

NK CELL CYTOTOXICITY IN INFLUENZA-INFECTED MICE WITH DIET

VARIATIONS

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Honors Thesis

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2017

Approved:

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Abstract

Obesity is epidemic that affects more than a third of the U.S. population. Additionally, the influenza virus is an infectious disease that affects millions of people and claims a quarter to half a million lives each year. The few studies that have been conducted on these two factors and their effect on immune response have found that obese influenza-infected mice had suppressed immune function in combatting the influenza infection as well as reduced expression of proinflammatory cytokines and decreased NK cell cytotoxicity. In this study, we expand on this topic and explore the effects of NK cell cytotoxicity with diet-induced weight change in influenza-infected mice. One of the goals of this study is to determine if beneficial weight change from a metabolically obese mouse to lean plays a significant role in restoring NK cell function. Upon analyzing the data, we found that weight change did not play a significant role affecting NK cell cytotoxicity. Furthermore, we found that the original weight status of the mouse had similar NK cell function and so mice that were originally lean, regardless of if they underwent weight change, had a significantly greater NK cell cytotoxicity than if the mouse was originally obese. I believe that these studies are relevant to global public health and preventative medicine because if we are able to determine how weight change effects the immune function in influenza-infected models, we will be better able to understand how to help certain individuals based on their weight status.

Introduction

Obesity and the Immune System

Beginning in the late twentieth century, the scientific community began to acknowledge obesity as a growing epidemic and a cause for many health risks such as cardiovascular disease,

diabetes, and cancer. Obesity trends have been growing globally as well as nationally and in the last decade alone the prevalence of adult obesity has increased 5.5% in the United States. The most recent (2011-2014) figures from the CDC indicate that 36.5% of U.S. adults and 17.2% of U.S. children are obese (1). More importantly obesity has shown to result in altered immune response leading to increased susceptibility to bacterial infection, chronic increases in pro-inflammatory response, and even increased vulnerability to viral infections (2). With a little over a third of the population dealing with the disease and its many health risk factors, obesity prevention and treatment plays a crucial role in the sustainability of a healthy population.

The immune system plays an integral part in humans as the primary protector of the body against foreign pathogens while also utilizing biological mechanisms for self-identification and maintenance. The immune system is divided into two classes: innate (non-specific) and acquired (specific) immunity. The innate immune system consists of several components such as physical barriers and mucous membranes, antimicrobial substances, phagocytes such as neutrophils and macrophages, and other leukocytes such as natural killer (NK) cells (3). The innate immune system is regarded as the general response by the body and uses germline-encoded pattern-recognition receptors (PRRs) for microbial recognition. PRRs can bind to microbial components known as pathogen-associate molecular patterns (PAMPs), essential for the survival of the microorganism, and allow for detection of the pathogen. PRRs are non-specific and independent of immunologic memory making the immune cells that utilize them a component of the innate immune system (4). The acquired (or adaptive) immune system is primarily characterized by its utilization of memory and past interactions to deliver immune response. This system recognizes foreign antigens projected on bacteria and virus-infected host cells and generates humoral and cell-mediated responses that are tailored to specifically destroy the pathogens carrying specific

antigens. These two responses also develop an “immunological memory” in order to handle subsequent attacks by the same pathogens (3).

Natural Killer Cells

Natural killer cells, also known as NK cells, are large, granular lymphocytes belonging to the innate immune system. NK cells are primarily developed in the bone marrow, but are found in the lungs, liver, lymph, and spleen, with the spleen being the target tissue for this study (5). NK cells serve several important functions most importantly in response to viral infections and tumor formations. In terms of viral infections, such as influenza, NK cells are recruited through two mechanisms. Either they are stimulated by direct engagement on their activating receptors by viral ligands or by the activation of chemokines and cytokines, such as interferon gamma (IFN- γ) and TNF- α , produced by other components of the immune system. These complex receptor interactions elude to the point that NK cells have more role in adaptive immunity than previously thought and why more research is being done about the specific roles NK cells play in this alternative branch of the immune system (5,6).

NK cells most prominent role is in the detection and destruction of virus-infected and cancer cells through their cytotoxic ability. NK cell cytotoxicity is a tightly regulated process and dictated by an array of receptors and signals that determine whether or not a cell in the body should be targeted by NK cells. The primary and most well understood mechanism is through a hypothesis known as the “missing self” hypothesis. Primarily all nucleated cells in the body have a major histocompatibility complex class I or MHC-I. MHC-I is present on the cell surface and displays intracellular peptides that the cells use for normal protein functions. This represents a display of normal function or “self” for a cell (5). Virus-infected cells and cancer cells can have a down regulation of MHC-I because it is thought that in order to avoid other immune responses

they decrease MHC-I translation. On a side note, NK cells have two classes of surface receptors, activating and inhibitory. It is the inhibitory receptors on the cell surfaces of NK cells which sense a down regulation of the MHC-I molecules. This mechanism is the indicator for NK cells that the cell is not functioning normally and has lost the sense of “self”. The second mechanism mentioned earlier is through the use of activating ligands on the cell surfaces of virus-infected and cancer cells. One example is that NK cells that carry a receptor such as Ly94H which recognizes ligands produced by virally-infected cells like m157 (6-8). When the NK cell encounters the correct ligand for its activating receptor it will activate its cytotoxic ability and be able to destroy the cell. NK cells exocytose intracellular granules such as perforin and other granzymes to create pores in the target cell membrane and induce apoptosis (9). Because there is an array of receptor and ligand interactions, NK cell mechanisms are still studied extensively seeing as how they work synergistically with other aspects of the immune system to recognize and ultimately destroy virus-infected and tumor cells.

Influenza and Obesity’s Effect on NK Cells

Influenza is an airborne infectious disease caused by the influenza virus. Every year, there is an influenza outbreak affecting three to five millions of people and resulting in up to half a million deaths worldwide (10). The influenza virus is classified as an RNA virus due to its genomic make-up and has three distinct classes: Influenza A, B, and C. Additionally, with obesity dramatically on the rise and affecting a large portion of the population, various studies have been conducted exploring the effect obesity has on one’s susceptibility to viral infections such as the influenza virus (11). For example, following the H1N1 pandemic in 2009 the California Department of Public Health found that obese adults were more hospitalized due to H1N1 and that extreme obesity was associated with increased odds of death by the virus (12).

Influenza's effects have also been studied with the immune system, specifically NK cells. Following an infection with the influenza virus, NK cells are recruited to lung and other infected tissues with various signals such as interferon gamma. These NK cells function to destroy the influenza-infected cells as well as help in adaptive immunity to the influenza virus. Various experiments, human and murine, have looked at NK cell function with influenza virus. One murine study found that certain receptors NKp46/NCR1 were essential in influenza response by NK cells. Knockout mice without these NK receptors had increased viral titers compared to the normal wild-type mice. Additionally, a human found during the pandemic of H1N1 that individuals had decreased numbers of peripheral blood NK cells compared to their healthy counterparts (8). However, much more research needs to be done to determine how influenza and obesity play a role in NK cell function.

The central literature that this study is developed from explores the immune response of diet-induced obese mice compared to normal lean mice. The study found that NK cell cytotoxicity and enumeration was significantly reduced in the influenza-infected mice in the lung and spleen. In this study we wanted to expand on this association and explore the effects of weight change on NK cell cytotoxicity. By creating two additional groups of mice, lean-to-obese and obese-to-lean, we would be able to potentially see if weight change could restore NK function (2).

Methods

Mice Growth and Diet

The mice used for the study were C57BL/6J mice obtained from Jackson Labs. Their genetic background makes them susceptible to diet induced obesity making them a good model

for this study. The mice are obtained at 6 weeks of age and are acclimated for one week at the UNC School of Medicine Animal Colony. The mice were then randomly placed into two groups to induce the initial weight status. One group was fed normal chow (low fat) to maintain a metabolically lean weight status while the second group was put on a high fat diet to induce obesity. Additionally, the mice were also infected with influenza in the first 22 weeks to create an influenza-infected mouse model for this study. At the end of 22 weeks, both groups were randomly divided into two additional groups. Half would remain on the same diet and half would switch to the alternate (low or high fat) diet to induce weight change. After 15 weeks on the weight altering diet, the mice were now in four distinct groups: always lean, always obese, lean-to-obese, and obese-to-lean. The mice were then euthanized and spleens were extracted. Jenny Rebeles of the Beck lab was the primary animal handler and caretaker for the mouse study population.

YAC-1 Cell Growth and Maintenance

To perform the NK cytotoxicity assay, target cells are required to interact with the spleen cells of the mice. The cells obtained as the target for the spleen NK cells were YAC-1 cancer cell line which is commonly used for NK cell studies. These cells were cultured in 75 cm² culture flasks containing 7.5 mL of RPMI 1640 (ThermoFisher) with 10% fetal bovine serum and 1% Pen-strep and stored in an incubator at 37°C with 5% CO₂. The cells were split in their logarithmic growth phase which peaked at 1 to 2 million cells per mL as this was the optimal growth volume before deterioration of the media and overcrowding in the cell plates. The YAC-1 cells were maintained throughout a six-month period from June 2016 to December 2016. The cells were split regularly during this time period and if viability was down, there was a batch of frozen YAC-1 cells stored in liquid nitrogen. Prior to being used in the cytotoxicity assay, the

YAC-1 cells were split 24 hours before to ensure that they were in an upward growth phase and not too old. Splitting these cells the day before allowed them to be fresh and ensure the greatest viability for the experimental assay.

Spleen Extraction and Cell Enumeration

To obtain the spleenocytes to use for the cytotoxicity assay, spleens were extracted from the mice the same day they were euthanized. The entire spleen was removed and placed in a 10% FBS RPMI media with Pen-Strep on ice. Any fat tissue or adiposity was separated from the spleen tissue and discarded. The end of a 5 mL syringe was used to crush and dissolve the spleen tissue in the spleen media. The aim was to obtain a single cell suspension of the spleen media, therefore this was done thoroughly until there were no visible tissue clumps remaining. The solution was then run through a 40 μ m nylon mesh cell strainer to separate the spleen tissue and have single cell spleen suspension in the spleen media. This solution was then centrifuged at 300 x G for 8 minutes at 8° C. One mL of ammonium-Chloride-Potassium (ACK) lysis buffer was added for 1 minute at room temperature to lyse red blood cells from the spleen solution which would interfere with spleen cell enumeration. Visible cell debris would form after this step and would be removed by pipette. This step was repeated 2 to 3 times with the ACK lysis buffer until there was no visible layer of blood after centrifuging. The spleen pellet was then suspended in 1 milliliter of spleen media and placed on ice until cell counting.

At this stage in the procedure the YAC-1 cells were also prepped so that both spleenocytes and YAC-1 cells could be counted using a hemocytometer at the same time. For the setup of the cytotoxicity assay, discussed in detail below, there were to be four sets of spleen-to-cancer cell ratios for each mouse spleen. In essence, there would be a ratio of 100:1, 50:1, 25:1, and 12.5:1 of spleen cells to cancer cells. Prior to running the experiment, optimization protocols

were run to determine the optimal number of target cells (YAC-1 cancer cells) to use. In the October 4, 2016 experiment 10,000 YAC-1 cells per well were used whereas in the December 20, 2016 experiment it was 40,000 YAC-1 cells per well. For spleenocytes the ratios were determined based off the YAC-1 target cells in the respective ratios listed above. These cells were counted by staining with Trypan Blue at a 1:1 ratio of cells to Trypan Blue. The cells per mL were calculated accordingly and the cell solutions, spleenocyte and YAC-1 cells, were diluted to equal their target cells per mL.

Cytotoxicity Assay

The assay used in this study was the CytoTox 96® Non-Radioactive Cytotoxicity Assay developed by Promega (13). This is an alternative and safer procedure compared to the previously used radioactive Chromium release cytotoxicity assays. The CytoTox 96 assay works by measuring lactate dehydrogenase (LDH) which is released upon cell death. LDH is an enzyme that converts lactate to pyruvate and is present in most cells including the YAC-1 tumor cells. The reagent solution, which is a part of the assay kit, contains tetrazolium salt or iodonitrotetrazolium violet (INT) which reacts through the released LDH to form a red formazan product. The amount of formazan red created is proportional to the amount of LDH released and is in turn proportional to the amount of cells that were lysed or killed (13). The absorbance of the wavelengths from each well are then read using a standard plate reader and these absorbance values are calculated with various controls to determine the relative cell death in each well. In terms of the study, the NK cells from the spleenocytes should be interacting with the YAC-1 tumor cells causing them to lyse and die. The conversion of INT to formazan product should then only be occurring due to NK cell cytotoxicity of YAC-1 cells which are releasing LDH upon death. The proportions calculated from the absorbance values should then give us the

percent cytotoxicity of their NK cells that each mouse in the four diet groups had. This would then allow us to compare cytotoxicities between the four groups and determine NK cell effectiveness.

The assay plates used were standard 96 V-bottom cell plates. The plates were all setup with either control or experimental wells in either triplicates or quadruplicates. The first set of wells were the experimental wells. These contained 100 uL of the YAC-1 target cells, 100 uL spleenocytes at the four ratios 100:1, 50:1, 25:1, and 12.5:1 of spleen-to-cancer cells (in the 12/20/16 experiment 100:1 ratio was removed and 6.25:1 ratio was added). Adjacent to each of these wells was the effector spontaneous LDH release wells which contained 100 uL of culture media, 100 uL of spleenocytes in order to determine how much LDH is released naturally from the spleenocytes themselves through leaking, apoptosis, or etc. Next a target cell (YAC-1) spontaneous and maximum LDH wells were prepared with 100 uL of culture media in the former and 100 uL of a lysis solution in the latter. Finally, culture background and volume correction control wells were setup with 100 uL of culture media with 100 uL spleenocytes and 100 uL culture media, 100 uL Spleen Media, and 20 uL lysis solution, respectively (13).

Example Cell Plate											
Target Spon- taneous	Culture Media Control		Effector Spontaneous #1					Effector Spontaneous #2			
			100:1	50:1	25:1	12.5:1		100:1	50:1	25:1	12.5:1
Target Max Release	Volume Corr. Control		Experimental #1					Experimental #2			
			100:1	50:1	25:1	12.5:1		100:1	50:1	25:1	12.5:1

Figure 1. Example setup of the cell plate with all six well types described above. Each 96 well plate can hold two mouse spleen samples.

Running the Cytotoxicity Assay

Once plate setup was completed, the plate was placed in an incubator (37°C, 5% CO₂) for four hours to allow time for the NK cells and target YAC-1 tumor cells to interact. In the 12/20/16 trial, the incubation time was increased to six hours to allow for more time the NK cells and tumor cells to bind. Forty-five minutes before the incubation time was finished, the lysis solution was added to all the maximum release and volume correction control wells. After the incubation time was complete, all the plates were spun in a centrifuge for 250 x G for 4 minutes. Then, 50 uL aliquots from all the wells were transferred to a 96 well flat-bottom plate where 50 uL of the CytoTox 96 Reagent was added to all the wells. This reagent contained the enzyme that would interact with LDH and turn red proportionately to the amount of LDH released. After a 30-minute rest period with the plate placed in room temperature under no light conditions, 50 uL

of Stop solution was added to each well to end the INT to red formazan reaction. Finally, the plate was placed in a plate reader and the absorbance values were recorded at 490 nm.

Calculations

After absorbance values were recorded, the percent cytotoxicity for each mouse was calculated incorporating the background and spontaneous release wells using the following formula (13).

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous}}{\text{Target Maximum} - \text{Target Spontaneous}} * 100$$

Equation 1. Formula used to calculate percent cytotoxicity for each mouse at the four effector-to-target cell ratios.

Statistical Design

An ANOVA test was run for the four mice groups using SAS statistical software. A t-test was run after combining the groups into two groups to determine significance. Differences were considered significant at a $P < 0.05$.

Results

The data obtained for the study primarily originates from two trials, a twenty mouse trial on October 4, 2016 and a three mice trial on December 20, 2016, with slight variations from each other in procedure outlined earlier. The percent cytotoxicity for each mouse were calculated with respect to their group type and effector-to-target cell ratios using the absorbance values. The tables for the two trials are shown below.

Table 1. Mouse data from trial 1 with mouse IDs, weight type, and percent cytotoxicity at each of the four effector-to-target cell ratios obtained on October 4, 2016

			% Cytotoxicity			
Date	Mouse #	Type	100:1	50:1	25:1	12.5:1
10/4/2016	21	Lean	5.66	12.45	18.28	2.87
10/4/2016	22	Lean	12.36	-5.05	6.79	0.09
10/4/2016	23	Lean	22.36	19.99	34.83	6.75
10/4/2016	24	Lean	52.98	22.69	13.82	18.46
10/4/2016	25	Lean	-23.18	123.44	77.14	53.74
10/4/2016	26	Lean to Ob	20.37	-94.85	26.40	-11.28
10/4/2016	27	Lean to Ob	35.79	4.39	0.21	-2.06
10/4/2016	28	Lean to Ob	55.00	23.32	0.85	-0.57
10/4/2016	29	Lean to Ob*	647.02	1931.79	987.42	66.89
10/4/2016	30	Lean to Ob*	56.29	-30.46	-91.39	-47.68
10/4/2016	31	Obese	-81.27	-160.11	-23.08	-11.89
10/4/2016	32	Obese	-87.18	-32.91	-34.19	-59.62
10/4/2016	33	Obese*	18.44	-4.12	7.32	0.15
10/4/2016	34	Obese*	-251.90	33.81	-14.76	-3.33
10/4/2016	35	Obese*	-91.30	-1.74	-26.96	-0.87
10/4/2016	36	Ob to Lean*	-603.48	-205.22	-184.35	-113.04
10/4/2016	37	Ob to Lean	-72.73	-12.38	131.14	-26.31
10/4/2016	38	Ob to Lean	-22.89	-18.76	-18.50	12.19
10/4/2016	39	Ob to Lean*	-480.36	-109.64	-150.36	5.00

10/4/2016	40	Ob to Lean*	-191.79	-143.21	-132.14	-122.50
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* — indicates the data values that are outliers and to be excluded from the test for significance

described below.

Table 2. Mouse data from trial 2 with mouse IDs, weight type, and percent cytotoxicity at each of the four effector-to-target cell ratios obtained on December 20, 2016						
			% Cytotoxicity			
Date	Mouse #	Type	50:1	25:1	12.5:1	6.25:1
12/20/2016	1	Lean	17.16	-6.21	-2.24	-6.90
12/20/2016	2	Obese	2.93	-5.43	-11.12	-5.17
12/20/2016	3	Ob to Lean	18.02	-11.12	-4.83	-10.26

The data from trial 1 and trial 2 were combined and outliers were removed using two methods. I first removed the outliers using the IQR rule. I also removed visual outliers that were unreliable by looking at the absorbance value readings that showed data that did not make practical sense. For example, some results from the plate readings showed maximum release of LDH in the YAC-1 cells lower than spontaneous release of LDH in the same concentration of YAC-1 cells. After removing these values, Table 3 shows the fifteen data points that remained and would be used in the statistical test.

Table 3. Data from trial 1 and 2 combined with outliers removed. This was the final data set used in the tests for significance.						
			% Cytotoxicity			
Date	Mouse #	Type	100:1	50:1	25:1	12.5:1

10/4/2016	21	Lean	5.66	12.45	18.28	2.87
10/4/2016	22	Lean	12.36	-5.05	6.79	0.09
10/4/2016	23	Lean	22.36	19.99	34.83	6.75
10/4/2016	24	Lean	52.98	22.69	13.82	18.46
10/4/2016	25	Lean	-23.18	123.44	77.14	53.74
10/4/2016	26	Lean to Ob	20.37	-94.85	26.40	-11.28
10/4/2016	27	Lean to Ob	35.79	4.39	0.21	-2.06
10/4/2016	28	Lean to Ob	55.00	23.32	0.85	-0.57
10/4/2016	31	Obese	-81.27	-160.11	-23.08	-11.89
10/4/2016	32	Obese	-87.18	-32.91	-34.19	-59.62
10/4/2016	37	Ob to Lean	-72.73	-12.38	131.14	-26.31
10/4/2016	38	Ob to Lean	-22.89	-18.76	-18.50	12.19
12/20/2016	1	Lean	-	17.16	-6.21	-2.24
12/20/2016	2	Obese	-	2.93	-5.43	-11.12
12/20/2016	3	Ob to Lean	-	18.02	-11.12	-4.83

The ANOVA statistical test was run and the analysis is displayed below. Figure 2 shows percent cytotoxicity of the NK cells versus the four mouse weight types for all the effector-to-target cell ratios. At a p-value of 0.05, the only set of data that was significant was the 100:1 ratio which had a p-value of 0.0021. Figure 3 shows the statistically significant graph of 100:1 effector-to-target cell ratio in greater detail.

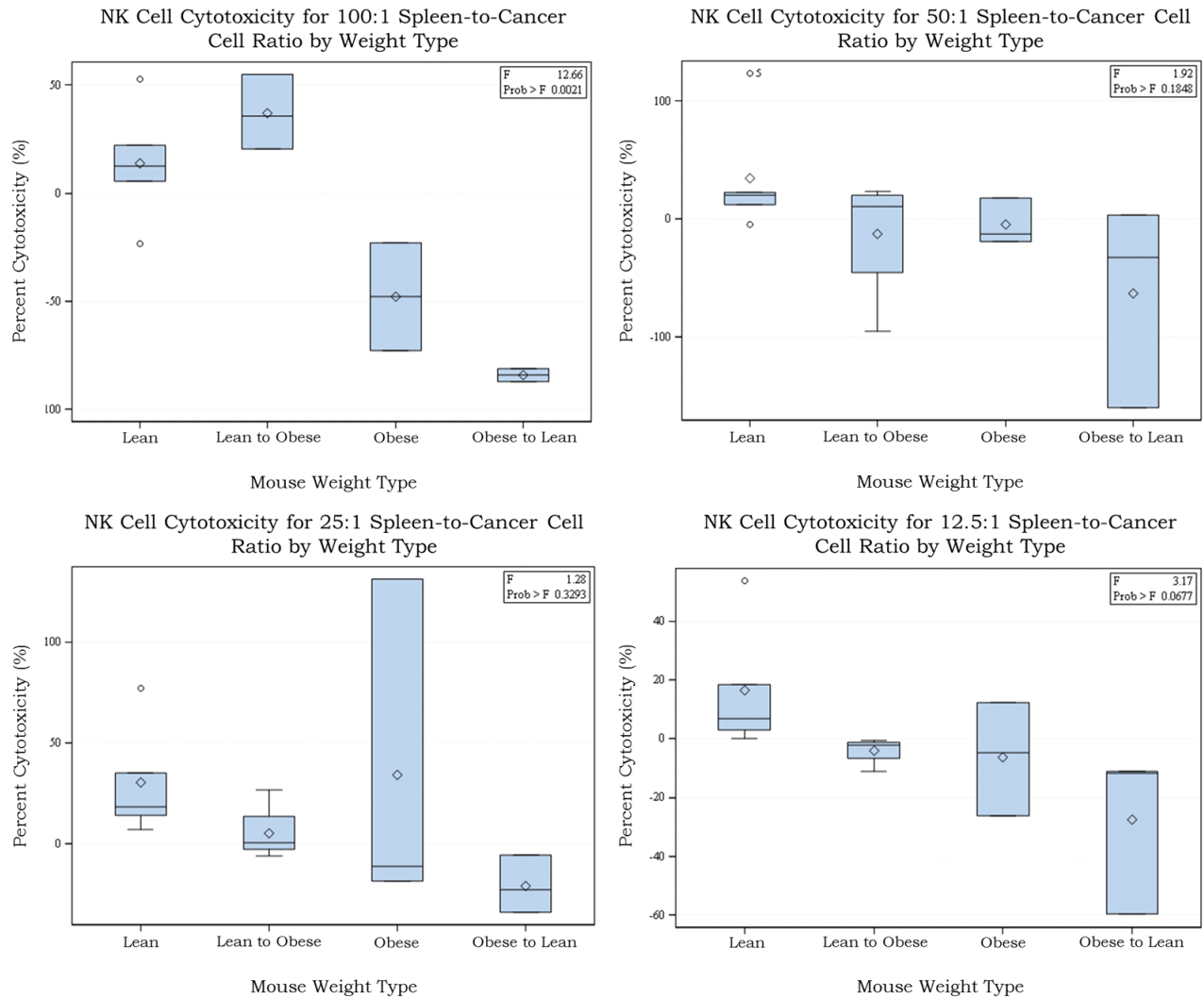


Figure 2. NK cell cytotoxicity for all 4 effector-to-target cell ratios and separated by weight type.

Although the 50:1, 25:1, and 12.5:1 cell ratios were not statistically significant it is worth noting the general downward trend of the graphs as you go from lean, lean-to-obese, obese, and finally to obese-to-lean.

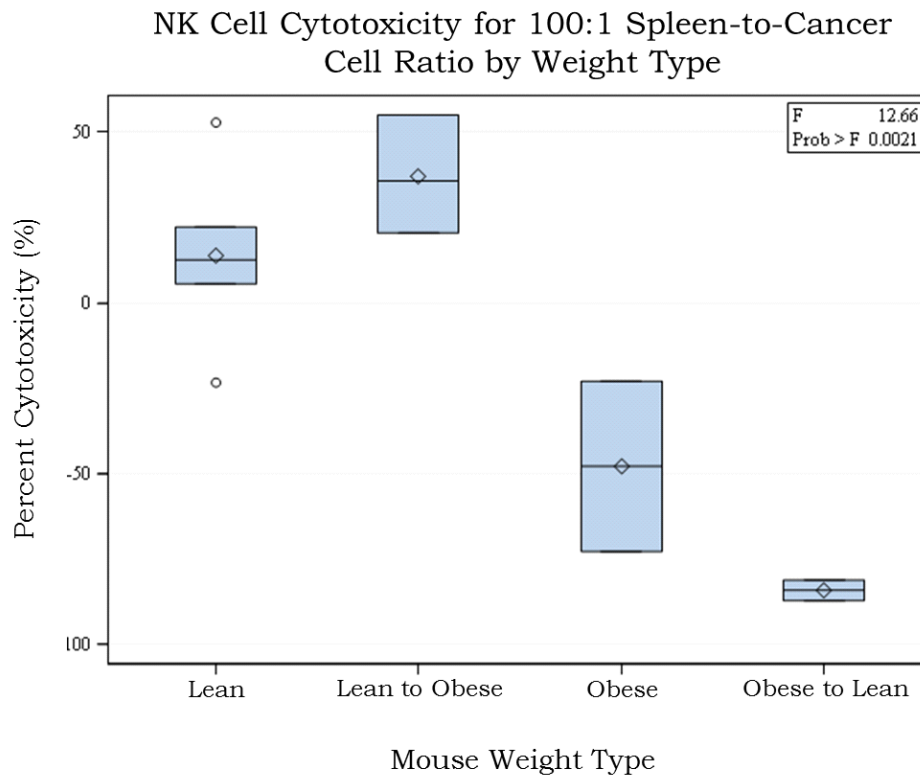


Figure 3. NK cell cytotoxicity for the 100:1 spleen-to-cancer cell ratio. The p-value is less than 0.05 indicating statistical significance.

The 100:1 effector-to-target cell ratio figure shows that there was a significant difference between the four mouse weight types in their NK cell cytotoxicity. Additionally, we can see that the lean and the lean-to-obese mice had greater cytotoxicity than the obese and obese-to-lean mice. To further explore this trend, I combined the lean and lean-to-obese mice in a group called “Lean Original” and the obese and obese-to-lean mice in a group called “Obese Original”. I ran a t-test to determine if there was a significant difference between these two groups when combined in a group with mice of the same original weight status. Figure 4 below shows percent

cytotoxicity of the NK cells versus the two combined original mouse weight types for all the effector-to-target cell ratios.

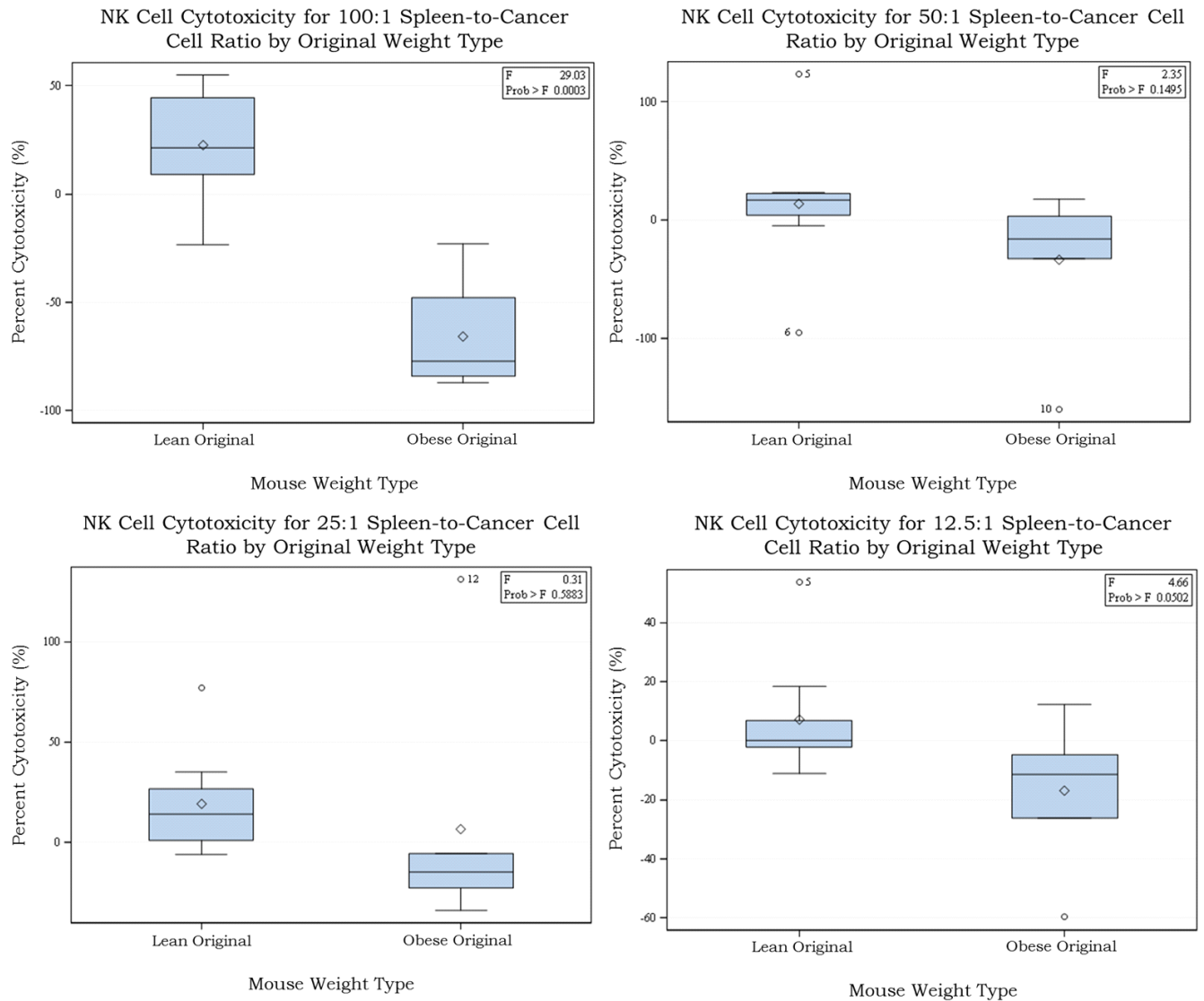


Figure 4. NK cell cytotoxicity for all 4 spleen-to-cancer cell ratios and separated by weight type. The weight types are combined into two groups with “Lean Original” and “Obese Original”.

Similarly, with a p-value of 0.05 the only set of data that was significant was the 100:1 effector-to-target cell ratio. Figure 5 shows the 100:1 effector-to-target cell ratio by itself because it was the only statistically significant data set.

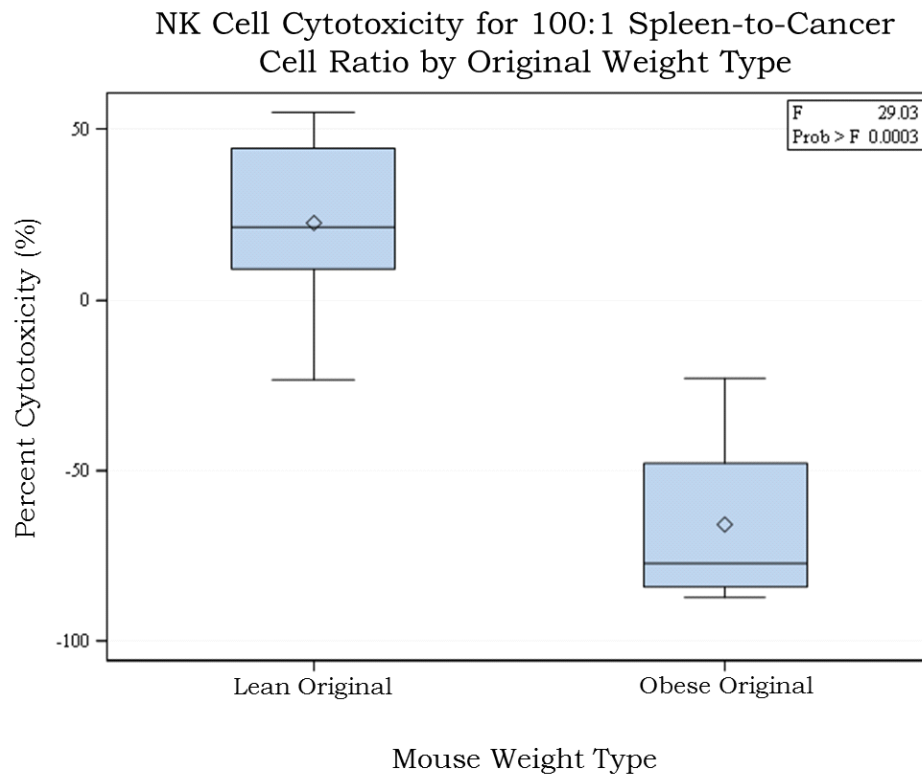


Figure 5. NK cell cytotoxicity for the 100:1 spleen-to-cancer cell ratio with the combined weight types. The p-value is less than 0.05 indicating statistical significance.

This figure shows that there is a significant difference in the cytotoxicity of the mice based on their original weight status at the start of the experiment. Mice that were originally lean had more cytotoxic NK cell activity compared to the mice that were originally obese regardless of the weight status change they underwent.

Discussion

Previous studies have established that the immune response to influenza vaccination is compromised in obese individuals. These studies also showed that NK cell cytotoxicity and enumeration were also decreased in the obese mice compared to lean mice (2). In this experiment, we wanted to expand on the study and attempt to establish an association similar to that described if there were weight changes in the mice. On top of the two groups of obese and lean mice, two additional groups were added to the study population to determine the effect of weight change on the immune response, specifically in NK cells. Four groups of mice were tested: always lean, lean-to-obese, obese-to-lean, and always obese. Because the original study had already established that always obese mice had decreased NK cell cytotoxicity and enumeration compared to the lean mice, I hypothesized that the obese-to-lean and the lean-to-obese groups would have greater NK cell cytotoxicity than the always obese mice, but less than the always lean mice. Similarly, I also hypothesized that the obese-to-lean mice would have greater NK cell cytotoxicity than the lean-to-obese mice. I hypothesized this because I assumed that the metabolic changes that follow obesity would have a negative impact on NK cell production and effectiveness and therefore make the mice that became obese have a lower NK cell cytotoxicity than the mice that became lean.

Upon interpreting the data, we can make the conclusion that the hypothesis was incorrect. Looking at the percent cytotoxicity of the four groups at the different effector-to-target cell ratios from Figure 2, we can see a general downward trend. The lean and lean-to-obese mice generally tend to have greater NK cell percent cytotoxicity than the obese and obese-to-lean mice. This correlation hints at the idea that the lean and lean-to-obese mice were similar in their NK cell effectiveness and had greater cytotoxicity than obese and obese-to-lean groups. With a p-value of 0.05, the only set of data that was significant was the 100:1 spleen-to-cancer cell ratio. For that

particular set of data, we can say the difference between the four groups NK cell percent cytotoxicity was statistically significant. This is different from my hypothesis because it shows that mice that were lean-to-obese had better percent cytotoxicity than the obese-to-lean whereas I had predicted that the opposite.

To further investigate this claim, I combined both the lean and lean-to-obese mice in one group as “Lean Original” meaning that these were mice that had lean weight statuses at the start of the study period. I combined the obese and obese-to-lean groups in a second group called “Obese Original” for similar reasons. I ran the ANOVA test again and the results were seen in Figure 4. Figure 4 showed a similar trend to that in Figure 2, with the lean mice groups have higher percent cytotoxicity than the obese groups. At a p-value of 0.05, the only data set again that was statistically significant was the 100:1 spleen-to-cancer cell ratios. It is worth noting that the 12.5:1 data set had a p-value of 0.052 which was close to the acceptable p-value, but not statistically significant. Analyzing the 100:1 cell ratio graph in Figure 5 in more depth, we see that the mice that were originally lean at the start of the study had significantly higher NK cell percent cytotoxicity than the mice that were originally obese at the start of the study period. This analysis shows that mice starting out lean had a more effective NK cells than the mice that started out obese regardless of the weight status change that they underwent. There could be a few possible reasons as to why the results were as shown.

Epigenetics, such as DNA methylation and histone acetylation, are heritable components in DNA that do not change the underlying genetic code, but can play a role in gene expression. Environmental factors and nutrition can play a role in these epigenetic factors and therefore could potentially result in altered gene expression. As we discussed earlier, NK cells rely on an array of cell surface receptors and signaling mechanisms to detect and initiate interactions with

virus-infected and cancer cells. One study found that KIR, an Ig-like receptor on NK cells which is one of the many receptors involved in a recognition mechanism to target cells is controlled by DNA methylation of the surrounding CpG islands of the KIR gene. Increased methylation resulted in decreased gene expression of this crucial surface receptor needed for NK cell functioning (14). On a similar note, a separate study looked at how obesity was associated with increased methylation of the LY86 gene. The LY86 is a lymphocyte antigen gene important for cell surface receptors of many immune cells showing the potential for DNA methylation to be a contributor to the altered NK cell response we saw in the study (15).

Another possible explanation for the results seen in this study may be due to NK cell turnover rate. A study looked at the dynamics and kinetics of splenic NK cell populations in different conditions. They concluded that “most splenic NK cells in adults are not dividing rapidly” and of the NK cell proliferation they do see, it is a small subset population of the entire NK cell population and the divisions are infrequent (16). Further inquiry into specific NK cell populations would be needed to be done before assumptions can be made about NK cell cytotoxicity. Determining whether NK cells would even proliferate and be effected by the metabolic changes occurring during the weight change would be a key focal point. However, as of now it seems that the NK cell populations generally remained constant throughout the weight change which is why the results showed that the original weight status of the mice was indicative of the NK cell cytotoxicity.

Future Applications and Possible Limitations

The future applications of this study could be very beneficial in a society which is suffering from the harmful effects of the growing obesity epidemic. Knowing that immune response is suppressed in obese influenza-infected mice is useful, but having no solution is

problematic. By exploring the topic of weight change on influenza-infected mice, we are attempting to determine if healthy lifestyle changes in order to induce weight change would be helpful in returning immune function. From a public health and preventative medicine standpoint, this is an important topic because it would allow for concrete evidence in inducing these weight changes to restore immune functionality, specifically NK cells. From the data gathered thus far, it does not seem like weight change has an effect on NK cell function, but further research is required to extensively research the intricacies of NK cells and their function in our immune systems.

There are some limitations to this study that should be considered when revising and repeating the study design. One would be to increase sample size. Because of the schedule and timing of mice being euthanized, I was only able to utilize about 23 samples for my study and then those only had 15 samples after removing outliers. Additionally, the NK cell population for this study was obtained from spleen tissue harvested from the mice. In a follow-up study, I would use NK cell populations from multiple sources such as lung and blood. There is literature which indicates that lung tissue in influenza-infected mice actually has separate NK cell populations than the other parts of the body. This could mean that different tissues have varying NK cell function and enumeration and could be an interesting area of study especially dealing with the influenza-infected models (17). On a similar note, the NK cells were not isolated from the spleenocytes which could have led to possible unwanted interactions/secretions within the spleenocytes that could manifest in the assay and alter readings. Spleenocytes consist of T-cells, B-cells, and other immune cells that potentially secrete cytokines and other signalers which can have an effect on NK cell function. By isolating the NK cells from the tissues, it would ensure that only the NK cells and the target (YAC-1) cells would be interacting. Additionally, studies

have shown the NK cells have two types of cytolytic activity, spontaneous and induced. Spontaneous is the baseline cytotoxic ability of the NK cell population whereas the induced is the additional NK cell cytotoxic ability that comes from inducers such as interferon gamma which activates NK cell activity. Developing a study with and without these inducers may allow us to study the full potential of NK cell cytotoxicity. Finally, I would increase the study duration to allow for the NK cell populations to proliferate under the new metabolic conditions of the mice. The study cited earlier discussed splenic NK cell turnover rate and infrequent subpopulation divisions (16). Allowing more time for the NK cell populations to adjust to the new metabolic status may give us insight in a follow-up study into how the NK cell population adjusts over time.

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