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Glycovariants of ManC-Lectibody, a Novel Immunotherapeutic Drug, and their Effects on ADCC Activity

Sreevatsa Vemuri

Existing cancer treatments have detrimental side effects that stem from drugs' inability to distinguish between healthy and cancerous cells. Scientists have researched immunotherapy, an alternative treatment which essentially enhances the innate immune system to eliminate diseased cells. ManC-lectibody is a novel immunotherapeutic drug that imitates an antibody and performs a process known as antibody-dependent cell-mediated cytotoxicity or ADCC within the humoral immune system. Previous research proves that ManC-lectibody is highly selective to oligomannose glycans, or sugars which are expressed along the surface of a cancer cell. Additionally, ManC-lectibody has been known to induce ADCC but there is no information regarding modifications to the drug's structure and how those modifications can influence ADCC activity. This study aims to find what glycovariants of ManC-lectibody will increase ADCC activity. It was hypothesized that the GnGn glycovariant, a humanized Fc glycosylation modification, would increase ADCC activity. An ADCC reporter assay served as the primary method for the research and quantified ADCC activity. Three glycovariants of ManC-lectibody were created: GnGn, Wild type, and N200Q. A dose response curve was created to measure the efficacy and potency of the glycovariants of the drug. The GnGn glycovariant of ManC-lectibody was the most efficacious against lung cancer cells (LLC cell line) but was the most potent against melanoma cancer cells (B16F10 cell line). A two-way ANOVA statistical test proved that the GnGn glycovariant was statistically significant and produced a strong effect against the lung, melanoma, and ovarian cancer cells, therefore indicating that the GnGn glycovariant makes the ManC-lectibody a more effective drug.

Keywords: ManC-lectibody, antibody-dependent cell-mediated cytotoxicity (ADCC), fragmented crystallizable region (Fc region), Fc receptors, lung cancer, ovarian cancer, melanoma

Introduction

Pharmaceutical companies around the world are developing many drugs to combat and cure diseases. From changing the genetic makeup of bacteria to creating chemical derived drugs, scientists are able to reduce the number of disease-related deaths around the world. While this may seem like a plausible solution in decreasing diseases, pharmaceutical drugs are tedious in their manufacturing process. Currently, the production of pharmaceutical drugs is not feasible for large scale application, thereby limiting drug quantities for patients[1]. In addition, current pharmaceutical drugs only target select diseases, requiring further research to be conducted to resolve other deadly diseases[2]. Most importantly, however, pharmaceutical drugs result in negative side effects, giving rise to severe implications to the human body[3].

Immunotherapeutic drugs are a specific type of pharmaceutical drugs that can help mitigate diseases by using the immune system. Essentially, "immunotherapy aims to harness the host's adaptive and innate

immune system" to ultimately "[eliminate] diseased cells[4]." While this approach is effective in "[eradicating] smallpox...typhoid, cholera, hepatitis, and more, it has been far less effective against cancer[4]." This problem has negatively affected cancer patients as there are very few immunotherapeutic drugs that are capable of reducing side effects. Not only this, prolonged exposure to tumors without a treatment would place the patient in danger. Several drugs have been created to combat this issue of selectiveness, including the novel drug ManC-lectibody. This drug imitates the function of an antibody and is capable of selectively targeting cancer cells without causing harm to normal cells[9]. However, drug improvements still need to be made in order to improve ManC's activity in decreasing cancer cells while still reducing detrimental side effects.

Brief Overview of the Humoral Immune System

The immune system is an essential human body system as it helps the "host eliminate toxic or allergenic substances" that can negatively affect the body[8]. An immune response is the reaction that takes place within the immune system to fight against foreign molecules[7]. There are several immune responses that contribute to the function of the immune system. However, when looking at the immune response associated with ManC-lectibody, the humoral immune response is the most prevalent due to the drug's function. Within the humoral immune system, there are two main identities involved: antibodies and antigens. Antigens are molecules that stimulate an immune response. For example, carcinomas, bacteria, and viruses are all molecules that initiate an immune response, therefore making them antigens[5]. Antibodies are Y-shaped molecules that have two main regions: the fragment crystallizable region (Fc region) and the fragment antigen-binding region (Fab region). The Fc region is constant, meaning that the structure is relatively the same in all antibodies[6]. This is because the Fc region binds to the same immune cells, such as B cells or effector cells, that kill antigens. However, the Fab region is antigen-specific, meaning that it changes to target specific foreign substances[6]. Because of the antibody's structure, the humoral immune system is antibody-mediated, meaning that the antibody serves as the primary form of communication between the immune cells and the antigens[5]. To facilitate the understanding of this system, the antibody can be seen as a bridge connecting the immune cells to the antigens so that the immune cells can target the antigens.

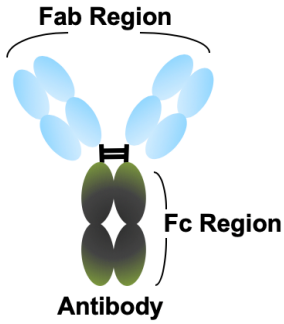


Figure 1. "Structure of an IgG1 antibody". An IgG1 antibody structure with two Fab regions and one Fc region (visual created by student researcher).

Literature Review of ManC-Lectibody

As noted by Dr. Nobuyuki Matoba, the creator of ManC-lectibody, the immunotherapeutic drug "simulates an antibody-cell interaction that attempts to decrease cancer cells by increasing antibody behavior[9]." Dr. Matoba is a researcher at the University of Louisville and holds the current research regarding ManC-lectibody. The drug consists of two portions: an Fc region of an IgG1 antibody and two ManC portions. The ManC portions are derived from a lectin (plant protein) known as Actinohivin. Actinohivin has been known for its specificity in targeting glycoproteins, which are essentially sugars that surround the human immunodeficiency virus (HIV)[10]. By modifying this molecule, Dr. Matoba and his team were able to create a molecule capable of selectively targeting similar glycoproteins that surround cancer cells. These glycoproteins are expressed by some cancer cells and are known as oligomannose glycans or high mannose glycans (HMGs) and serve as biomarkers. Biomarkers are indicators that mark the presence of substances, in this case cancer cells[11].

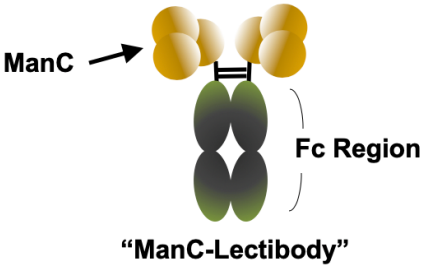


Figure 2. "Lectibody".
This figure illustrates the structure of ManC-lectibody. Two ManC portions are combined to an Fc region of an IgG1 antibody by the process of dimerization (Seber Kasinger, Dent, Mahajan, Hamorsky, & Matoba, 2019).

Previous research from Dr. Matoba's team shows that the drug can distinguish between cancer cells and healthy human cells mainly due to ManC-lectibody's high affinity for high mannose glycans[9]. High mannose glycans are expressed in higher yields on cancer cells when compared to normal cells, thereby making the drug selective to only diseased cells[9]. As mentioned earlier, since the high mannose glycans mark the cancer cell, they serve as biomarkers[11]. However, Dr. Matoba's team refers to them as glycobiomarkers since the biomarkers are composed of sugars[9]. Dr. Matoba's research was mainly concerned with determining whether the drug recognizes glycobiomarkers on the cancer cell's surface and his team only examined ManC-lectibody's effects on a specific ovarian cancer cell line[9].

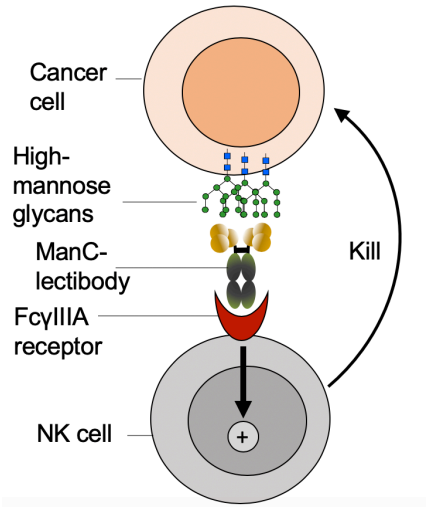
Similar approaches for the discovery of glycobiomarkers have been prominent in immunotherapy research. Muchena J. Kailemia, Dayoung Park, and Carlito B. Lebrilla, researchers from the Department of Chemistry at the University of California in Davis, California, found that the importance of glycobiomarkers is significant as it not only helps identify cancer in early stages, but it can also indicate the severity of the cancer[12]. The varied intensity of the glycans found on the site of interaction between the antibody and the cancer cell indicate the sugar presence on the surface of the cell. Presumably, larger quantities of glycans may mean that there is severe carcinoma behavior while smaller quantities of glycans may show signs of a growing tumor. This coincides with Dr. Matoba's research on ManC-lectibody since it demonstrates how the intensity of high mannose glycans can stimulate ManC-lectibody to eliminate cancer cells faster due to the more recognition of sugars on the cell's surface. Moreover, Kailemia and her team also discuss a glycosylation modification made to the Fc region on an antibody. Glycosylation is an enzymatic process that attaches glycans, or sugars, to the antibody[12]. In Kailemia's research, the modification to the glycosylation site on the antibody's DNA terminated the enzymatic reaction. This modification resulted in a "GnGn glycovariant", or a sugar variant of the drug, which is created when the glycosylation process is halted in the middle of the enzymatic pathway[9],[12]. The GnGn glycovariant is not found in nature, therefore requiring the genetic modification[12]. The Fc region has only one site for glycosylation and by modifying that site on a regular antibody, the researchers were able to find that there was a "enhanced recognition of glycans[12]." Given this, a GnGn glycovariant could improve ManC-lectibody's effectiveness since it could increase glycan recognition, which would, in theory, increase the drug's function.

Another aspect of the glycosylation process is that it can be reversed to cause severe changes to the Fc region's function. A study done by Michael Kelliher and Ramiah Jacks, lead researchers in the Department of Chemistry at DePaul University in Chicago, shows that by removing essential sugars from the Fc region, the antibody reduces its signaling abilities with immune cells. Decreasing "the activity of the Fc region... enables[s] the receptors to attract antigens," but it also stimulates less signaling between the Fc region and the effector cells[13]. With this, an "aglycosylation modification," as defined by Kelliher and his team, could serve as a drawback to ManC-lectibody's activity. This modification is specifically called a N200Q, or NQ, modification as it removes an amino acid at the two-hundred position and replaces it with a dysfunctional amino acid[13]. For the current research, however, the use of an aglycosylation modification or NQ glycovariant of ManC-lectibody may be beneficial since it could serve as a comparison trial to the GnGn glycovariant. Comparing the effects of both glycovariants will demonstrate how an Fc modification may improve the effectiveness of the drug. As mentioned before, ManC-lectibody has never been modified, thus contributing to existing research on the drug. This is

an important step in developing a drug as the most effective version of the drug is used in clinical trials.

Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)

The Fc region holds a cell interaction process known as antibody-dependent cell-mediated cytotoxicity, or ADCC. Maria Carmen Ochoa and her fellow researchers, in their immunology-based research publication, examined the process of antibody dependent cell-mediated cytotoxicity (ADCC) in depth. The authors contribute to the basis of knowledge about ADCC by contending that "antibodies attach to the surface of malignant cells, binding to proteins, which first stimulates the effector cell to enact on the malignant tumor[14]." ADCC induction occurs when there is a transmission of signals from the Fc portion of an antibody to the immune cell (see Figure 3). ManC-lectibody has been known to induce ADCC but has yet to be tested with the proposed glycovariants; that is the glycosylated and aglycosylated versions of the drug[9]. In theory, fewer cells present should indicate more signaling due to the higher cell-death response from the effector cells. In this research study, the immune cell used will be a cell line known as the Jurkat T cell line due to its "intensive use in modeling an immune system environment" without the use of an animal[15].



Brief Overview of Lung, Ovarian, & Melanoma Cancers

The glycovariants, or sugar versions, of ManC-lectibody will be tested on three types of mice-derived cancer cell lines: ovarian, lung, and melanoma cancer cell lines. The drug has been minimally tested on these cell lines and would contribute to the existing research on the drug if the glycovariants prove to be successful[9]. Additionally, the use of cell lines from immunocompetent mice will help simulate the immune system response while also allowing ADCC to occur. Starting with ovarian cancer, it is a type of cancer that begins in the female reproductive organ. It inhibits the ovaries and causes a tumor to arise. Ovarian cancer goes often undetected until it spreads to the rest of the body, specifically to the pelvis and abdomen[16]. Current research suggests that the cure rate for the fatal disease is around 30% and that preventing its spread is difficult due to the unnoticeability of it[17].

Along with ovarian cancer, lung cancer is another detrimental disease that has become the leading cause of cancer deaths within the United States[18]. It first develops by affecting a lymph node and can cause fatalities as the carcinoma spreads throughout the rest of the lung[18]. Finally, melanoma is a type of skin cancer that develops when melanocytes grow rapidly and causes dysfunction to the cell. This disrupts melanin, the pigment that gives the skin its color[19]. Current research proves that ManC-lectibody does inhibit these three cancer cells to stop its successive reproduction, but there is no research that shows if certain modifications to the drug will either sustain or improve its effect in killing the cancer cells[9].

Figure 3. "ManC-lectibody in relation with ADCC". The Fc region of ManC-lectibody sends a signal to the immune cell via the Fc gamma receptor and tells the immune cell to kill the cancer cell (Seber Kasinger, Dent, Mahajan, Hamorsky, & Matoba, 2019).

Gap, Value, Significance, & Research Goal

The research question has been centered around ManC-lectibody and the subsequent glycovariants of the drug while still playing a role in the larger context of cancer therapy. For this experimentation, the research question developed was: Which glycovariants of ManC-lectibody will increase ADCC activity? It has been hypothesized that the GnGn glycovariant of the drug will increase ADCC activity while still maintaining a high cell-death response. Although the drug has been known to selectively target cancer cells, it has never been modified, specifically with a glycosylation and aglycosylation modifications to create glycovariants[9]. Furthermore, an ADCC reporter assay has never been performed to quantify the effects of the glycovariants[9]. Finally, the drug has yet to be tested on different versions of ovarian, lung, and melanoma cancer cells[9]. While all these gaps are specific to the development of drug, ManC-lectibody is significant to cancer therapy since it will be the first-in-class immunotherapeutic drug to selectively target high mannose glycans. This will alleviate the issue of side effects that stem out from many cancer therapies. Additionally, the drug is "highly producible in a scalable plant-based system", thus allowing for widespread manufacturing of the drug[9]. Due to the drug's novel behavior and potential application, it is important to find the most effective version of the drug.

Method

A true experimental quantitative method was conducted to determine whether glycovariants of the drug would increase ADCC activity. ADCC activity was measured through an ADCC reporter assay, which quantified the signals between the effector cells (Jurkat T cell line) and the Fc region of the drug. Quantitative data was necessary as it aided in proving that each glycovariant increased ADCC activity and also helped determine the efficacy and potency of the glycovariant in terms of inducing ADCC. To employ this method, three main protocols were followed: cell splitting and plating, the addition of glycovariants, and the ADCC reporter assay.

Cell Splitting & Plating

Three cell lines were used: ID8, LLC, and B16F10 cell lines. These cell lines represent the specific version of ovarian (ID8), lung (LLC), and melanoma (B16F10) cell lines. The first protocol consisted of cell splitting and plating, which essentially means to culture, count, and plate cancer cells to ensure that the cells are in "optimal condition for the experiment[9]." This step is important because without healthy cells, the data may fluctuate and may have outliers, which could be detrimental to understanding the glycovariant's effects. To initiate this protocol, all the cancer cells were pre-cultured and only had to be split and counted. The enzyme Trypsin was used to separate the cells from a flask to make the cells confluent. According to lab scientists Frauke Haenal and Norbert Garbow, making the cells confluent "are important quality control parameters in cell-based assays" as they "determine the health of [the] cells[20]." After adding Trypsin, the cells were split based on the number of cells in the flask. Cell splitting refers to separating the cells from one flask to another so that the cells are healthy and confluent for future experiments. A hemocytometer, or cell counting device, was placed under a microscope at 40x magnification and helped count the cells. This was important as it "[normalized] the number of cells[20]." Once counted, the cell quantity was used in calculations to find the volume of cells and medium needed in order to plate the cells. Cell medium is the basic growth factors needed for the cells to stay healthy[20]. Cell plating refers to transferring the

cells into a ninety-six well plate. Ten-thousand cells were plated per well and the well plate was kept in an incubator at 37 degrees Celsius as this was the optimal temperature for cell growth[9]. This same process was done for the effector cells (Jurkat T cell line).

Addition of the Glycovariants

Through genome editing of ManC-lectibody, a DNA manufacturing company was able to create the glycovariants for the experiment. In order to achieve a true experimental method, the experiment needed a control. The wild type glycovariant served as the control and was essentially an unmodified version of the drug. After receiving the glycovariants in liquid form, they were added in different concentrations, through a serial dilution, to the well plate containing the cancer cells. A serial dilution splits the drug concentration by half, starting from an initial concentration of 50 nanomolar (nM). Nanomolar concentrations are used in many pharmacological experiments and help show the small amount of drug needed to produce a desirable effect[17]. A total of three trials were done for each cancer cell line as this helped with the statistical analysis.

ADCC Reporter Assay

The signaling between ManC-lectibody and the effector cells was measured using an ADCC reporter assay. According to Dr. Matoba and his team, "the assay measures ADCC not by cell death but rather by the expression of a reporter gene, known as luciferase[9]." Luciferase is expressed by the effector cell when signals travel from ManC-lectibody to the effector cell. When these signals travel across the Fc receptor, they activate the receptor which then stimulates the effector cell to produce luciferase. Higher expression yields of luciferase indicate greater Fc receptor activation[9]. Essentially, if more luciferase is present, then there are higher levels of signaling between the drug and the effector cell. In order to find the reporter gene, a luciferase substrate was added to the well plates. When this substrate binds to luciferase, it catalyzes to produce light, which can be quantified by the luminescence value. Once this value was obtained, it was converted into a fold induction value. This value simply shows the increase of the glycovariant's effects at different concentrations, which, when graphed, shows the minimal concentration needed to produce a strong effect[9]. Both the fold induction and luminescence values have no units and are used to quantify the signaling between the modified drug and effector cells. As deriving the fold induction value using an equation is a tedious process, a mathematical software known as GraphPad was used to automatically calculate fold induction when given raw luminescence values.

A non-linear regression data plot was created to show the drug's dose response for each glycovariant. This procedure is well known by researchers in the field of pharmacology[21]. When interpreting a dose response curve, there are two factors that determine the drug's effect: the top value and the EC50 value. The top value represents the efficacy of the drug and the EC50 value expresses the drug's potency. A drug's efficacy refers to its maximum, attainable strength when introduced into a stimulating environment while potency is defined as the drug's capability of producing an effect at a given concentration. Pharmacologists research drugs that are highly potent, meaning that the drug elicits the maximum effect at low concentrations[21]. In terms of the dose response curve, the efficacy of the drug would be the value at which the highest curve begins to level off while the EC50 is the concentration at which the drug gives half of its maximal response. For this experiment, the graphs will show the glycovariant's efficacy and potency needed to induce ADCC at a given concentration.

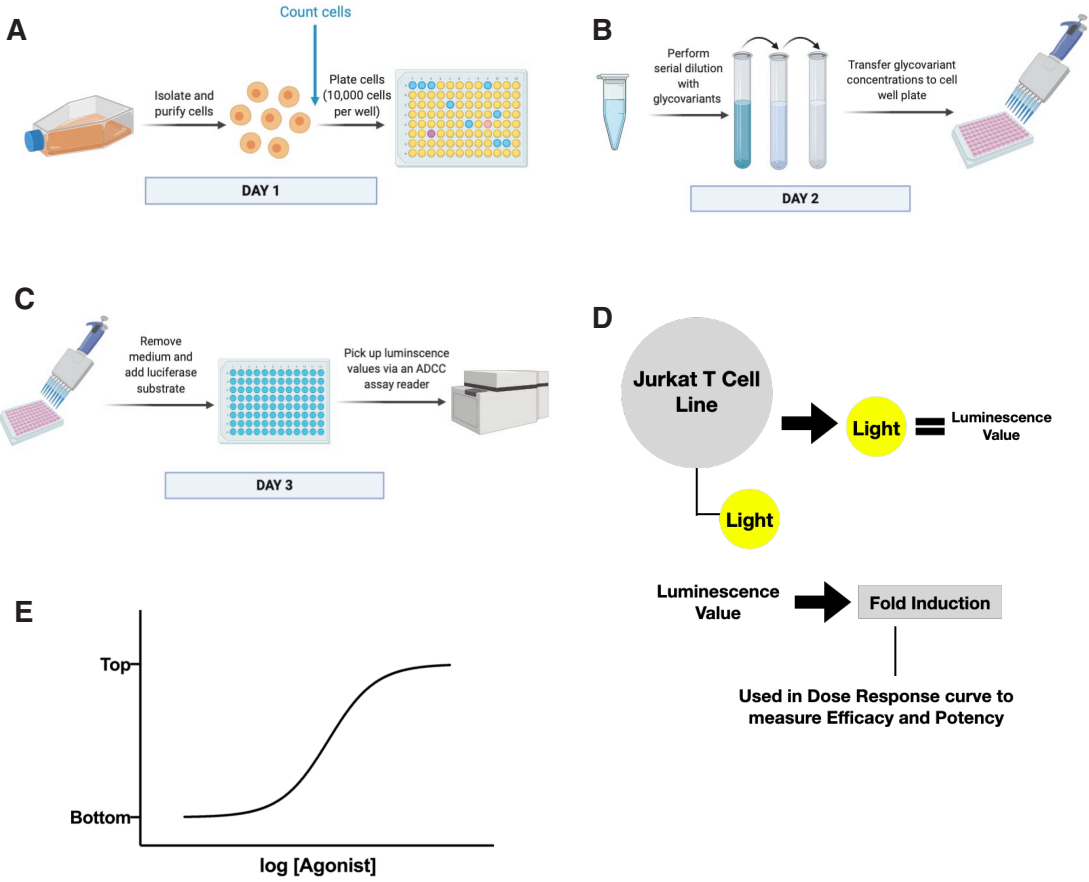


Figure 4. "Schematic Overview of the Methodology".

(A) All cancer cells were isolated and purified through the use of trypsin and a centrifuge. Once cells were confluent, they were counted using a hemocytometer. The quantitative number of cells was used to calculate the volume of cells and medium needed to achieve 10,000 cells per well. (B) The glycovariants were obtained in liquid form and were kept in an Eppendorf tube until time for the serial dilution. The serial dilution was performed in a sterile and new well plate to minimize error. After the concentrations were achieved, the glycovariants were transferred to the well plate containing the cells. (C) The old medium was removed carefully from each well as the cells settled on the bottom of each well. The luciferase substrate, which was part of a BioAssay Kit for ADCC assays, was then added in microliter volumes. The well plate was placed in the ADCC assay which transcribed light into luminescence values. (D) This diagram shows a sample ADCC assay. The Jurkat T cell line is engineered to produce light when the Fc receptor is activated. This light is converted into luminescence values, which then is converted into fold induction values. Fold induction values are used in dose response curves to help measure the drug's efficacy and potency in terms of inducing ADCC. (E) A sample dose response curve from GraphPad that displays the principles of top and EC50 values (Figures 4A-4D were created by the student while Figure 4E was obtained from GraphPad).

Results

Discussion & Analysis of Methods-Generated Data

To correctly determine whether a glycovariant had an effect on ADCC activity, the top and EC50 values were examined for all trials. A dose response curve was created for each trial along with a table that showed raw values. The GnGn glycovariant was examined closely as this was the hypothesized glycovariant. As mentioned before, the other glycovariants will be discussed as a comparison to the GnGn glycovariant to accurately depict the effects.

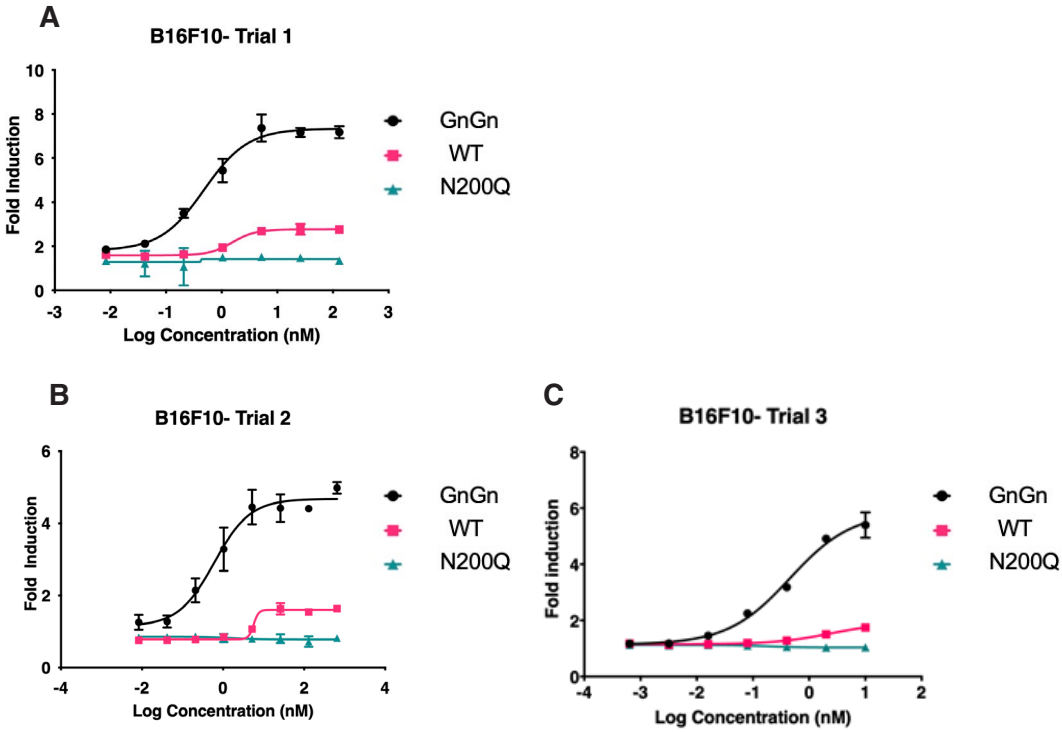


Figure 5. "Glycovariants of ManC-lectibody induce ADCC on B16F10 (melanoma) cancer cells."

(A) The GnGn glycovariant of ManC-lectibody reaches the highest peak for fold induction at a value of 7.335 for Trial 1. (B) Trial 2 indicates that the GnGn glycovariant has a higher Fc receptor activation as this version is able to induce ADCC with maximal effects at low concentrations. (C) Both the WT and N200Q (NQ) glycovariants fail to reach a fold induction peak beyond 4, depicting that they minimally activate the Fc receptor. *The NQ glycovariant did not elicit a strong enough effect; therefore, the EC50 value could not be calculated.

Table 1. Top Values of Glycovariants for B16F10 Cell Line (Melanoma cancer cells)

Trials	GnGn	WT	NQ
1	7.335	2.767	1.419
2	4.677	1.603	0.856
3	5.854	1.918	1.121

Table 2. EC50 Values of Glycovariants for B16F10 Cell Line (Melanoma cancer cells)

Trials	GnGn	WT	NQ
1	0.485	1.550	N/A*
2	0.605	5.683	N/A
3	0.453	2.412	N/A

Figure 5 illustrates the dose response curve for each glycovariant on the B16F10 cell line, with Trial 1 showing the most efficacy. Although the GnGn glycovariant is the most efficacious in inducing ADCC on the B16F10 cell line, this does not mean the glycovariant is capable of mediating cell cytotoxicity by directly acting on carcinoma. However, it does provide insight that the GnGn glycovariant activates the Fc receptor, meaning that there is ADCC activity within in the system. This supports the hypothesis that the GnGn glycovariant will increase ADCC activity by activating the Fc receptor. Collectively, the B16F10 cell line does prove that the glycovariants of ManC-lectibody can elicit Fc-mediated cytotoxicity via the effector cells but the drug is the most effective when it has a glycosylation modification.

The EC50 values of all glycovariants for the B16F10 cell line deviates significantly from the other cell lines. The cause of this behavior is unknown and would be a future topic of interest. For the GnGn glycovariant, the average EC50 value was 0.514. With regard to ADCC, this means that at a concentration of 0.514 nM, the glycovariant can produce half of the highest level of signaling. When compared to the other glycovariants, the GnGn version had a relatively low EC50 value, meaning that it was highly potent, since an EC50 value measures potency. This is important because many pharmaceutical drugs are highly potent, which make them effective at their function. While the WT glycovariant also has a relatively low EC50 value, it was not as strong as the GnGn glycovariant as indicated by the lower top value amount (Table 1).

The data above suggests that the LLC cell line had a slight increase in the top values and a significant increase in the EC50 values when compared to the B16F10 cell line. This could be taken under the assumption that the glycovariants have different effects when in contact with different cell lines. This could possibly be because of the varied expression of HMGs on each cancer cell or different glycans being produced from the cell. Stowell, Ju, and Cummings found that "protein glycosylation varied from cell line to cell line" and that one specific sugar alteration marked each cell line[22]. Additionally, they mentioned that diseased cells rarely produce other glycans, implying that the drug enacts differently on different cell lines[22]. Given this, glycovariants of the drug should specifically target one cell line that expresses HMGs consistently and makes the drug highly potent. The GnGn glycovariant had the highest efficacy in Trial 2 (see Figure 6B and Table 3) but was highly potent in Trial 1. The EC50 value did deviate from Trials 2 and 3, indicating that it could be a data outlier. But the findings stay consistent with the research question and prove again that the GnGn glycovariant increased ADCC activity. Dr. Matoba's team has tested an existing lung cancer drug known as Cetuximab and compared its ADCC activity to the WT version of the drug. Though the data is unpublished, they concluded that the WT version of the drug induces ADCC with a higher efficacy and potency compared to Cetuximab. Since the GnGn glycovariant is more effective than the WT version, the GnGn glycovariant could easily serve as a better targeted therapy.

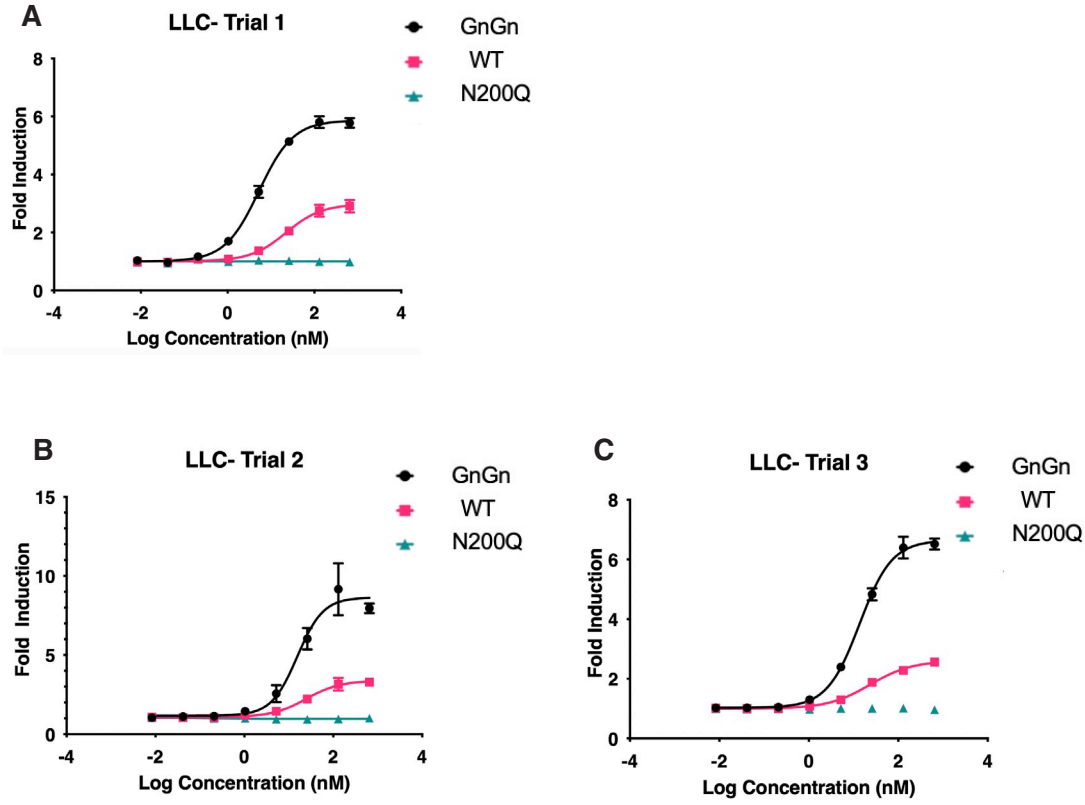


Figure 6. "Glycovariants of ManC-lectibody induce ADCC on LLC (lung) cancer cells."

(A) The GnGn glycovariant when introduced to the LLC cell line shows a similar dose response when compared to the GnGn glycovariant from the B16F10 cell line. (B) Trial 2 shows the GnGn glycovariant when it is the most efficacious at inducing ADCC via Fc receptor activation. (C) The WT does not show any potency or efficacy, implying how the removal of an essential sugar negatively affects the immune-mediated mechanism of the drug.

Table 3. Top Values of Glycovariants for LLC Cell Line (Lung cancer cells)

Trials	GnGn	WT	NQ
1	5.865	2.978	1.003
2	8.640	3.384	1.035
3	6.644	2.606	1.000

Table 4. EC50 Values of Glycovariants for LLC Cell Line (Lung cancer cells)

Trials	GnGn	WT	NQ
1	5.301	22.310	0
2	15.480	24.650	0
3	15.590	23.410	0

Table 5. Top Values of Glycovariants for ID8 Cell Line (Ovarian cancer cells)

Trials	GnGn	WT	NQ
1	4.876	2.878	0.984
2	5.208	2.801	1.064
3	4.940	1.665	1.091

Table 6. EC50 Values of Glycovariants for ID8 Cell Line (Ovarian cancer cells)

Trials	GnGn	WT	NQ
1	8.266	28.000	0
2	6.412	32.640	0
3	16.000	21.580	0

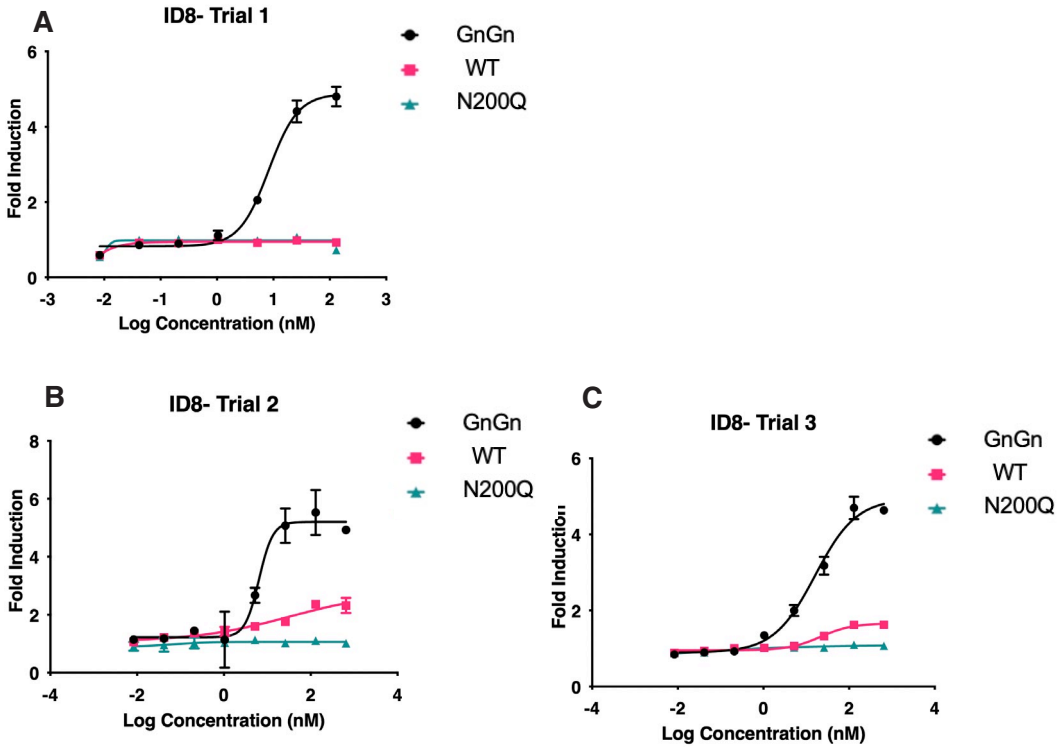


Figure 7. "Glycovariants of ManC-lectibody induce ADCC on ID8 (ovarian) cancer cells.

(A) Trial 1 shows no ADCC induction from the WT and NQ glycovariant as well as a relatively low ADCC induction for the GnGn glycovariant. (B) The WT glycovariant produces no curve to accurately portray the dose response. (C) There is a strong variation of EC50 values for the ID8 cell line, with Trial 3 having an EC50 value of 16.00. This may mean that the drug functions differently when in contact with ovarian cancer cells.

Table 7. Two-way ANOVA Test for Top Values

Source of Variation	% of Total Variation	P value	Significant?
Interaction	3.124	0.1542	No
Cell Line	2.678	0.0620	No
Treatment	86.80	<0.0001	Yes

Table 8. Two-way ANOVA Test for EC50 Values

Source of Variation	% of Total Variation	P value	Significant?
Interaction	8.415	0.0229	Yes
Cell Line	55.48	<0.0001	Yes
Treatment	26.49	<0.0001	Yes

As shown in Table 5, the highest top value recorded for the ID8 cell line was 5.208. This value is slightly less than both the LLC and B16F10 cell lines, indicating that the GnGn glycovariant minimally induced ADCC activity in ovarian cancer. However, this seems to be a data error because in Dr. Matoba's research, they found that ovarian tissue is overly expressed with HMGs[9]. In theory, the drug should display the highest ADCC activity when in an environment of ovarian cancer cells. However, this was not the case as it had the lowest efficacy among the three cell lines. Human error when pipetting and transferring could account for this difference. It is possible that the type of ovarian cell line used could have altered the data. Furthermore, the EC50 value slightly increased but was not consistent among the three trials. These trends can be attributed to the glycovariants as they could have affected the transmittance of signals. They could also be a result of effector cell dysfunction, meaning that the Jurkat T cell line could not kill the carcinoma. When objectively looking at the data, there seems to be increased ADCC activity with the GnGn glycovariant, which supports the hypothesis. However, this behavior of the glycovariant will be an aspect of future research.

Statistical Analysis

A two-way ANOVA test was done to statistically compare the effects of each glycovariant across all cell lines. The ANOVA test consisted of two variables: the glycovariant and the cell line. Using a two-way ANOVA test, one can determine the source of variation in the experiment to establish where the effects may come from. A P value was calculated for each glycovariant and this P value was compared to the biological P value of 0.05. This helped determine whether or not the glycovariant had a significant effect. An ANOVA test was done for both the top value and the EC50 value. The GnGn glycovariant was specifically examined as this was the hypothesized glycovariant. The ANOVA results for the top values of each glycovariant are shown in Table 7 and display four main components: percent of total variation, P value, P value summary (compares calculated P value to 0.05), and significance. If the calculated P value is less than 0.05, than the glycovariant's effect is significant. As listed in Table 7, there was about 2.7% variation from the cell line and about 87% variation from the glycovariant, indicating that each glycovariant causes the varying effects rather than the cell line. This was expected as the glycovariants should induce an effect on the various cell lines. The calculated P value for the cell line part of the ANOVA test was 0.062, which

makes this variable not significant whereas the calculated P value for the glycovariant part was less than 0.0001. This means that the different glycovariants are significant and show statistical difference, which was also expected. Furthermore, a multiple comparisons table was created to show all the possible combinations of glycovariants and lists their mean difference, P value, and whether or not they were statistically different. All GnGn glycovariants, when compared to the WT and NQ glycovariants, had P values less than 0.05, therefore meaning that the GnGn glycovariant was statistically different and had achieved in increasing ADCC activity.

When examining the EC50 ANOVA test, only the GnGn and WT glycovariants were compared as the NQ was not potent enough to produce an EC50 value. The percent of total variation for the cell line was about 55.5% while the glycovariant percentage was about 26.5%, which differed from the previous ANOVA test (see Table 8). Given this, the source of variation for the EC50 values could be dependent on the cell line rather than the treatment as seen in the top values ANOVA test. It was assumed that the EC50 value of each treatment should be similar across any cell line; however, the EC50 value recorded in the first trial of the B16F10 cell line shows deviation. Both the cell line and treatment variables resulted in P values that were significantly less than 0.05, showing that the EC50 values were statistically significant. The GnGn treatment was statistically significant against the WT treatment in all cell lines except the B16F10 cell line, implying that the GnGn treatment induces a different effect in melanoma cancer cells.

Conclusion

ManC-lectibody is an engineered molecule capable of selectively targeting diseased cells. This form of targeted therapy mitigates detrimental side effects to the human body while also enhancing the immune system to fight against foreign molecules. Dr. Matoba and his team have proved that ManC-lectibody induces ADCC and that the "mechanism of action of ManC-lectibody is mostly immune-mediated[9]." Dr. Matoba's research surrounded the standard version of the drug, which had no alterations. This research serves to fill a gap in the existing body of knowledge by modifying ManC-lectibody to potentially enhance its function of inducing ADCC. Earlier research suggested that altering the glycosylation site in the Fc region of an antibody changes the structure of the antibody, thereby changing its function[8]. Based on this information, three glycovariants of the drug were created that specifically changed the Fc region of ManC-lectibody: the GnGn, WT, and N200Q. It was hypothesized that the GnGn glycovariant would increase Fc receptor activation which would simultaneously increase ADCC activity. Using an ADCC reporter assay, this research was able to prove that the GnGn glycovariant of the drug increases ADCC activity when in contact with melanoma, lung, and ovarian mouse cancer cell lines.

Since the drug is still in testing phases, it is important to understand the data accurately. Though the GnGn glycovariant increases ADCC activity, it varies across cell lines. Additionally, the data shows inconsistencies with the EC50 values, suggesting further testing on certain cell lines. Despite these technical issues, the glycovariants add to the gap in the existing knowledge about ManC-lectibody and serve as the first modifications made to the drug.

The novelty of this study is that it is the first to enhance ManC-lectibody's function as an immunotherapeutic drug. Although the gap in the research is specific to improving ManC-lectibody's function, it can be applied to a bigger gap of finding a way to facilitate the production and implementation of pharmaceutical drugs. Many drugs are not applicable for large-scale manufacturing. However, the production of the drug is "rapid and scalable [through a] plant-based transient overexpression system[9]." Perhaps the most notable gap that ManC-lectibody fills is the fact that if approved by the Federal Drug Administration (FDA), it will serve as the first-in-class immunotherapeutic drug to selectively target HMGs. Because of this, it is vital that the most effective version of the drug is created. With this research, the GnGn glycovariant surpasses the standard drug and has an enhanced function in inducing ADCC, serving as the primary contender for approval.

Limitations

Since ManC-lectibody is still in testing phases, it is important to consistently get accurate results. In

this research, there was some human error as well as data outliers that negatively impacted the data. Additionally, the glycovariants produced different trends among the data, which sparked new understandings about the behavior and function of the modified drug. Another concern is that the drug may perform differently during in vivo tests. Although the primary use of mouse-derived cancer cell lines was to mitigate this, the in vivo tests could have some error that would impact the current knowledge about the drug. Finally, this study only examined the glycovariants effects on ID8, LLC, and B16F10 mouse cell lines and not human cell lines. The glycovariants could induce a different effect on human cancer cells, which could either hinder or improve ManC-lectibody's function.

Future Directions

The glycovariants of ManC-lectibody will be tested on immunocompetent mice through an in vivo study to better simulate an immune system environment. Furthermore, the EC50 variation that occurred would be further studied to see if the cause of this variation is the glycovariants or human error. This is specific to the B16F10 cell line as the GnGn glycovariant had the most EC50 deviation on melanoma cancer cells. In addition, other types of ADCC assays will be performed on the glycovariant to examine other aspects of the modified drug. Most importantly, however, ManC-lectibody will be researched on its ability to selectively target Covid-19 as this virus has HMG-type glycans that coat its shell23]. ManC-lectibody has been tested on the SARS-CoV virus, which is similar to the novel Covid-19 virus, and Dr. Matoba's team have found that it is unable to neutralize the virus, meaning that ManC-lectibody cannot prevent the virus from spreading. However, no research exists on ManC-lectibody's ability to induce ADCC on infected cells to possibly kill the cells before the virus reproduces and spreads (See Figure 8).

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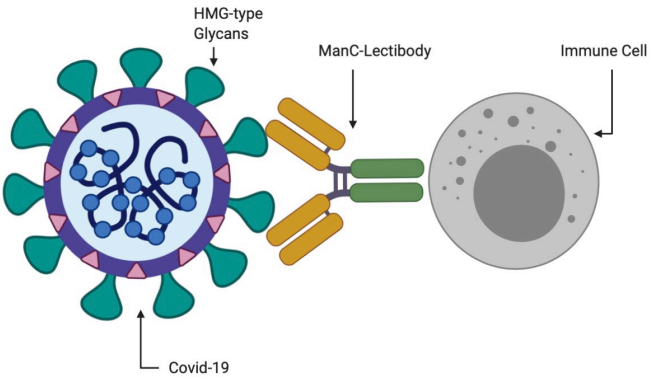

Figure 8. "Glycovariants of ManC-lectibody against Covid-19 using ADCC"
ManC-lectibody has the potential to induce ADCC on infected cells. The spike proteins that stem out of the Covid-19 virus are glycoproteins that contain oligomannose-type glycans, or HMG-type glycans. Since ManC-lectibody is selective to HMGs, it could possibly target the virus and act as an immune-mediated drug to kill infected cells.

References

1 Pharmaceutical Statistics. (n.d.). Retrieved September 29, 2019, from https://www.mailman.columbia.edu/become-student/degrees/masters-programs/master-science/pharmaceutical-statistics.

2 Pharmaceutical Drugs. (n.d.). Retrieved September 29, 2019, from https://www.rand.org/topics/pharmaceutical-drugs.html.

3 Pathak, N. (2019, January 23). What Are the Pros and Cons of Immunotherapy? Retrieved September 29, 2019, from https://www.webmd.com/cancer/immunotherapy-risks-benefits#1.

4 Naran, K., Nundalall, T., Chetty, S., & Barth S. (2018). Principles of Immunotherapy: Implications for Treatment Strategies in Cancer and Infectious Diseases. Frontiers in Microbiology, 9, 3158. https://doi.org/10.3389/fmcib.2018.03158

5 Janeway, C. A. (1970, January 1). The structure of a typical antibody molecule. Retrieved September 19, 2019, from https://www.ncbi.nlm.nih.gov/books/NBK27144/.

6 Blundell, P. A., Le, N., Allen, J., Watanabe, Y., & Pleass, R. J. (2017). Engineering the fragment crystallizable (Fc) region of human IgG1 multimers and monomers to fine-tune interactions with sialic acid-dependent receptors. The Journal of biological chemistry, 292(31), 12994–13007. https://doi.org/10.1074/jbc.M117.795047

7 Nicholson L. B. (2016). The immune system. Essays in biochemistry, 60(3), 275–301. https://doi.org/10.1042/EBC20160017

8 Chaplin D. D. (2010). Overview of the immune response. The Journal of allergy and clinical immunology, 125(2 Suppl 2), S3–S23. https://doi.org/10.1016/j.jaci.2009.12.980

9 Seber Kasinger, L. E., Dent, M. W., Mahajan, G., Hamorsky, K. T., & Matoba, N. (2019, August). A novel anti-HIV-1 bispecific bNAb-lectin fusion protein engineered in a plant-based transient expression system. RetrievedSeptember 19, 2019, from https://www.ncbi.nlm.nih.gov/pubmed/30729651

10 Chiba, H., Inokoshi, J., Nakashima, H., Ōmura, S., & Tanaka, H. (2004). Actinohivin, a novel anti-human immunodeficiency virus protein from an actinomycete, inhibits viral entry to cells by binding high-mannose type sugar chains of gp120. Biochemical and Biophysical Research Communications, 316(1), 203–210. doi: 10.1016/j.bbrc.2004.02.036

11 Strimbu, K., & Tavel, J. A. (2010). What are biomarkers?. Current opinion in HIV and AIDS, 5(6), 463–466. https://doi.org/10.1097/COH.0b013e32833ed177

12 Kailemia, M. J., Park, D., & Lebrilla, C. B. (2017). Glycans and glycoproteins as specific biomarkers for cancer. Analytical and bioanalytical chemistry, 409(2), 395–410. https://doi.org/10.1007/s00216-016-9880-6

13 Kelliher, M. T., Jacks, R. D., Piraino, M. S., & Southern, C. A. (2014). The effect of sugar removal on the structure of the Fc region of an IgG antibody as observed with single molecule Förster Resonance Energy Transfer. Molecular Immunobiology, 60(2), 103–108. Retrieved from https://www.sciencedirect.com/science/article/pii/S016158901400 0856?via=ihub

14 Ochoa, M.C., Minute, L., Rodriguez, I., Garasa, S., Perez-Ruiz, E., Inogés, S., Melero, I. and Berraondo, P. (2017), Antibody-dependent cell cytotoxicity: immunotherapy strategies enhancing effector NK cells. Immunol Cell Biol, 95: 347-355. doi:10.1038/icb.2017.6

15 Chen, J.-L., & Nong, G.-M. (2018). Advances in application of Jurkat cell model in research on infectious diseases. Chinese Journal of Contemporary Pediatrics , 20(3), 236–242. Retrieved from http://www.zgddek.com/EN/Y2018/V20/I3/236

16 Ovarian cancer. (2019, July 25). Retrieved September 29, 2019, from https://www.mayoclinic.org/diseases-conditions/ovarian-cancer/symptoms-causes/syc-20375941.

17 Lengyel E. (2010). Ovarian cancer development and metastasis. The American journal of pathology, 177(3), 1053–1064. https://doi.org/10.2353/ajpath.2010.100105

18 Lung cancer. (2019, August 13). Retrieved September 29, 2019, from https://www.mayoclinic.org/diseases-conditions/lung-cancer/symptoms-causes/syc-20374620.

19 Melanoma Skin Cancer Statistics. (2019, August 14). Retrieved September 29, 19AD, from https://www.cancer.org/cancer/melanoma-skin-cancer/about/key-statistics.html.

20 Haenel, F., & Garbow, N. (2014). Cell Counting and Confluency Analysis as Quality Controls in Cell-Based Assays, 1–5. Retrieved from https://www.perkinelmer.com.cn/labsolutions/resources/docs/011833_01_APP.pdf

21 Li, J., McKeown, L., Ojelabi, O., Stacey, M., Foster, R., O'Regan, D., Porter, K. E., & Beech, D. J. (2011). Nanomolar potency and selectivity of a Ca^{2+} release-activated Ca^{2+} channel inhibitor against store-operated Ca^{2+} entry and migration of vascular smooth muscle cells. British journal of pharmacology, 164(2), 382–393. https://doi.org/10.1111/j.1476-5381.2011.01368.x

22 Stowell, S. R., Ju, T., & Cummings, R. D. (2015). Protein glycosylation in cancer. Annual review of pathology, 10, 473–510. https://doi.org/10.1146/annurev-pathol-012414-040438

23 Liu, C., Zhou, Q., Li, Y., Garner, L. V., Watkins, S. P., Carter, L. J., Smoot, J., Gregg, A. C., Daniels, A. D., Jervey, S., & Albaiu, D. (2020). Research and Development on Therapeutic Agents and Vaccines for COVID-19 and Related Human Coronavirus Diseases. ACS central science, 6(3), 315–331. https://doi.org/10.1021/acscentsci.0c00272