the proteasome. Ubiquitin-specific protease (USP14) a deubiquitinase, associates with the proteasome and is responsible for removing ubiquitin chains from a protein targeted for degradation (21). Recent work suggests Usp14 may be involved in antigen presentation (11). Three inhibitors are tested to study their impact on antigen presentation, 1B10, 1D18 and IU1. IU1is a known USP14 inhibitor (15), while 1B10 and 1D18 are structurally similar to IU1 and tested for their ability to alter USP14 (11).

NEDD8-Conjugation Pathway

Neural precursor cell expressed developmentally down-regulated 8 (NEDD8), is a small protein that binds covalently to other proteins and alters their function. Preliminary research from our group suggests the conjugation of NEDD8 to proteins of interest may be necessary for antigen presentation and involved in proteasome-mediated degradation. Although the function of NEDD8 is not fully understood, we wanted to directly address the impact of NEDD8 inhibition by treating cells with MLN-4924 (Pevonedistat)(9). MLN-4924 blocks the binding of NAE1 enzyme to NEDD8, therefore, inactivating NEDD8 conjugation (10).

The study of antigen presentation is important for development of cancer treatment and understanding the onset for autoimmune disease. These three pathways are studied with the mentioned inhibitors to better comprehend their impact on antigen presentation and overall MHC Class I levels at the cell surface. The knowledge gained by conducting these experiments will be useful for manipulating the process of antigen presentation and alter outcome for disease treatment.

Thesis Statement

The goal of this project is to gain a deeper understanding of the impact Unfolded Protein Response, USP14 and Nedd8 have on presentation of antigens at the cell surface. Using various inhibitors to these pathways, change the MHC Class I levels on the cell surface, which is analyzed via cell staining and flow cytometry. We hypothesize that MHC Class I levels will slightly decrease for murine tumor cell lines, SaI/Ak and 4T1.

Materials and Methods

Abs and reagents

Brefeldin A (BFA) was from Millipore. The stock concentration was 5 mM and kept frozen in DMSO to be diluted to a final concentration of 5 uM for use. 1-[1-(4-Fluorophenyl)-2,5-dimethyl- 1H-pyrrol-3-yl]-2-(1-pyrrolidinyl) ethanone (IU1) was from Cayman Chemical. The stock concentration was 10 mM and kept frozen in DMSO to be diluted to a final concentration of 20 uM for use. Compounds N-(2-(1-(4-fluorophenyl)-2,5-dimethyl-1H-pyr- rol-3-yl)-2-oxoethyl)-N-methyl-2,3-dihydrobenzo[b][1,4]dioxine-6-sulfonamide (1D18) and 1-(1-(3-chloro-4-fluorophenyl)-2,5-dimethyl-1H-pyrrol-3-yl)-2- (piperidin-1-yl)ethan-1-one (1B10) were from Enamine. Both stock concentrations were 10 mM and kept frozen in DMSO to be diluted to a final concentration of 5 uM for use. Sulfamic acid, [(1S,2S,4R)-4-[4-[[(1S)-2,3-dihydro-1H-inden-1-yl]amino]-7H-pyrrolo[2,3-d]pyrimidin-7-yl]-2-hydroxycyclopentyl]methyl ester (MLN 4924) was from Cayman Chemical. The stock concentration was 10 mM and kept frozen in DMSO to be diluted to a final concentration of 5 uM for use. Anti-Mouse CD16/CD32 (93) Purified (Fc Block), anti-mouse H-2Kk (AF3-12.1.3) coupled to biotin, anti-mouse H-2Kd/H-2Dd (34-1-2S) coupled to PE, and streptavidin coupled to APC are from Thermo Fisher Scientific. 8-Formyl-7-hydroxy-4-methylcoumarin, 7-Hydroxy-4-methyl-2-oxo-2H-chromene-8-carbaldehyde (4u8C) [4] was from Sigma Aldrich. The stock concentration was 10 mM and kept frozen in DMSO to be diluted to a final concentration of 30 uM for use. 1-(2,6-Dichlorobenzylideneamino)guanidine acetate salt (Guanabenz acetate) [7] was from Millipore. The stock concentration was 100mM and kept frozen in DMSO to be diluted to a final concentration of 2.5 uM for use. PKR Inhibitor III (GSK2656157) [5,6] was from Calbiochem. The stock concentration was 10 mM and kept frozen in DMSO to be diluted to a final

concentration of 5 uM for use. Finally, (2E)-3-phenyl-N-[2,2,2-trichloro-1-[[(8-quinolinylamino)thioxomethyl]amino]ethyl]-2-propenamide (Salubrinal) [8] was from Millipore. The stock concentration was 10 mM and kept frozen in DMSO to be diluted to a final concentration of 40 uM for use.

Cell culture

Murine tumor cell lines, SaI/Ak cells and 4T1 mouse cells were a kind gift from Suzanne Ostrand-Rosenberg (University of Maryland Baltimore County). SaI/Ak and 4T1 cells were resuspended in Iscove's Modififed Dulbecco's Modified (IMDM) media supplemented with 7.5% fetal Bovine Serum (Atlanta Biologics) and BLANK mM Glutamax (Thermo Fisher).

Acid wash and flow cytometry

Tumor Cells were chilled on ice for ten minutes, centrifuged at 200 RCF and resuspended in acid wash buffer at 100 uL per $1\ x\ 10^6$ cells and incubated on ice for two minutes. Cold RPMI 1640 media, supplemented with HEPES buffer is added to the mixture to neutralize the acid. The cells are washed and resuspended in warm IMDM at $1\ x\ 10^6$ cells/mL. Cells were then plated in 24-well plate and incubated for six hours with inhibitor at calculated concentration for 1mL at $1\ x\ 10^6$ cells/mL. BFA, an inhibitor of secretory pathway, was used as positive control. After six hours of treatment, cells were stained with MHC class I antibodies. SaI/Ak were stained with anti-mouse H-2Kk (AF3-12.1.3) coupled to biotin, anti-mouse H-2Kd/H-2Dd (34-1-2S) coupled to PE, and streptavidin coupled to APC antibodies. 4T1 were stained using anti-mouse H-2Kd/H-2Dd (34-1-2S) coupled to PE antibodies. SaI/Ak and 4T1 cells align with different staining antibodies (11). Cells were plated and washed with 0.1% BSA/HBSS, then resuspended in 30 uL of Fc Block diluted 1:300 in HBSS/BSA for fifteen minutes at 4C. After blocking Fc receptors, cells were washed with 0.1% BSA/HBSS, both

SaI/Ak and 4T1 cells are resuspended with 30 uL of correct label antibody (1:300) for 30 minutes. Followed wash SaI/Al cells are incubated in a mixture of PE/APC antibody's diluted (1:300) in HBSS/BSA for 30 minutes. All cells were then washed in HBSS/BSA and resuspended in 100 uL of 0.1% BSA/HBSS. Treated cells were analyzed by flow Cytometry using an Accuri C6 flow Cytometer (BD Biosciences). Flow cytometer data is evaluated using Accuri C6 software and graphed using Prism 7. Collected data was analyzed by using Mean Fluorescent Intensity (MFI), which attempts to normalize data for comparison between many trials.

Statistics calculation

Statistical t-test calculations were performed with GraphPad Prism software.

Results

MHC Class I levels were analyzed via flow cytometry using fluorescently labeled monoclonal antibodies specific for MHC class I proteins. To determine if acid washing of cells was effective in reducing MHC class I levels at the cell surface, we measured MHC class I levels immediately after the acid wash procedure and after incubating cells for six hours under normal tissue culture conditions. In each case, MHC class I levels were compared to mock-treated cells. (Figure 3). Following acid wash of SaI/Ak cells, levels of D^d and K^k MHC class I molecules are reduced compared to mock treated cells (Figure 3A and 3B) but six hours after culture, levels of each MHC class I protein increase. Similar results are observed when examining both D^d and K^d proteins on 4T1 cells (Figure 3C). This data confirms that treatment with low pH buffer of tumor cells leads to a reduction of MHC Class I levels at the cell surface which will can be restored following culture.

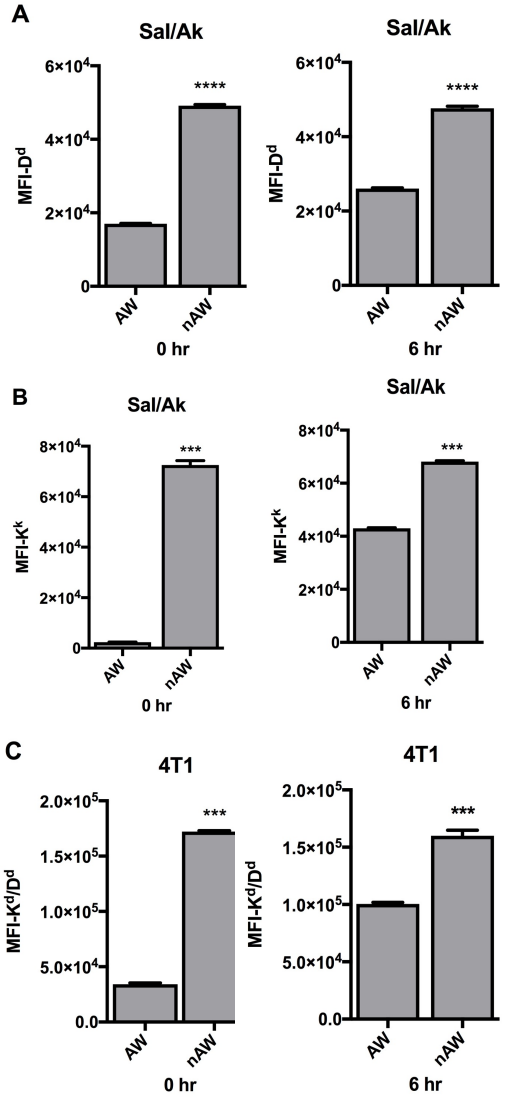

Figure 3. Acid wash treatment degrades MHC Class I at the cell surface.
A. D^d MHC Class I levels measured for SaI/Ak cells, washed with low pH buffer, and analyzed immediately post-treatment (0 hr) and after incubation (6 hr). Values compared to mock cells without acid wash treatment. **B.** Similar to (A) above, except K^k MHC Class I levels measured for SaI/Ak cells. **C.** Similar to (A) above, except D^d and K^d MHC Class I levels measured for 4T1 cells. Analysis performed by flow cytometry. ***$p < 0.001$, ****$p < 0.0001$

To determine if the UPR had an impact on MHC class I antigen presentation in SaI/Ak and 4T1 tumor cells, we tested the ability of UPR modifying compounds 4u8C, Guanabenz acetate, Calbiochem and Salubrinal to alter the recovery of MHC class I levels at the cell surface following acid-wash. 4u8c blocks IRE1α signaling, Guanabenz acetate and Calbiochem are PERK inhibitors that continue protein translation and Salubrinal phosphorylates eIF2a to halt protein translation and enhances the UPR signal. The impact of each inhibitor/activator was measured after six hours of incubation.

Figure 4A and 4B highlight the impact inhibitors had on SaI/Ak cells. The graphs indicate a slight decrease in MHC Class I in the SaI/Ak cell line when treated with inhibitor. However, UPR modulators had no effect on MHC class I levels in the 4T1 cell line.

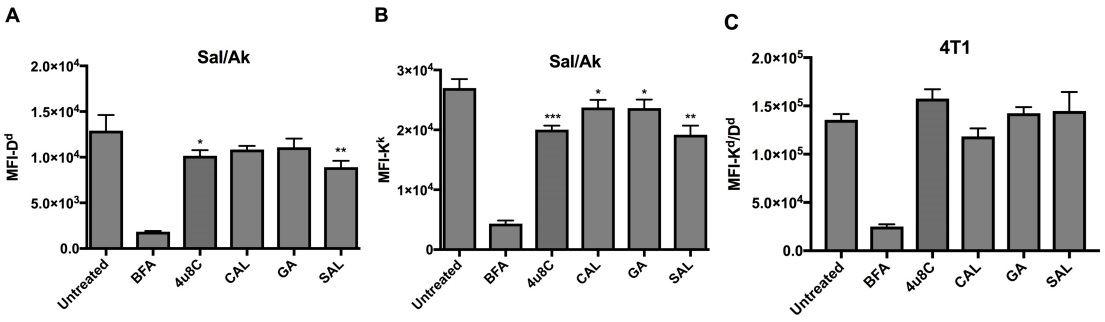

Figure 4. Impact of UPR inhibitors has upon MHC class I levels in SaI/Ak and 4T1 cells.

A. D^d MHC Class I levels measured for SaI/Ak cells, cells washed with low pH buffer, and treated with 4u8C, Guanabenz acetate, Calbiochem and Salubrinal. Cells were analyzed after incubation (6 hr). Values compared to acid wash cells without treatment. **B.** Similar to (A) above, except K^k MHC Class I levels measured for SaI/Ak cells. **C.** Similar to (A) above, except D^d and K^d MHC Class I levels measured for 4T1 cells. Analysis performed by flow cytometry. ***$p < 0.001$, ****$p < 0.0001$

USP14, a vital deubiquitinase involved in proteasome-mediated degradation, was investigated using inhibitors 1B10, 1D18 and IU1. MHC class I recovery following acid wash procedure was measured in the presence of various Usp14 inhibitors. The impact of inhibitor treatment was measured after six hours of incubation. In SaI/Ak cells, there was a statistically significant decrease in D^d levels following Usp14 treatment, but no change in MHC class I was noted for K^k nor for either MHC class I proteins in 4T1 cells (Figure 5). These data indicate that inhibition of Usp14 have minimal to no impact on MHC class I antigen presentation.

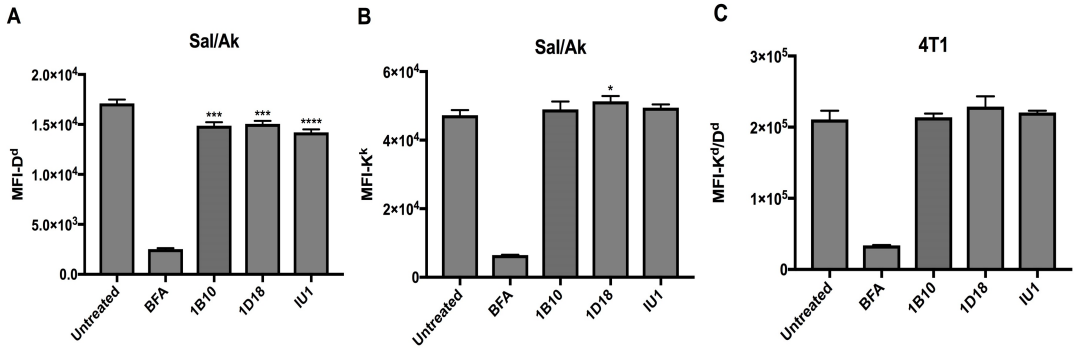

Figure 5. USP14 inhibitors had a minimal impact on MHC Class I levels.

A. D^d MHC Class I levels measured for SaI/Ak cells, cells washed with low pH buffer, and treated with 1B10, 1D18 and IU1. Cells were analyzed after incubation (6 hr). Values compared to acid wash cells without treatment. **B.** Similar to (A) above, except K^k MHC Class I levels measured for SaI/Ak cells. **C.** Similar to (A) above, except D^d and K^d MHC Class I levels measured for 4T1 cells. Analysis performed by flow cytometry. ***$p < 0.001$, ****$p < 0.0001$

Neural precursor cell expressed developmentally down-regulated 8 (NEDD8), a protein that conjugates to other proteins and alters their function, signals protein degradation, and is necessary for cell division. The inhibitor of interest, MLN-4924, prevents NEDD8 activation and downstream effects of Nedd8. The impact of inhibitor treatment was measured after six hours of incubation following acid washing of cells. Figure 6A and 6B show the significant effect MLN-4924 has upon Sa1/Ak cells. The diagram indicates the inhibitor slightly decreased MHC Class I levels for Sa1/Ak cells. No change was seen in 4T1 cells (Figure 6C). These data demonstrate the inhibitor had minimal impact on decreasing MHC Class 1 levels in 4T1 cells.

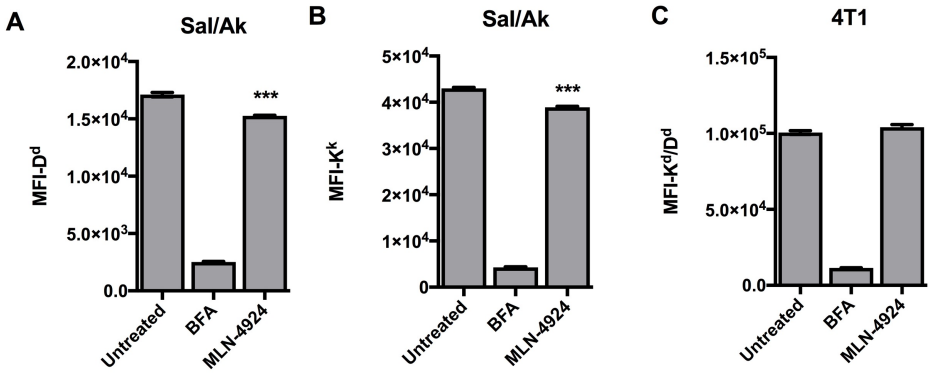

Figure 6. MLN-4924 had differing impact on MHC Class I between SaI/Ak and 4T1 cells.

A. D^d MHC Class I levels measured for SaI/Ak cells, cells washed with low pH buffer, and treated with MLN-4924. Cells were analyzed after incubation (6 hr). Values compared to acid wash cells without treatment. **B.** Similar to (A) above, except K^k MHC Class I levels measured for SaI/Ak cells. **C.** Similar to (A) above, except D^d and K^d MHC Class I levels measured for 4T1 cells. Analysis performed by flow cytometry. ***$p < 0.001$, ****$p < 0.0001$

Discussion

Antigen presentation is a complex process which is dependent on many cellular processes, including protein synthesis, protein degradation, and protein trafficking. However, it is unknown to what extent other aspects of cell biology impact antigen presentation. Here, we examined three different cellular pathways: the unfolded protein response, USP14-mediated protein deubiquitination, and NEDD8 activation to determine if they play a role in direct MHC class I antigen presentation. Chemical modifiers of each pathway were used to determine if treatment resulted in alteration of MHC class I levels. We tested these chemicals in murine tumor cell lines, which are known to elicit T cell responses *in vivo*. Activating or enhancing T cells is a central tenet of emerging immunotherapies used in the clinic. Therefore, our findings with tumor cells can be extended to clinical applications.

Scholarly work in literature has been minimal in addressing the role of these pathways in direct antigen presentation. Granados et al. (12) analyzed the impact of ER stress upon MHC Class I presentation by treating cells with tunicamycin to induce ER stress. It was found that peptides derived from ER-resident proteins are presented during UPR whereas presentation of peptides from cytosolic proteins is unaltered (12). However, these studies did not address which UPR activating pathways may be involved. The UPR inhibitors used during the experiment, 4u8C, Guanabenz acetate, CAL and Salubrinal had a slight decrease on MHC Class I levels in Sa1/Ak, but no change in 4T1 cells (Figure 4). We also examined total class I levels and did not specifically address the origin of peptides for antigen presentation. It is possible that total class I levels remain unchanged with treatment, but the types of peptides presented do.

USP14 has a role in proteasome-mediated degradation. USP14 tags a protein to be dragged and bound to the 19S proteasome (14). Lee et al. (15) determined that a functional USP14 is directly involved in antigen presentation and it was supported by Palmer et. al. (11). Peptides for antigen presentation are mostly derived from defective ribosomal products (DRiPs) (11). DRiPs proteins are those that are degraded as soon as translation is complete (11, 24). Most proteins presented at the cell surface are derived from DRiPs (24). USP14 is believed to play a role in the presentation of DRiPs. Our USP14 inhibitors 1B10, 1D18 and IU1, had no impact on MHC Class I levels in either Sa1/Ak or 4T1 cells when analyzed via flow cytometry (Figure 5), however, as previously stated, we did not directly test the source of peptides for antigen presentation. Indeed, Palmer et al found that total MHC class I levels were unaffected by USP14 inhibition in the model cell line used in their study, similar to data reported here with tumor cells. It is therefore possible that DRiP presentation was diminished in both tumor cell lines, but total MHC class I levels remain unaltered.

Nedd8 is a protein that is conjugated to other proteins during post-translational modification. The covalent bond that forms during Nedd8 conjugation is thought to disrupt the protein confirmation, stability, intracellular compartmentalization and binding affinity to substrate (17). If the proteins function is disrupted, it will likely undergo rapid degradation (17). Vijayasimha et al. suggest that inhibition of Nedd8 decreases presentation of peptides derived from defective ribosomal products (DRiPs) (10). Therefore the inhibition of Nedd8 with MLN-4924 was tested. The results indicated a slightly decrease in MHC Class I levels in Sa1/Ak cells, but no effect on 4T1 cells (Figure 6). MLN-4924, also known as Pevonedistat, is currently in clinical trials for the treatment of Mantle Cell Lymphoma (MCL). It would be interesting to determine what, if any, alterations of anti-tumor $CD8^+$ T cell responses occur in patients

undergoing treatment. Such data would be useful in determining the extent of DRiP presentation in clinically important diseases.

Overall, the chemical inhibitors tested here had either no or a minor impact on total MHC class I levels, suggesting that the UPR, USP14 deubiquitination, and Nedd8 protein conjugation do not play a role in direct antigen presentation. Despite these findings, it is important to distinguish between loss of MHC class I and the types and abundances of peptides which may be presented at the cell surface. It is entirely possible that these chemical inhibitors altered antigen presentation in subtle ways that may not be obvious by simply measuring MHC class I recovery. Future studies should not only examine this issue, but also which MHC class I allomorphs are impacted by drug treatment or not. Throughout our study, we assumed all MHC Class I have equal value, which is not necessarily correct (13). A complete understanding of this process is necessary for the successful development of therapies to treat multiple diseases which have a $CD8^+$ T cell component, such as Type I diabetes and tumor immunotherapy.

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