Evaluation of a Monoclonal Antibody for Studying MHC class I Antigen Presentation In-Vitro

by
Eden VanderHoek

A THESIS

submitted to

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degree of

Honors Baccalaureate of Science in Kinesiology
(Honors Scholar)

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Human epidermal growth factor receptor 2 (HER2), a member of the receptor tyrosine kinase (RTK) family, is frequently dysregulated in a variety of cancers. Overexpression of the receptor leads to increased cell proliferation and resistance to cell death due to downstream signaling effects. Proteasomal degradation of HER2 results in the generation of HER2-peptide, which is then presented by major histocompatibility complex class I (MHC-I) molecules to the immune system. In this study, we evaluated the effectiveness of a customized antibody (RL01) to monitor the changes in presentation of HER2-peptide on Epstein–Barr virus (EBV)-immortalized B-cell lymphoblastoid (JY cells) cell surface. The RL01 antibody detected the antigenic peptide-MHC complex in cells that were exposed to high concentrations of the HER2 peptide. RL01 antibody showed more specificity in HER2-overexpressed JY cells following stripping of existing MHCI on the cell surface. Our findings suggest that RL01 has been moderately successful in detecting the desired peptide under specific conditions.

Key Words: immunotherapy, antigen presentation, MCH1, HER2, HER2-peptide.

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Honors Baccalaureate of Science in Kinesiology project of Eden VanderHoek presented on June 5, 2020.

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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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Eden VanderHoek, Author

1. Introduction

1.1 Cancer Immunotherapy

Cancer causes the death of around 10 million people globally every year (WHO), and stems from a mutation or DNA damage at the cellular level. This often results in increased cell proliferation and evasion of cell death (American Cancer Society). The difficulties arising from cancer are often due to the immune system's inability to functionally recognize the aberrant cells (Drake et al, 2006). Additionally, many cancer treatments kill healthy cells in addition to the cancer cells they aim to target (Liu et al, 2015).

The usual immune response to an aberrant cell involves detection of the diseased cell by the adaptive immune system, and innate immune cells with phagocytic or otherwise destructive properties to induce tumor-cell death (American Cancer Society). The nature of cancer, however, makes it difficult to fight successfully without exogenous treatments. This is due to the increased rate of cell division, innate abilities of some cancers to "[commandeer] immune cells" to aid successful spread (Ouzounova et al, 2017) and the ability of some tumor cells to evade recognition by the immune system (Drake et al, 2006).

As understanding of the immune system has progressed in medicine, the field of cancer research has developed immunotherapy techniques that have shown promise as treatment options for patients facing cancer diagnoses (Rosenburg, 2001). These techniques present a more personalized strategy for treating cancer – an approach that is becoming more important as medicine further considers individual differences when defining wellness and classifying disease (Immunotherapy - the Science behind Hope). Immunotherapy utilizes immune components to help the body effectively self-treat cancer (American Cancer Society), triggering

the immune system to attack tumor cells rather than using other exogenous treatments like chemotherapy.

Cancer immunotherapy development traces back to as early as 1893, when live bacteria was used as an immune stimulant to treat tumor growth (Yang, 2015). An important element of the eukaryotic adaptive immune system are antibodies, which play a role in the defense against cancer cells and pathogens (American Cancer Society), but antibodies are also useful tools that can be used both in the laboratory and the clinic. Antibodies are unique and highly specific for particular macromolecules (Alberts et al, 2002). The science behind immunotherapy is dependent upon the use of these “monoclonal antibodies”. Once successful in binding to target antigens on tumor cells, antibodies mark cells for destruction, or prevent macromolecules from performing their functions (Scott et al, 2012).

Another form of immunotherapy relies on the use of monoclonal antibodies to modulate immune cell function rather than targeting them for killing. Currently, there are many ongoing trials in the field of immunotherapy, including the recent federal funding of a clinical trial surrounding 30 rare cancers at the Knight Cancer Institute at Oregon Health and Science University. This trial is termed a “combination checkpoint blockade,” and employs the use of two monoclonal antibodies (Riley, 2013). The antibodies, “ipilimumab” and “nivolumab,” target CTLA4 (cytotoxic T lymphocyte associated molecule-4) T-cell antigens and PD-1 (programmed cell death) receptors, respectively. Checkpoint inhibitors block negative signals given to tumor-specific T cells - immune cells that can eliminate cells that have become infected or transformed. Negative signals given to T cells through proteins such as CTLA4 and PD-1 turn off T cell function. By blocking these negative signals, checkpoint inhibitors allow for continued T cell-mediated destruction of tumor cells. The goal of this immunotherapy trial is to develop intensive treatment

strategies for patients possessing rare forms of cancer by evaluating the overall response rate to different immunotherapy treatments used. The study also aims to estimate survival rates and effectiveness of each treatment (Rojas-Burke, 2017). Other studies are proving immunotherapy to be an increasingly effective treatment, as well as a useful approach to extending the overall survival rate of patients diagnosed with aggressive forms of cancer. For example, UCLA conducted a trial of patients possessing advanced non-small cell lung cancer treated with an immunotherapy drug known as Pembrolizumab. This drug acts as a checkpoint inhibitor, preventing the interaction of membrane proteins between immune cells that essentially deactivates the immune system. The development of the drug has progressed remarkably, lifting the 2012 5-year survival rate of 5.5% to 15%, currently (Garon et al, 2019). The use of checkpoint inhibitors has also improved the survival rate of patients with melanoma (Marconcini et al, 2018), among other advanced and aggressive cancer varieties.

Currently, extensive research is being conducted for the use of antibodies as a tool to fight diseases like cancer. Different monoclonal antibodies have been approved by the FDA for use in immunotherapy approaches targeting cancers such as non-Hodgkin's lymphoma (NHL), metastatic breast cancers with over-expression of HER2, colorectal cancer and a few variations of leukemia (Waldmann and Morris, 2006).

1.2 MHC Class I Antigen Presentation

The MHC class I antigen presentation pathway is the mechanism by which cells of the body can alert cytotoxic T cells to the presence of intracellular pathogenic infection or oncogenic transformation (Palmer et al, 2017). Antigens are short peptide fragments derived from bigger proteins synthesized within the target cell. These peptides are generated and presented on MHC class I molecules after initial proteins are broken down into smaller fragments via

cytoplasmic proteolysis. Each peptide fragment is then loaded onto MHCI molecules in the endoplasmic reticulum and taken to the cell membrane to be presented (Kotsias et al, 2019), creating a “cognate antigen” (Lu et al, 2012). These peptide-MHC complexes (pMHC) are presented to CD8+ cytotoxic T-cells, which then kill infected or cancerous cells (Palmer and Dolan, 2013).

In cancer, enhanced antigen presentation results from mutations that increase specific protein production. Genes that are continuously transcribed can lead to an overabundance of proteins that, in turn, amplify signalling pathways (Bizari et al, 2006). For example, overexpression of human epidermal growth factor receptor-2 (HER2) has been observed in 15-20% of invasive breast cancers due to amplification of the ERBB2 oncogene (Burstein, 2005, Bizari et al, 2006). Normal properties of HER2 are to signal cell proliferation and survival; however, with excessive expression and overactive signaling, HER2 can lead to very aggressive cancers that shorten survival outcomes and increase the risk of recurrence (Iqbal and Iqbal, 2014).

A plethora of laboratory research has been done on modulating antigenic peptide presentation, particularly for MHC class I antigens. For example, chemical inhibition of a ubiquitin-specific protease 14 (Usp14) has been shown to diminish direct antigen presentation by MHC class I molecules (Palmer et al, 2017). The ubiquitin-specific protease mediates degradation of the precursor protein to create peptide-MHC complexes for presentation. This observation, along with the lack of Usp14 interference with model protein production, suggests that functional Usp14 enhances antigenic peptide presentation in MHC class I molecules. Contrary to the results from inhibition of Usp14, a study by Cram et al observed that chlamydial infection increases presence of self-antigen presentation in a lymphoblastoid cell line. The chlamydia appeared to limit accumulation of model host proteins and increase presentation of the resulting

antigenic peptides, which are defective and rapidly degraded. The bacteria synthesize a necessary component to altering antigenic peptide presentation, as presentation levels could be reversed by preventing bacterial protein synthesis. This is thought to be a mechanism of preventing antigenic peptide presentation from chlamydia-derived proteins, along with subsequent death from binding of cytotoxic T-cells.

Antigen presentation is an important cell characteristic that allows for targeted cell killing in cancer therapies. As checkpoint-inhibitor therapies become more common for enhancing T cell activity against tumors, it will become more critical to understand how these T cells recognize transformed cells. If antigen presentation can be modulated through methods similar in nature to those discussed above, immunotherapeutic outcomes have the potential to be enhanced as well.

1.3 Measuring Peptide Presentation

Peptide presentation is measured in order to determine whether or not methods of modulation have been successful. This can be indicative of effective methods that alter presentation of specific antigenic peptides that can create an immune response. In order to determine effectiveness of treatments that potentially alter MHC class I antigen presentation, we need a way to measure antigenic peptides bound to MHC class I molecules. Peptide presentation can be measured through the use of a monoclonal antibody customized to bind to a specific peptide-MHC complex. A common antibody used to study antigen presentation is known as 25D-1.16 (Porgador et al, 1997). This antibody recognizes the peptide SIINFEKL, which is derived from chicken ovalbumin protein, when it is presented on a mouse MHC class I molecule. This antibody was used to determine the importance of Usp14 in antigen presentation (Palmer et al, 2017). Another recently developed antibody recognizes a peptide derived from the West

Nile virus E protein presented by human MHC class I molecules. This antibody was utilized to determine the role of *Chlamydia*-induced antigen presentation enhancement (Cram et al, 2016). In both cases, the specific antigen studied was derived from a model protein and therefore may not be reflective of actual tumor antigen presentation. It is therefore imperative that we validate our previous findings using an antibody which can measure relevant peptides presented by tumor cells if we are to understand and enhance tumor immunotherapy.

1.4 Importance of Antibody Validation

Antibody validation involves determining if an antibody actually detects a target antigen. It involves extensive research and experimentation on cell cultures with antibody staining and different methods of analyzing the staining. It is highly important that an antibody is specific to the surface protein that it is designed to detect; this ensures that any conclusions made about tests or experimental results are accurate and that they can be applied correctly to the formation of treatment (Andersson et al, 2017). Regulating antigen presentation is a crucial component of cancer immunotherapy, as discussed prior. Thus, an antibody failing to bind the correct substrate could mislead conclusions of experimentation (Taussig et al, 2018) and potentially affect patient outcomes from treatments. Many research experiments build off of previous well-supported studies and have broad clinical implications that influence treatments and patient outcomes, making it crucial that an antibody detects the proper antigen before being relied on for further research.

One instance of insufficient antibody validation occurred in research surrounding breast cancer treatment development. In approximately 70% of all breast cancer variants, the cells overexpress a membrane estrogen receptor termed estrogen receptor alpha (ERα) (Andersson et al, 2017). After the discovery of a new receptor, estrogen receptor beta (ERβ), there was a

new hope for developing “improved endocrine therapies,” which unfortunately never materialized throughout 20 years of research using 13 anti-ERβ antibodies. After the failure of the research to thoroughly evolve and produce new treatment strategies, the validation of the utilized antibodies was reevaluated, and revealed that only one 1 of the 13 supposed ERβ antibodies truly detected the receptor (Andersson et al, 2017). The rest, when exposed to ERβ -negative cells, stained them to produce a false-positive for the receptor. False-positives can lead researchers and physicians to believe a cell possesses certain characteristics, which can dictate research and treatment approaches. This could not only have a direct effect on treatment success and patient outcome, it also, in this case, delayed the progression of knowledge of breast cancer treatments.

Another instance of insufficient antibody validation that is worthy of note is one that has stimulated controversy in the scientific community; namely within the realm of exercise science. The irisin hormone has, since 2012, been thought to have a “potent physiological role” in mediating beneficial effects of exercise (Gizaw et al, 2017). It is thought that, after higher concentrations are induced by physical activity, irisin may increase energy expenditure by promoting the browning of white adipose tissue. This would, in theory, lead to increased energy expenditure via thermogenesis (Gizaw et al, 2017). Irisin was seen as a potential route to further advancements for solving the obesity epidemic, as well as metabolic diseases like type II diabetes. Problems surrounding irisin research stemmed from the kit used for blood testing in initial studies, which relied on polyclonal antibodies that had not been tested against potential cross-reacting proteins found in the blood (Albrecht et al, 2015). The contradictory results of studies done on the hormone using these antibodies initiated skepticism about its role and relevance in the human body. The recent study by Albrecht et al in 2015 found the polyclonal antibody to be “inappropriate” and many studies to have “ignored the possibility of

cross-reacting proteins." The study concludes that up to 45 previously-published articles reported on "unknown cross-reacting proteins," rather than irisin levels, once again highlighting the importance of validated antibodies in research.

The potential to mislead clinical research and treatment of cancers and its ability to also impact patient outcomes makes it highly necessary to have a validated tool for detecting antigens on different forms of cancer. Many forms of cancer have similar expression of surface peptides that possess downstream signaling properties, and cause changes to cellular behavior such as increases in cell proliferation (Iqbal and Iqbal, 2014). Without ensuring the legitimacy of an antibody for use in research and immunotherapeutic treatment, the ability of the treatments to be effective is significantly diminished and targeting tumor cells becomes exponentially more difficult.

1.5 Goal of Current Research

Antigen modulation, as discussed, has an important role in the targeted binding of cytotoxic T-cells of the immune system. Given that many studies are centered around the use of model antigens, it is imperative that actual tumor antigens are evaluated prior to applying laboratory findings to clinical research in immunotherapy. This provides the opportunity to measure presentation of bonafide tumor antigens.This project aims to validate a specific antibody, termed RL01, for its effectiveness in detecting the antigenic HER2 peptide in cells with overexpression of HER2 receptor. HER2 is overexpressed in a few different types of cancer, and leads to downstream signaling that triggers excessive cell growth and proliferation (Iqbal and Iqbal). If the customized antibody is successful in detecting the peptide, it could become a reliable tool for detecting HER2 peptide presentation in clinical research. In this study RL01 was able to detect the HER2 peptide; however, very high concentrations of the peptide were necessary to

be able to discern between cells with the peptide-MHC complex and those without. Therefore, it will be necessary to see if this antibody can detect the peptide in a more physiologically relevant setting prior to it being used in clinical research for more practical applications.

2. Results

2.1 RL01 Antibody Fails to Detect Presented HER2 Peptide in Lymphoblastoid Cells with HER2 Over-expression.

To test if the RL01 monoclonal antibody could recognize HER2 peptide presented by human MHC class I, we needed to generate a cell line expressing HER2 protein. JY lymphoblastoid cells do not express HER2 (data not shown) and we therefore made a stable JY cell line expressing HER2 by transfecting cells with DNA plasmids encoding the HER2 protein. After establishing the cell lines, each lymphoblastoid cell line was stained with different concentrations of RL01 antibody solution and analyzed via flow cytometry. The stainings of JY-HER2 cells did not show higher fluorescence values post-staining than the parental line of JY cells (Figure 1). The difference in mean fluorescence index was not significant enough to suggest that RL01 successfully stains HER2 peptide without more controlled cell conditions.

2.2 HER2 Peptide Pulsing Increases Chances of RL01 Function.

A peptide pulse experiment was conducted after initial trials had resulted in failed detection of the HER2 peptide-MHC complex by the RL01 antibody. JY cells were incubated in a solution of HER2 peptide before being introduced to the antibody. Peptide pulsing the JY cell line led to increased staining activity by the RL01 antibody (Figure 2). The results were more successful in stainings that utilized higher antibody concentrations. These data show that RL01 is capable of

recognizing the HER2 peptide when it is bound to MHC class I molecules on JY cells, and that effective detection can be increased by increasing the concentration of available HER2 peptide.

2.3 Acid Stripping of Existing Peptide-MHC Complexes Increases the Sensitivity of Staining.

Acid stripping of cells was carried out to minimize other possible complexes interfering with or competing for the binding of available antibodies. This experiment was used to determine whether cells would have increased HER2 peptide detection by the RL01 antibody if potentially confounding complexes were removed. Cells that were stripped of existing peptide-MHC complexes showed increased sensitivity of staining by RL01 following a peptide pulse with HER2 peptide (Figure 3). This is likely due to a decrease in antibody binding interference by other antigens or peptide-MHC complexes present on the cell line, and helped increase the efficiency of RL01 binding to the desired peptide-MHC complex. By removing other MHC class I molecules and binding sites, this procedure ensured that RL01 was staining the correct peptide. More accurate results were obtained about changes to peptide-MHC complex presence on JY cell membranes.

3. Discussion

Different experimental procedures are being used to test the staining by this antibody, including incubation of cell cultures in a concentrated antibody solution. Prior to staining, methods of modulating the expression of HER2 peptide on a cultured human cell line were carried out. These methods included incorporation of a peptide pulse procedure, where cells were incubated in peptide concentrations before being introduced to the antibody. Another experiment involved stripping cells of existing membrane proteins prior to the peptide pulse and antibody incubation.

Following all procedures, it was determined that RL01 does detect the presence of the HER2 peptide, but that conditions of the cells play a large role in dictating the success of detection.

Initial antibody staining procedures resulted in insignificant differences in HER2 peptide detection on both JY and JY-HER2 lymphoblastoid cells, regardless of antibody concentration used (Figure 1). After staining with RL01 antibody following incubation in HER2 peptide solution, the antibody did begin detecting the presence of the peptide on JY cells; however, conditions needed to be optimized, as the antibody was only successful under high concentrations (Figure 2). The acid stripping of peptide complexes from the JY cells was performed in order to further enhance the success of RL01 antibody in terms of HER2 peptide detection. This experiment resulted in significant increases in HER2 peptide detection. The results from this research are not consistent with other data obtained from similar studies using the same antibody (Jain et al, 2013). RL01 is not as effective in HER2 antigenic peptide detection as it was initially hoped it to be. We would expect to see a higher detection of the peptide in the JY-HER2 cell line that expresses the antigenic peptide of interest; however, that was not the case in initial procedures of antibody staining without highly optimized conditions, as it required very high peptide concentrations.

The results of this research are important in the broad spectrum of immunological research, as it involves testing actual tumor antigens against an antibody, rather than model antigens. This provided information on the validity of this antibody for potential use in future tumor research and clinical applications. Perhaps the most important consideration for future research in immunology is a continuation of the importance placed on antibody validation. Lack of thorough validation has proven to lead multiple fields astray and delay the production of treatment options for the ever-present population of individuals with cancer, in addition to wasting resources. This

project was centered around the goal of legitimizing a specific antibody for its use as a tool in the cancer immunotherapy realm of medicine, and aims to exemplify a necessary step in this area of research. The field of medicine has the potential to learn from the mistakes made throughout the history of immunotherapy research, which remain important in the wake of immunotherapy's rising popularity as a form of cancer treatment. RL01 has been only moderately successful in detecting the presence of the desired peptide for this research, and further experiments will seek to optimize the conditions in which this antibody can stain and detect the HER2 peptide on cell membranes in more physiologically relevant settings.

4. Materials and Methods

4.1 Antibody and reagents

The monoclonal antibody RL01 was a gift from Emergent Technologies. The antibody was directly coupled to the dye Alexa 647 (Molecular probes) following the manufacturer's instructions. The peptide KIFGSLAFL (corresponding to amino acids 415 to 423 of the HER2 protein) was from Bio-Synthesis Inc.

4.2 Cells and transfections

JY cells were cultured in RPMI 1640 supplemented with Glutamax, a HEPES buffer (all from Invitrogen) and 7.5% fetal bovine serum (Atlanta Biologicals). Cells were grown at 37°C in 6% CO_2. For cellular transfection, $5x10^5$ JY cells were resuspended in 20 µL Amaxa solution SF and mixed with 200ng of HER2 expression plasmid. Cells were electroporated using the Amaxa nucleofector program DS-138 and returned to culture. HER2+ cells were selected by magnetic bead sorting (Miltenyi Biotec).

4.3 MHC-I Peptide Stripping

Cells were initially placed on ice for 10 minutes before being centrifuged at 100Xg for 4 minutes to form a cell pellet. Once a pellet was formed, media was flicked from the plate and the cell plate was vortexed. The cells were treated with 100µL of citric acid wash buffer (0.13M citric acid and 0.056M dibasic sodium [pH 3.0]) per 10^6 cells for 2 minutes on ice. The acid was then neutralized with 13mL of cold RPMI media (without serum) and centrifuged for 4 minutes at 100Xg. The plate was vortexed and cells were suspended in warm complete media prior to proceeding with the peptide pulse and antibody staining procedures (see sections 4.4 and 4.5).

4.4 Antibody Staining

The JY and JY-HER2 cells were counted using Trypan Blue dye. Cell density was determined for each culture flask and an appropriate volume of cell solution was pulled from each flask for antibody staining before a small sample of cells were passed into new flasks to remain in culture. Cells pulled for antibody staining were divided into wells of a round-bottom 96-well plate, with approximately 10^5 cells per well. All cells were washed using 200µL/well of a 0.1% buffer solution of BSA/HBSS and centrifuged at 2000RPM for 1 minute. The plate was flicked to remove excess supernatant and vortexed to break up the cell pellets. The washing procedure was repeated twice prior to proceeding with antibody application and incubation. 30µL of diluted antibody (RL01) solution was then added to each well, and the plate was incubated at 4℃ for 30 minutes. Following the incubation, the cell plate was centrifuged at 2000RPM for 1 minute, flicked of supernatant, and washed twice with Hanks' Balanced Salt Solution (HBSS). Cell fluorescence was then analyzed via flow cytometric analysis (see section 4.5).

4.5 Flow Cytometry

Each cell line was incubated in varying concentrations of antibody solution for 30 minutes prior to analysis with flow cytometry. Flow cytometric analysis was performed using an Accuri C6 flow cytometer (from BD Biosciences) to determine binding affinity of RL01 to HER2 peptide on JY and JY-HER2 cells. Data were then analyzed using Accuri C6 software.

4.6 Data Analysis

Data was exported to and graphed with Prism 8 application software. Data from all procedures (sections 4.3 and 4.4) were analyzed for significance using statistical t-test analysis. Each experiment was performed a minimum of three times and the results are depicted in figures 1-3.

5. Figure Legends

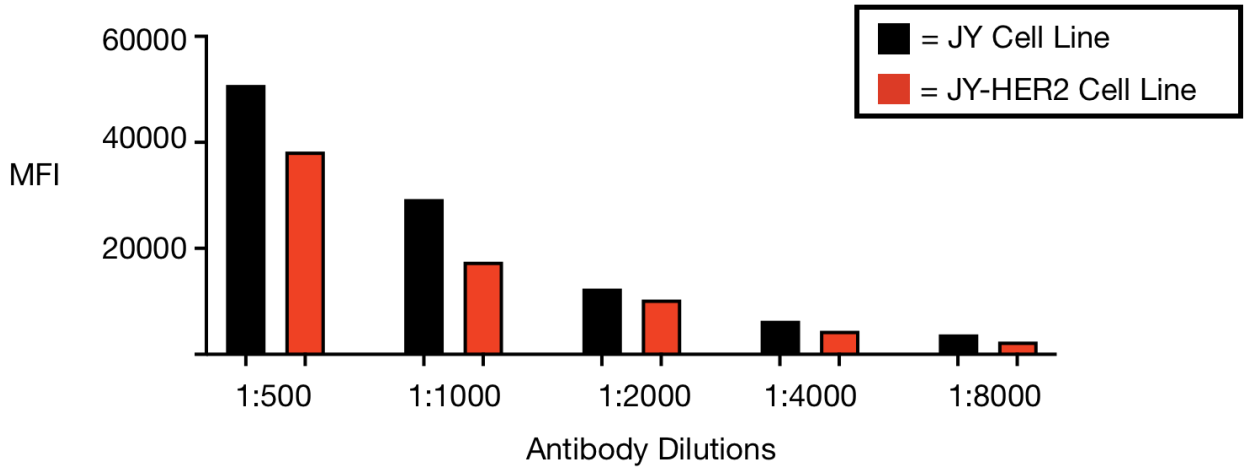

Figure 1: **RL01 Antibody Does Not Detect HER2 Peptide in Lymphoblastoid Cells with HER2 Over-expression.**

JY parental cells and JY-HER2 cells were stained with multiple RL01 antibody concentrations (1:500, 1:1000, 1:2000, 1:4000, 1:8000) for 30 minutes and analyzed via flow cytometry. MFI (mean fluorescence intensity) values post-staining were calculated from three independent experiments.

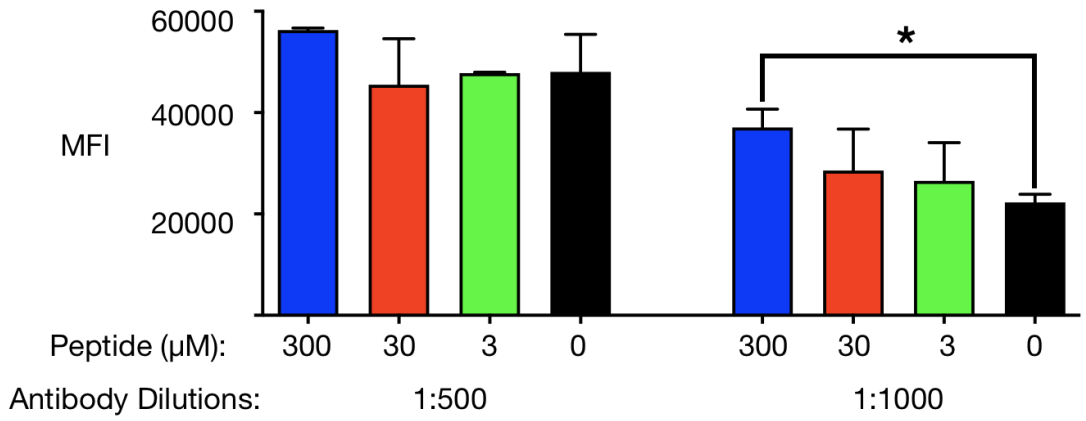

Figure 2: Peptide Pulsing Provides a Condition in which RL01 Antibody Recognizes HER2 Peptide.

The JY parental cells were incubated in a solution of different concentrations of HER2 peptide (300 μM, 30 μM, 3 μM, 0 μM) for 30 minutes prior to staining with 1:500 or 1:1000 dilution of RL01 antibody. The intensity of fluorescence in each of the samples was analyzed by flow cytometry MFI (mean fluorescence intensity) values post-staining were calculated from three independent experiments. * $P<0.01$

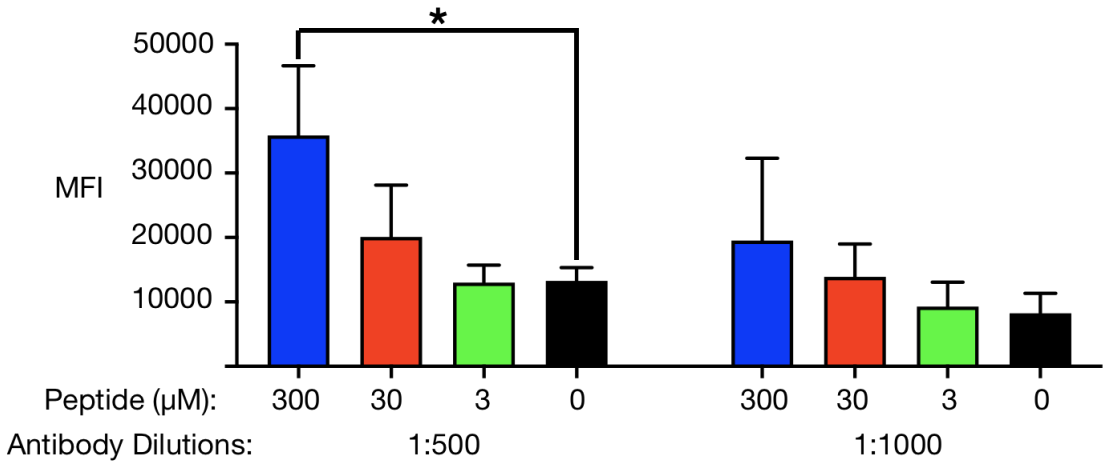

Figure 3: Removing of Existing Peptide-MHC I Complexes Increases Sensitivity of RL01 in Detecting HER2 Peptide on the Cell Surface.

The JY parental cells were stripped of existing peptide-MHC I complexes by an acid buffer (pH 3.0) prior to a 30-minute incubation with a range of HER2 peptide concentrations (300 μM, 30 μM, 3 μM, 0 μM). Cells were then stained with 1:500 or 1:1000 dilution of RL01 antibody. The intensity of fluorescence in each of the samples was analyzed by flow cytometry MFI (mean fluorescence intensity) values post-staining were calculated from three independent experiments. * *P<0.01*

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