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Exploring Interleukin 21 and Its Role in Humoral Immunity in the Mouse Model of Influenza Infection

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EXPLORING INTERLEUKIN 21 AND ITS ROLE IN HUMORAL IMMUNITY IN THE MOUSE MODEL OF INFLUENZA INFECTION

by
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Honors in the Major program in Biomedical Sciences
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ABSTRACT

In summary, this study will be focused on Il-21 and its implications in the antibody response in influenza. The isotype classes primarily involved in this process will also be examined. This will be accomplished by looking at the serum of mice and analyzing the present influenza specific antibodies using ELISA. Another goal was to optimize the ELISA in order to make it sensitive enough to catch small differences in the results. This topic is important due to its implications for improving influenza vaccinations and preventions as current vaccines are not 100% effective. Influenza contributes to significant disease and death around the world every year and each piece of this puzzle is significant in order for the scientific community to be able to eventually make strides to improve the burden of this disease.

ACKNOWLEDGEMENTS

The candidate thanks Dr. Kai McKinstry for his patient guidance and support as well as the opportunity to learn and conduct research. The candidate also thanks Kunal Dhume and Caroline Finn for their assistance with procedures. The candidate additionally thanks the members of the committee. The candidate would lastly like to thank the College of Medicine for the award of the HIM Scholarship.

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BACKGROUND

Influenza A Virus

The influenza A virus (IAV) causes serious illness and deaths in humans every year. Especially due to its unpredictability it continues to be a severe global health threat around the world [6]. IAV contributes to great morbidity, mortality and healthcare costs each year. In the United States, the annual economic cost of influenza is estimated to be around $8 billion [13]. The annual influenza seasonal outbreaks cause between 3 and 5 million instances of severe illness and about 500,000 deaths per year [9]. The virus continuously mutates, making targeted treatment and prevention difficult. The viral surface antigens on the influenza virus, hemagglutinin and neuraminidase, frequently mutate, leading to the different forms of IAV such as H1N1 and H3N2 [7]. For instance, a devastating 2009 H1N1 pandemic hit at the same time the scientific community was preparing for a possible H5N1 pandemic [6]. This shows how the constant mutations of IAV can lead to completely unexpected breakouts and prove to cause great challenges for global health. These variations in the viral surface glycoproteins are what cause the antibodies produced against one strain to be ineffective against other strains. There are two major drives of mutation in IAV. These are antigen shift and antigenic drift. Antigenic drift is the process of changes to viral epitopes through point mutations [22]. Antigenic shift is a result of a complete exchange of HA genes. This only occurs among influenza A viruses. [22]. These challenges prove more reason for research into the mechanisms of the influenza infection, the antibody response, as well as possible vaccine strategies.

The constant mutations of IAV provide the foundation for the main challenge concerning IAV vaccines. Scientists must continuously try to prepare for pandemics as well as try to predict

antigenic drift and make recommendations on strains to focus on for the upcoming vaccines for the season. The Global Influenza Surveillance and Response System is a group of over 120 laboratories that work to survey influenza trends globally and collectively help to form the recommendations for each season [6]. Additionally, these recommendations have to be made far in advance so there is substantial time for antigenic drift to contribute to changes in the dominant strain, leading to ineffective vaccines [6]. For the 2017-2018 flu season, the Center of Disease Control (CDC) estimated the flu vaccine efficacy to be approximately 36% [8]. This low percentage of protection lead to great hospitalizations, secondary infections as well as deaths [8].

Innate and Adaptive Immunity

Immune responses in the body are divided into 2 categories: the innate immune system and the adaptive immune system. The innate immune system is viewed as the first line of defense against pathogens and consists of physical barriers such as the skin, as well as cytokines, interferons and other nonspecific immune system components [11]. The adaptive immune system, on the other hand, focuses on antigen specific defenses. B cells and T cells work together to neutralize pathogens. These two components both contribute towards fighting the IAV infection.

Antibody Response and Immunoglobulin Classes

Neutralizing antibodies against hemagglutinin and neuraminidase are crucial in providing protection against influenza infection [10]. This is homotypic protection, since these antibodies will be effective against the same strain of IAV, but not different strains. These neutralizing

antibodies are part of the humoral immune response to IAV [11]. Antibodies can also act to induce cytotoxicity or the complement cascade to eliminate pathogens in another way [11].

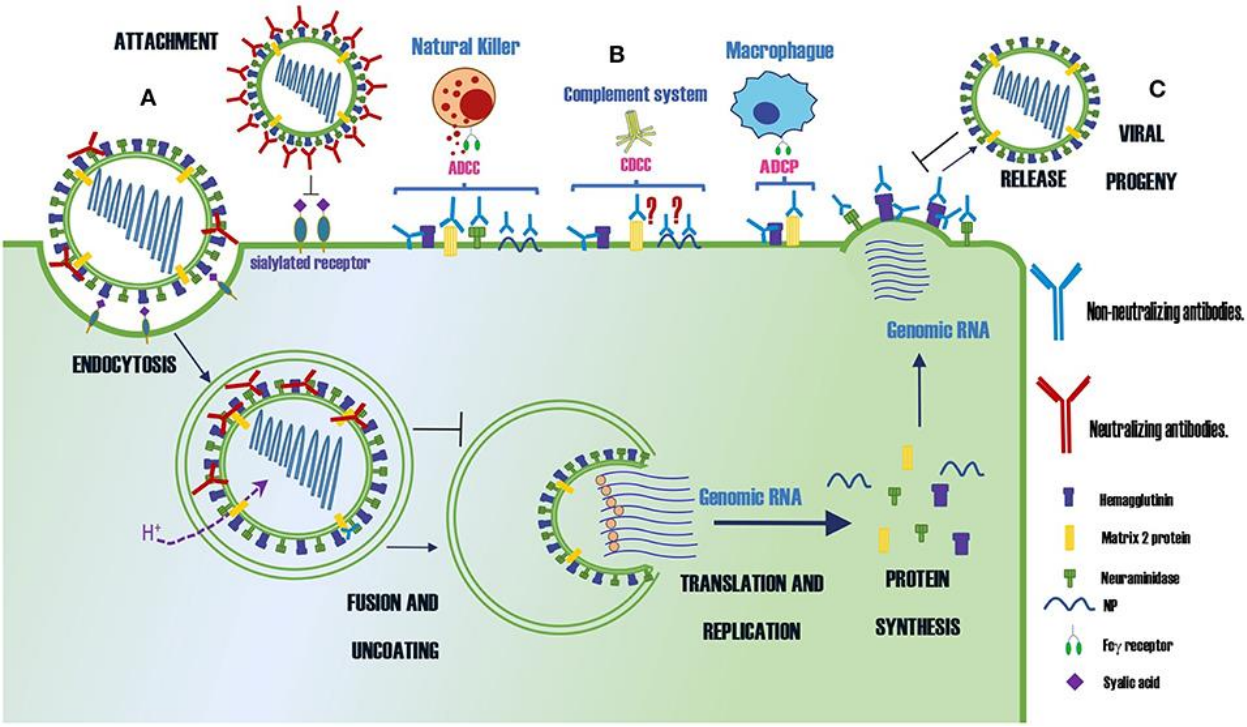


Figure 1. Summary of the protection of neutralizing and non-neutralizing Abs specific for influenza. (A) Abs that neutralize the infection (B) Abs that control the infection by indirect mechanisms as ADCC, CDCC or ADCP (C) Abs that prevent virus budding. [From Padilla-Quirarte, Frontiers of Immunology] [25].

Antibodies can act in two ways, one of them being the homotypic protection offered by neutralizing antibodies. The other is their involvement in heterosubtypic immunity. Heterosubtypic immunity is immunity generated by a particular IAV subtype that can offer protection against a challenge with another IAV subtype. There has been some evidence to show that antibodies can play a role in IAV heterosubtypic immunity in addition to homotypic immunity [18]. Non-neutralizing antibodies bind to viral proteins but do not neutralize virus directly. They can be cross-reactive and therefore produce more general protection, opposed to homotypic protection [23].

There has also been evidence showing that alone, non-neutralizing antibodies are relatively ineffective at providing heterosubtypic immunity [19]. Additionally, influenza virus-specific CD8+ T cells alone are ineffective at providing this immunity [19]. However, when they are combined, they cooperatively provide significant immunity to a heterosubtypic challenge [19]. This suggests that antibodies do have a role in heterosubtypic immunity.

Immunoglobulins (Ig) are heterodimeric proteins. They are comprised of two light and two heavy chains, with a constant domain as well as a variable chain [12]. The variable domains are subject to somatic hypermutation to allow for affinity maturation after antigen exposure [12]. There are five different major groups of heavy chain C domains [12]. These are what determine the isotype class. The main immunoglobulin isotypes are IgM, IgG, IgA, IgD, and IgE. Additionally, IgG can be further categorized into four subclasses: IgG1, IgG2, IgG3, and IgG4 [12]. These different immunoglobulins have a variety of functions within the immune system. In humans, IgG is divided into 4 subclasses: IgG1, IgG2, IgG3, and IgG4. In mice, there are 5 subclasses: IgG1, IgG2a, IgG2b, and IgG2c [24].

IgM antibodies are made initially by B cells so they are present in higher levels during the start of infection [12]. Initially, B cells will produce IgM but later in the infection the plasma cell can class switch so it begins producing immunoglobulins of a separate isotype class. This is important because each of the isotype classes govern different functions in the immune response. IgM antibodies have relatively low-affinity but high avidity, meaning they are more poly-reactive than other isotypes, but can respond to a variety of antigens. It functions by opsonization and triggering the complement cascade.

IgA antibodies are associated with mucosal areas and secretions such as saliva and breast milk, and are found in particularly high concentrations in these areas. These antibodies are crucial for protecting vulnerable mucosal surfaces in the body from pathogens [12].

IgE is present in very low concentrations in the serum [12]. This immunoglobulin class is mainly associated with allergic and parasitic immune responses.

Lastly is IgG, the most prevalent immunoglobulin isotype found in the serum. The further divisions of this class into IgG1-4 were named in descending order of their prevalence in blood of a healthy individual [12]. This group of antibodies has a variety of functions, one of which is activating complement. IgG1-3 subclasses all can fix complement by binding to C1q, the first component of the complement cascade [12]. Certain subclasses of IgG may be particularly associated with a particular pathogen or disease process [12]. For influenza in particular, it has been shown that expression of IgG2a antibodies has been associated with a Th 1 response and the presence of IgG2a in particular was correlated with increased clearance of virus and protection against lethal influenza challenge [16]. IgG antibodies also directly neutralize some pathogens and subclass can play a part in the efficacy of this neutralization in different disease processes in this instance as well [12]. Knowing which isotype classes are involved in different infections and processes is key to understanding how to best prevent and treat them.

Interleukin 21

Interleukin-21 is a cytokine that has regulatory effects on different immune cells [5]. There has been evidence to show that the induction of antibody production through IL-6 is mediated by IL-21 [5]. It was shown that IL-21 production by CD4+ T cells is necessary for IL-6

to promote B cell antibody production [5]. This suggests the importance of both IL-21 and IL-6 in the humoral response in the influenza infection. IL-21 mainly activates STAT3, which in turn down-regulates B cell lymphoma 6 and up-regulates B lymphocyte–induced maturation protein 1 [15]. This is what then encourages plasma cell differentiation which then promotes antibody production [15]. It has also been shown that optimal follicular helper T cell (Tfh) formation requires IL-21 and IL-6 [2]. Tfh cells are a subset of CD4+ T cells that help B cells produce antibodies by playing a key role in the formation of germinal centers [2]. There are two ways to create antibodies, one being T cell dependent and the other being T cell independent. The first way is when B cells are triggered by Tfh cells. This is what needs to happen to create long lived antibody responses [20]. The other process is B cells being triggered by T cell independent antigens and it is not always T cell dependent. This form produces an antibody response that is not nearly as strong as that of the T cell dependent process of antibody production [21].

Previous research in our lab has been shown that $IL21R^{-/-}$ mice do not fare well during heterosubtypic infection as compared to wild type mice. There are several possibilities for a heterosubtypic response failing. One of these would be the T cell response failing, and the other would be the antibody response failing. The IL21 receptor binds to the IL21 and this activates STAT1 and STAT3. The STAT3 pathway is crucial to generate an antigen specific antibody response as well as a long lived antibody response [26]. Analyzing IL21's role in antibody protection could provide insight into if IL21 role in heterosubtypic immunity. It could also indicate whether or not vaccines should try to induce IL21 signals in order to increase immunity. This is significant because heterosubtypic immunity is key to helping prevent the damage caused by pandemics.

The vehicle of IL21 for this project was chosen because there has been some discrepancy shown in regards to IL21's role in the flu antibody response. Previous research has shown decreased antibody production in flu of both the IgG1 and IgG2c classes in IL21 knockout mice [5]. On the other hand, there has been some results showing that IL6 knockouts do not show decreased IgG production in flu [30]. Because of how IL21 is supposed to directly mediate the IL6 associated antibody production, this suggests that perhaps IL21 is not necessary for an optimal antibody response [5]. The goal for this project was to more clearly define the role of IL21, as well as optimize the ELISA test.

Aims of the Project

The overall goal of this research is to look at how interleukin 21 is involved in influenza and the antibody response in the infection.

The first component of the project will be optimizing the enzyme-linked immunosorbent assay (ELISA) for the experiments. This will involve testing different concentrations of coating virus, as well as different dilution series. The goal of this is to figure out the most effective experimental procedure to get the optimal readings while minimizing background signal. It is also the goal to try to make it as sensitive as possible in order to detect small differences between groups. It is also important in order to conserve resources by not using more coating virus than is needed. IL21r-/- mice were looked at as a platform for the optimization because there is some debate in the literature as to how IL21 affects the antibody response.

Additionally, different isotype classes of influenza specific antibodies will be studied in relation to their prevalence in IL-21 receptor knockout (IL-21 $R^{-/-}$) mice versus wild type mice.

The isotype classes that will potentially be studied will be Immunoglobulin G (IgG) subtypes IgG1, and IgG2a. This will allow for insight into IL-21's effect on the production of these particular antibodies.

Lastly, the IgG2c subtype will be looked at in comparison to previous data published through our lab using IgG2a. IgG2c is an alternate to IgG2a that is present in B6 mice which are used in our lab. This is in order to see how the subtypes compare and to see if IgG2c could be used in future experiments in our lab. Looking at the different isotypes is of interest because this shows how different components interact with the flu virus and knowing these pathways is important for understanding how the antibody response to flu works and furthering vaccine development.

METHODS

Mouse Model

While several small animals can be infected with influenza experimentally, mice have been one of the most popular choices for research in this field [1]. The mouse model is a powerful tool to explore the influenza infection and to reflect the human disease. While there are some downsides to this model, the relatively easy access to mice, the comparatively low cost and the variety of genetic variations and knockouts make mice a common animal model for influenza research [1]. Additionally, there are a wide variety of immunological reagents available for use with the mouse model which makes it an attractive choice [1].

The mice to be used are B6N.129-IL21R$^{-/-}$ mice from The Jackson Laboratory. These mice are developed from backcrossing the 129 strain with the B6 strain. The control mice will be Wild Type B6 mice.

The other type of mice to be used are B6 T-bet/Eomes Double Knockouts, as well as B6 T-bet Knockouts.

Mice are infected with PR8 IAV (H1N1) and blood is harvested either through cardiac puncture in the case of a terminal bleed, or a submandibular bleed. This harvest is done on Day 28 of the primary infection because this is when we know that the antibody present is long lived and T cell related. The blood is then let to sit so it clots then centrifuged for 15 minutes at 1000-2000g, then the serum is pipetted out and frozen at -20 °C until use in ELISA assays.

ELISA

The main lab technique that will be used in this investigation is the Enzyme Linked Immunosorbent Assay (ELISA). This technique was chosen due to its versatility, sensitivity and ability to quantify the amount of target antibody in a sample. There are many different kinds of ELISAs including the indirect ELISA, direct competitive ELISA, and the antibody sandwich ELISA [3]. For this study, an indirect ELISA will be used.

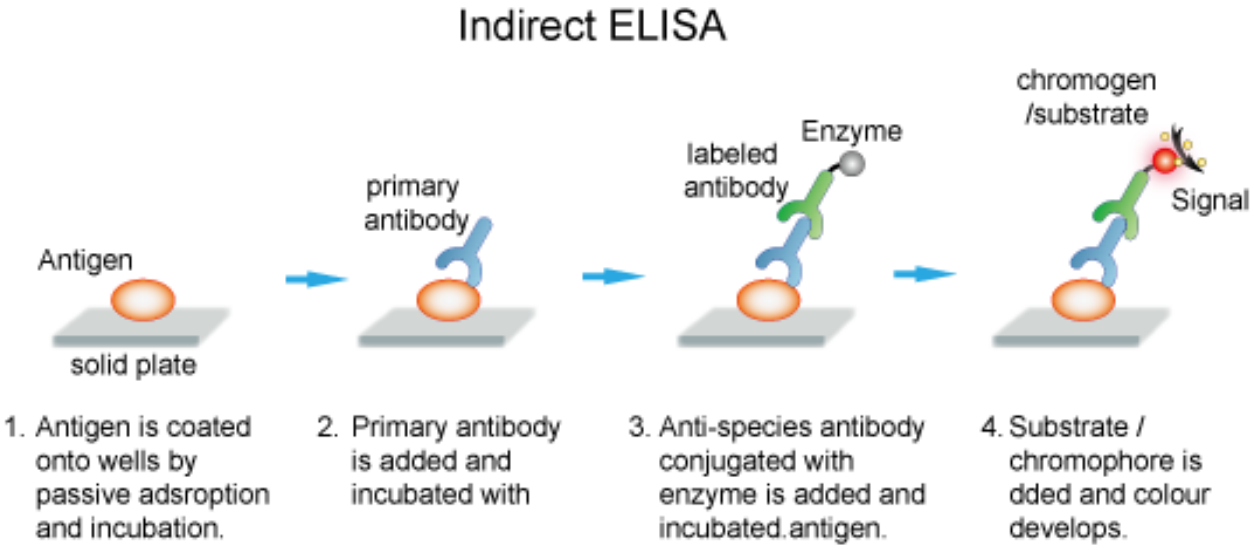


Figure 2: There are 4 main steps to the indirect ELISA. The first is the coating antigen. The next step is the primary antibody being added. The third is the secondary antibody being added. The secondary antibody is conjugated with an enzyme. The last step is the substrate that reacts with the enzyme being added and the color developing [From ELISA Guide - Creative Diagnostics] [29].

The flu virus will be used to coat the plate so that only the antibodies specific to influenza bind. This virus comes from single use aliquots of the virus that are used when infecting the mice. All the aliquots were freeze thawed together so they should be very similar. This will then allowed to incubate for one week. Then the sample solution containing the target antibody will be allowed to incubate in the plate for one week. Next, a secondary antibody that binds to the primary antibody will be added in a 1/2000 concentration. This secondary antibody is conjugated to an enzyme and this will then be incubated for 3 hours. In between these steps are washing

steps involving 5 washes with PBS/Tween. Washing is done in order to remove any unbound material. This is crucial in order to achieve accurate results because without proper washing, there would be high background signal related to the unbound antibody. Finally, a substrate solution will be added activating the reaction that will produce a color change. OPD is the substrate in this reaction and this is found in the substrate buffer that is used. The enzyme is horseradish peroxidase (HRP) which is conjugated to the secondary antibody. These two react and the resulting product forms a color that gives a visualization to the amount of antibody present in each well.

This reaction will then be halted with an acid stop with 25% H_2SO_4. This is done in order to halt the HRP activity and therefore stop the reaction between the substrate and enzyme. The ELISA will be read by using a plate reader measuring OD at 492 nm. This allows for a determination and quantification of the amount of sample present in each well of the ELISA plate.

When looking at different isotypes in the ELISA, we ensured to keep the same isotypes on the same plate. This is because mixing different isotypes on one plate could lead to acid stopping at a non-ideal time for one group of samples. This would lead to increased background signal for one group looking at one isotype, while another group looking at another isotype might not be allowed to react for long enough. Looking at one isotype per plate helps to alleviate these issues.

Controls and Data Analysis

End point titers were calculated using the method of the double mean blank. This is done by averaging the OD of the blank wells and then doubling that. This is a common method for determining end point titers. For controls in this experiment, there were both negative and positive controls to help ensure accuracy of the ELISA results. Conjugate blanks were included in every plate in order to show what should be a negative control because the secondary antibody was not included so there should be no enzyme substrate reaction, and therefore a baseline level of OD. A positive control was also included and this was the serum of a wildtype mice who are known to have the antibodies being tested present, and therefore should yield a positive result.

Statistical analysis

Statistical analysis was done using GraphPad Prism. Standard error bars were used on the graphs. Additionally, a two-tailed t-test was used to determine if the difference in end point titers was significant. In the case that there were more than two end point titers in one graph, an ANOVA test was done to determine statistical significance. Ns marking on the graph indicated a not statistically significant finding. * indicated a p value of $< .05$, ** indicated a p value of < 0.01, *** indicated a p value of < 0.001 and **** indicated a p value of < 0.0001.

RESULTS

As a part of preparing for this project, some experimental optimization will be done since the ELISA has several components that can be changed for each experiment and can be the difference between acquiring data and being able to draw conclusions from the experiment, and a failed experiment and unhelpful data. The amount of coating virus will be experimented with as well as different dilution series in order to try and identify end-point titers with more accuracy.

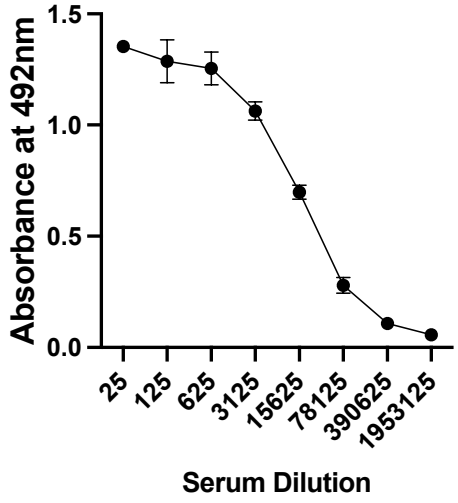
The first component of this project was to optimize the ELISA to try to increase effectiveness of the assay and to minimize potential errors. This was necessary because while the ELISA can be a very beneficial test, it can also be prone to several errors. Because the ELISA uses a dilution series to determine the end point titer, it is important to use an ideal dilution series as well as an appropriate amount of coating virus on the plate. For instance, if you used only two dilutions, one of ½ and another of 1/1000000, this would result in many different samples having the same end point titer. The ELISA can be tricky because it is easy to miss more subtle differences by using a dilution series that is too broad. The dilution series needs to strike the balance between being narrow enough to catch the differences in samples, but not being too narrow where you do not reach the end point titer. First of all, several different virus dilutions were tested in order to see how much antigen was needed to achieve a strong result without using excess virus.

When looking at the graphs shown below, we saw clear differences in the curves but the end point titers are more clinically relevant and simpler to analyze so this is why they were included.

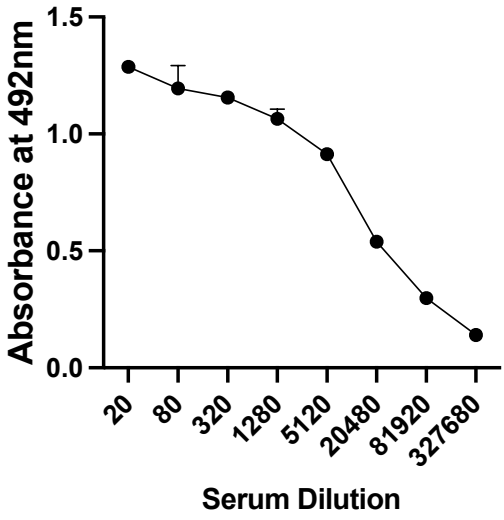
Coating Virus

A limited amount of coating virus exists in the lab so it was important to see how much was necessary to achieve the desired result. Figure 3 shows the ELISA results including the absorbance graphs and end point titer graphs for the testing of 3 different virus dilutions as well as 2 different dilution series. This was done using wild type mice. Total IgG was assessed and measured by absorbance at 492 nm. After looking at all of the graphs and end point titers collected, it can be seen that the virus dilution 1/100 yielded the most ideal absorbance curves where there is a slow decrease, then a steeper slope down, followed by a more gradual declining curve at the end. However, the titer value for 1/1000 dilution was very similar so we decided to look further and test a 1/100 versus a 1/500 dilution. In this part, two different dilution series were tested. Dilution Series 1 starts with a 1/25 and then decreases by 1/5 continually after that. Dilution Series 2 starts with a 1/20 dilution and then decreases by ¼ continually after that. The dilutions are shown in the x-axis of the absorbance graphs in Figure 3.

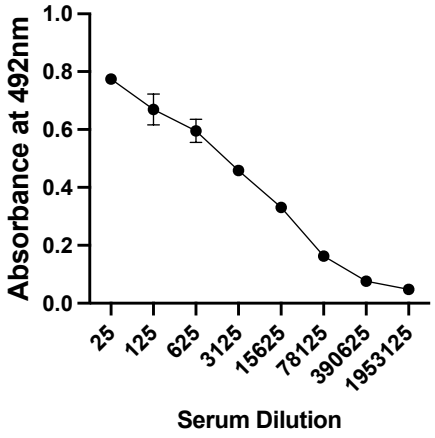
Dilution Series 1 Virus Dilution 1/100



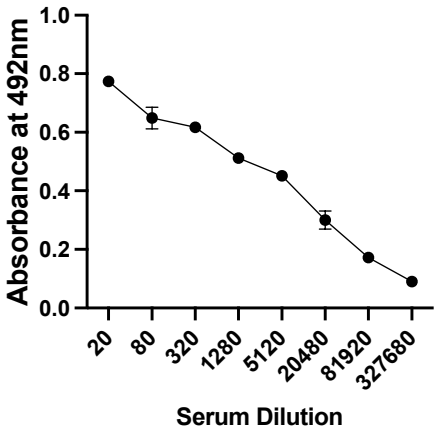
Dilution Series 2 Virus Dilution 1/100



Dilution Series 1 Virus Dilution 1/1000



Dilution Series 2 Virus Dilution 1/1000



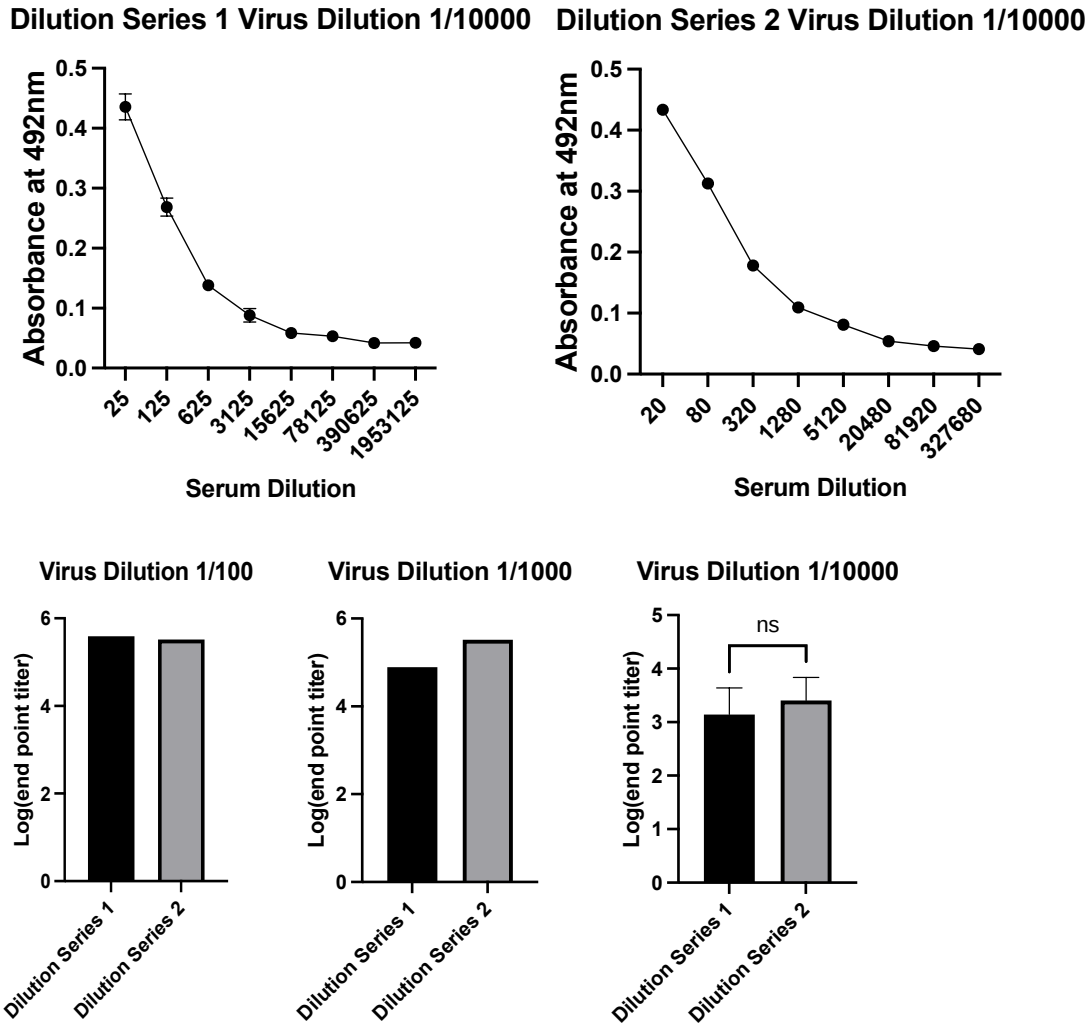
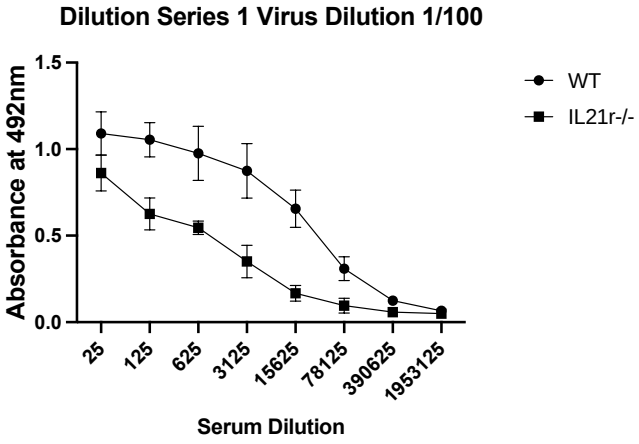
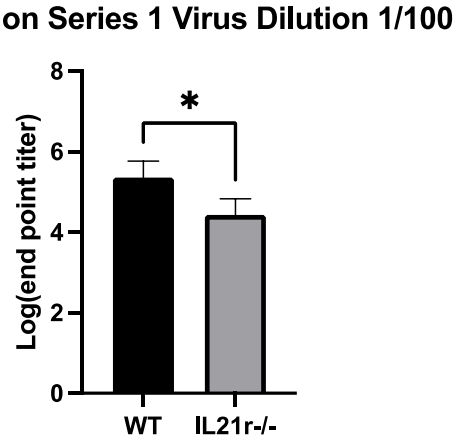
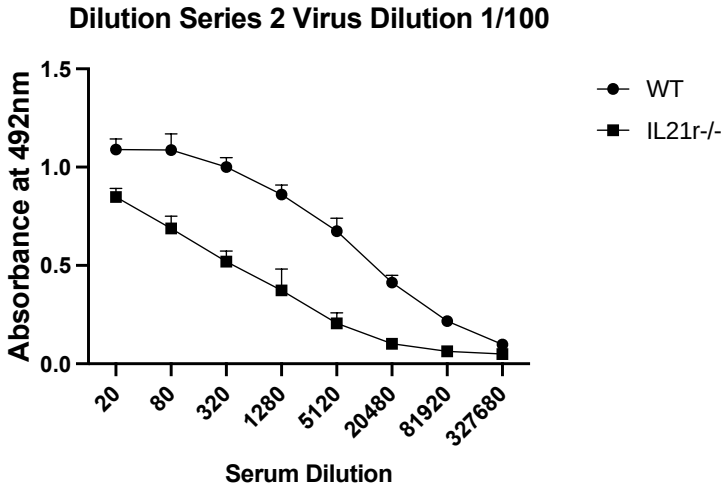
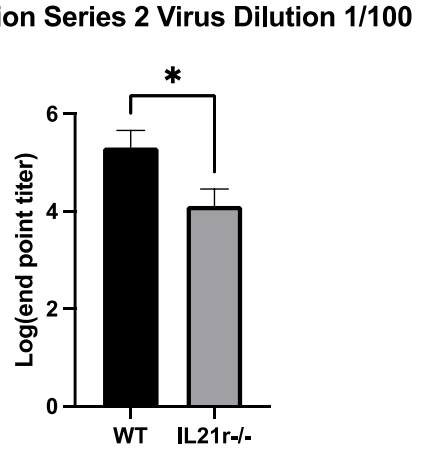


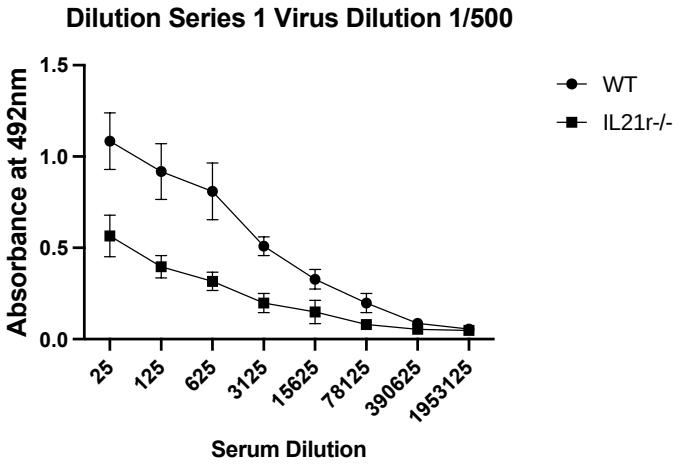
Figure 3: Absorbance and titer graphs for total IgG for various virus and serum sample dilutions are shown. Two mice were used in each group.

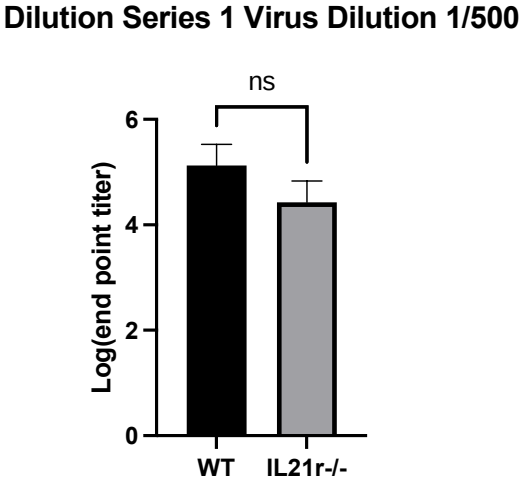

Dilution Series 1 Virus Dilution 1/100
Absorbance at 492nm
1.5
1.0
0.5
0.0
25
125
625
3125
15625
78125
390625
1953125
Serum Dilution
WT
IL21r-/-


Dilution Series 1 Virus Dilution 1/100
Log(end point titer)
8
6
4
2
0
*
WT
IL21r-/-


Dilution Series 2 Virus Dilution 1/100
Absorbance at 492nm
1.5
1.0
0.5
0.0
20
80
320
1280
5120
20480
81920
327680
Serum Dilution
WT
IL21r-/-


Dilution Series 2 Virus Dilution 1/100
Log(end point titer)
6
4
2
0
*
WT
IL21r-/-


Dilution Series 1 Virus Dilution 1/500
Absorbance at 492nm
1.5
1.0
0.5
0.0
25
125
625
3125
15625
78125
390625
1953125
Serum Dilution
WT
IL21r-/-


Dilution Series 1 Virus Dilution 1/500
Log(end point titer)
6
4
2
0
ns
WT
IL21r-/-

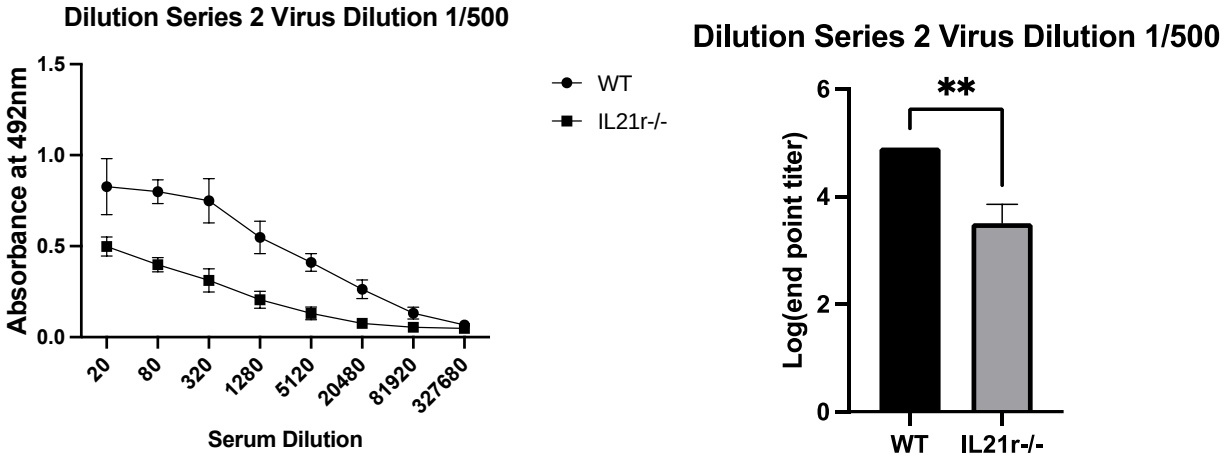
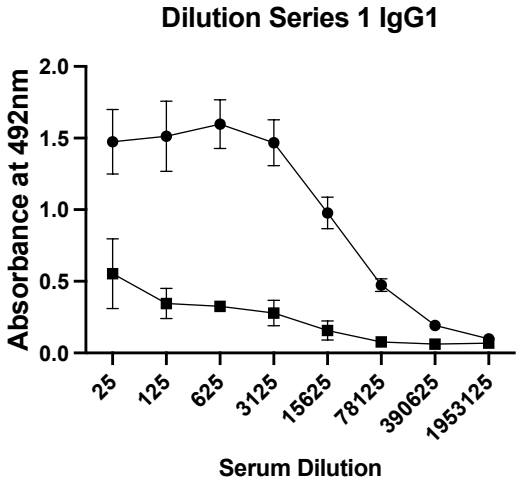
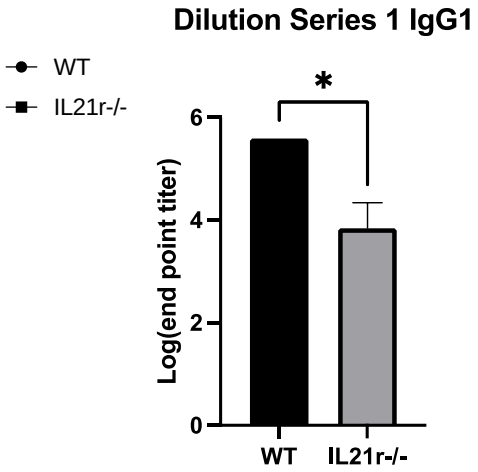


Figure 4: Absorbance and titer graphs for total IgG for both WT and IL21r-/- mice using two serum dilution series as well as two virus dilutions are shown. Three mice were used for each group.

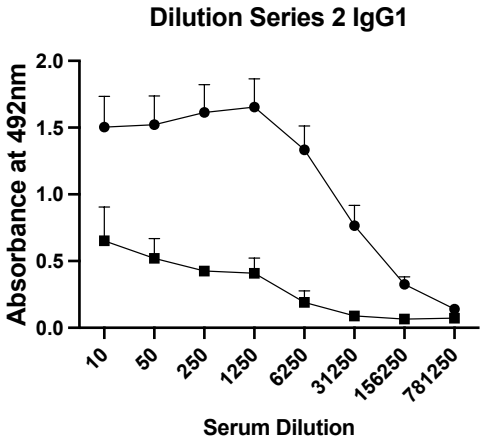
In this experiment, different virus dilutions and different dilution series were tested, and the same parameters were also tested using IL21R-/- mice in order to compare and see the differences. Once again, the total IgG was assessed using the absorbance at 492 nm. Through looking at the absorbance graphs in Figure 4, it can be seen that the 1/100 virus dilution still resulted in the most ideal ELISA curve. Also the graphs show that the 1/100 virus dilution with the dilution series starting with a 1/25 antibody dilution was resulted in the most ideal but we decided to look further into other potential dilution series which can be seen in Figure 5, in order to ensure the most ideal and efficient dilution series was being used for further experiments. Also in Figure 4, it can be seen that the IL21R-/- mice showed significantly lower serum IgG when compared to the wild type mice. This corroborates other research that emphasizes IL21's importance in the antibody response [5].

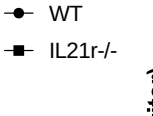
Serum Dilution Series

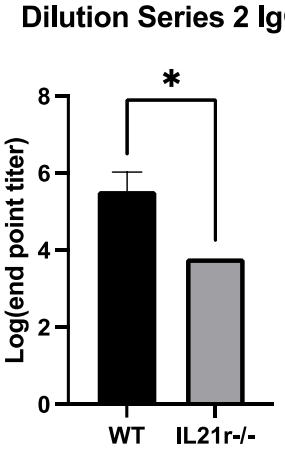

Dilution Series 1 IgG1
Absorbance at 492nm
2.0
1.5
1.0
0.5
0.0
25
125
625
3125
15625
78125
390625
1953125
Serum Dilution

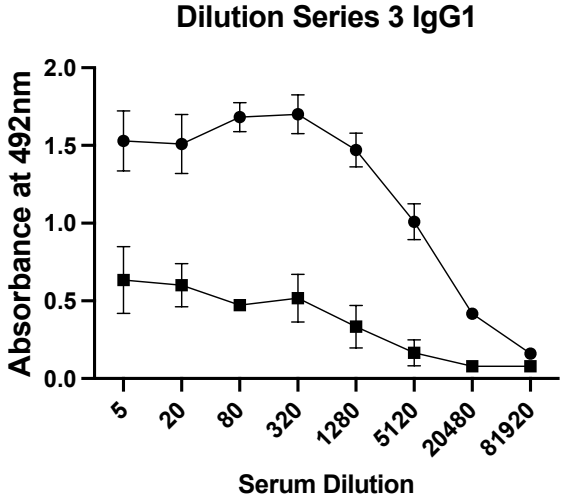

WT
IL21r-/-

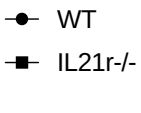
Dilution Series 1 IgG1
*
Log(end point titer)
6
4
2
0
WT
IL21r-/-


Dilution Series 2 IgG1
Absorbance at 492nm
2.0
1.5
1.0
0.5
0.0
10
50
250
1250
6250
31250
156250
781250
Serum Dilution


WT
IL21r-/-


Dilution Series 2 IgG1
*
Log(end point titer)
8
6
4
2
0
WT
IL21r-/-


Dilution Series 3 IgG1
Absorbance at 492nm
2.0
1.5
1.0
0.5
0.0
5
20
80
320
1280
5120
20480
81920
Serum Dilution


WT
IL21r-/-

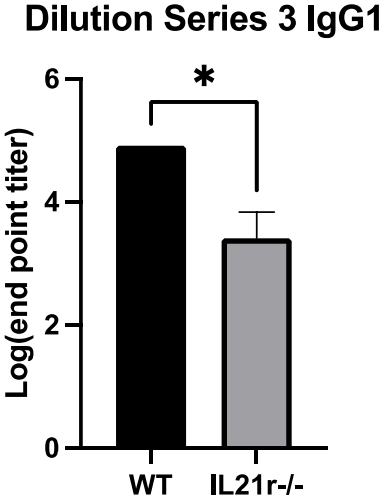
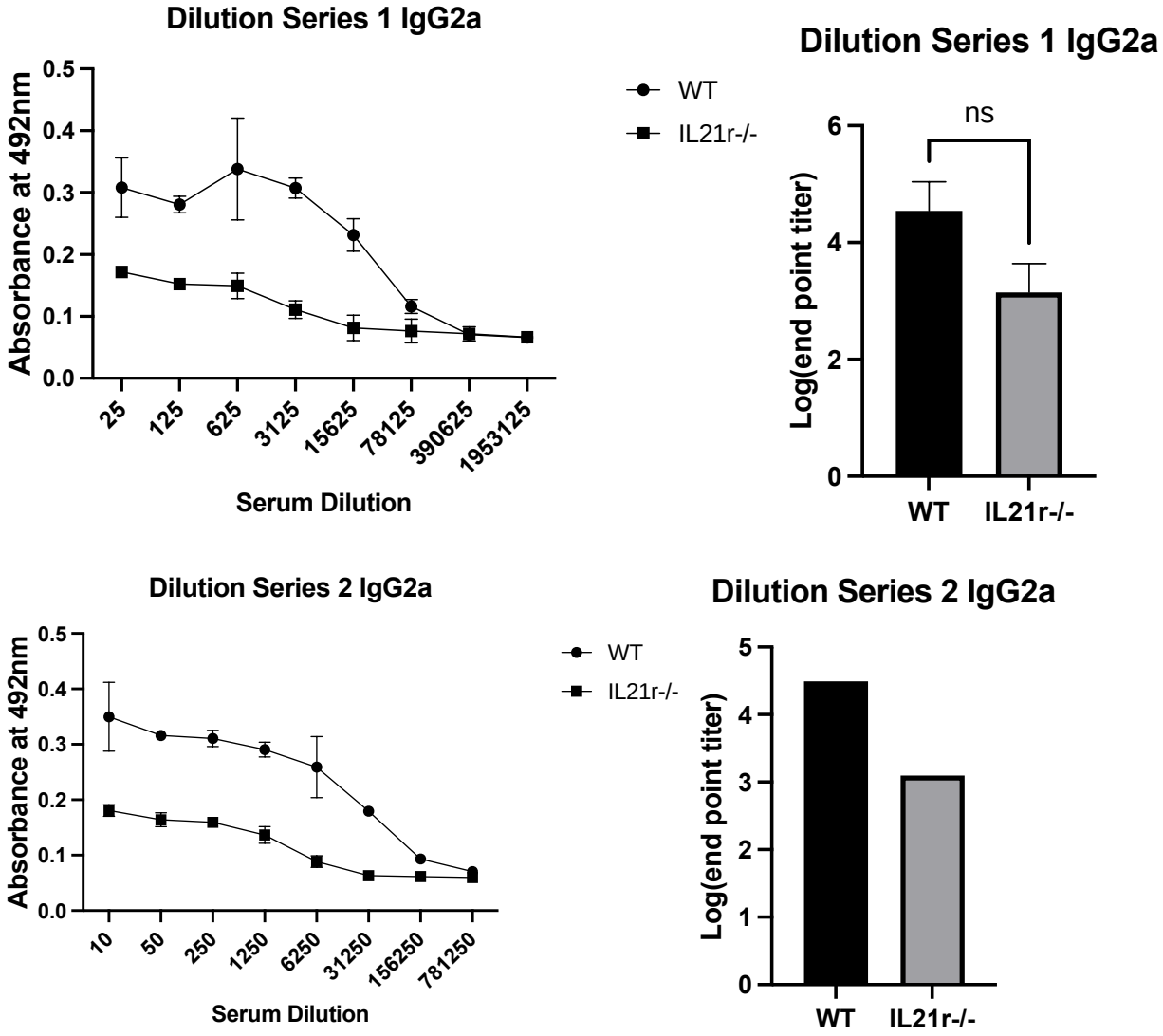

Dilution Series 3 IgG1
*
Log(end point titer)
6
4
2
0
WT
IL21r-/-

Figure 5: Absorbance and titer graphs for IgG1 in both WT and IL21r-/- mice for three dilution series are shown. Two mice were used in each group.



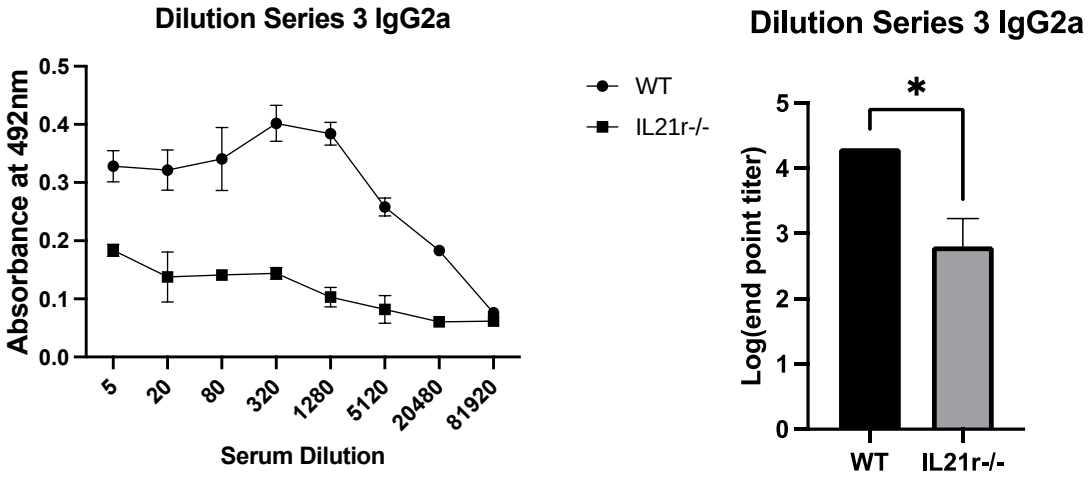


Figure 6: Absorbance and titer graphs for IgG2a in both WT and IL21r-/- mice using three dilution series are shown. Two mice were used for each group.

In figures 5 and 6, different dilution series were tested. Dilution Series 1 was again starting with 1/25 and then decreasing by 1/5. Dilution Series 2 in this experiment was starting with 1/10 and decreasing by ¼. Finally, Dilution Series 3 started with 1/5 and then decreased by ¼ continually. As shown in Figures 5 and 6, 2 additional dilution series were tested and compared with the series that fared best in the past tests, which was Dilution Series 1. Additionally, in this experiment the two IgG subtypes IgG1 and IgG2a were examined. This was in order to see how the dilution series did when looking at the different subtypes that may be present in much smaller quantities. The graphs showed that still, Dilution Series 1 produced an effective graph and there is no reason to use the dilution that uses a greater amount of serum because we do not want to use excess sample if it is not needed because the serum samples are limited and valuable resources. This experiment also showed a repeated result of decreased IgG production in the IL21r-/- mice. Since Figure 4 showed an overall decreased total IgG concentration in the IL21r-/-, it makes sense that both subtypes IgG1 and IgG2a are decreased in the IL21r-/- when compared to the wild type mice. This shows that IL21r-/- is beneficial to the

production of both IgG1 and IgG2a since significantly lower concentrations of both are found in the IL21r-/- mice.

IgG2c and IgG2a

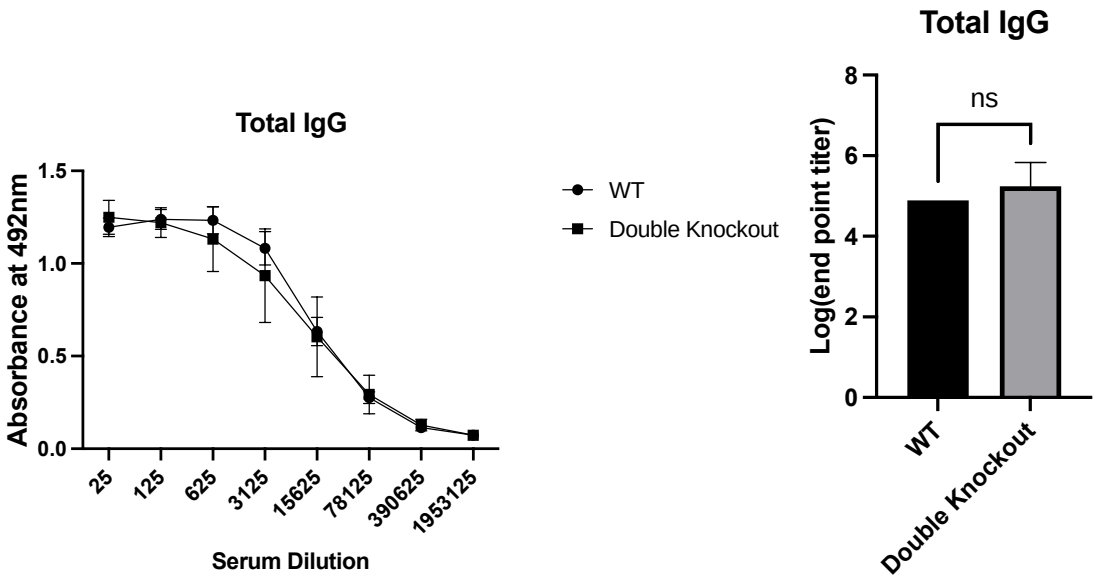


Figure 7: Absorbance and titer graphs for total IgG in both WT and double knockout mice are shown. Six mice were used in each group.

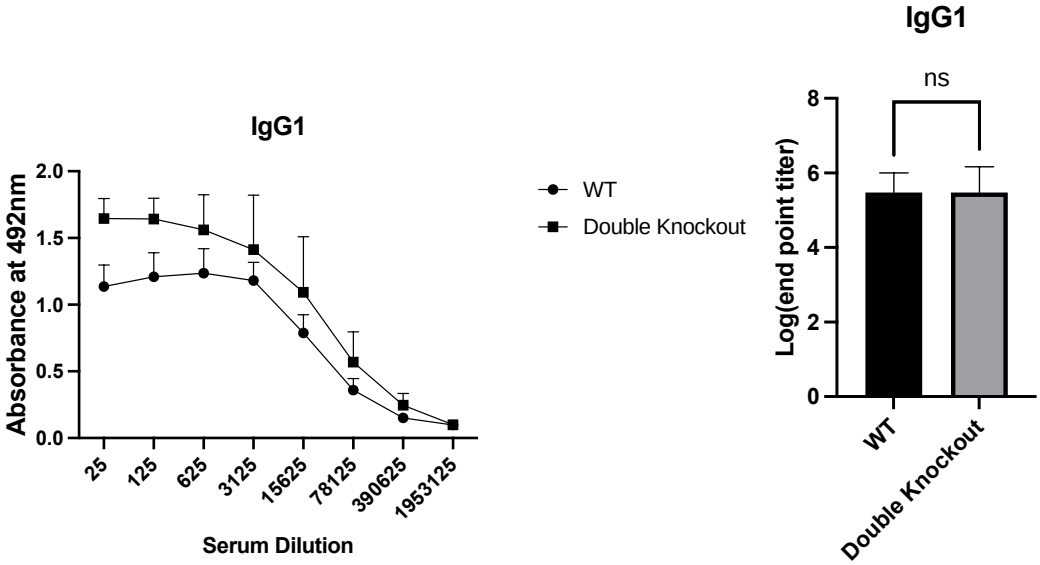


Figure 8: Absorbance and titer graphs for IgG1 in both WT and double knockout mice are shown. Six mice were used in each group.

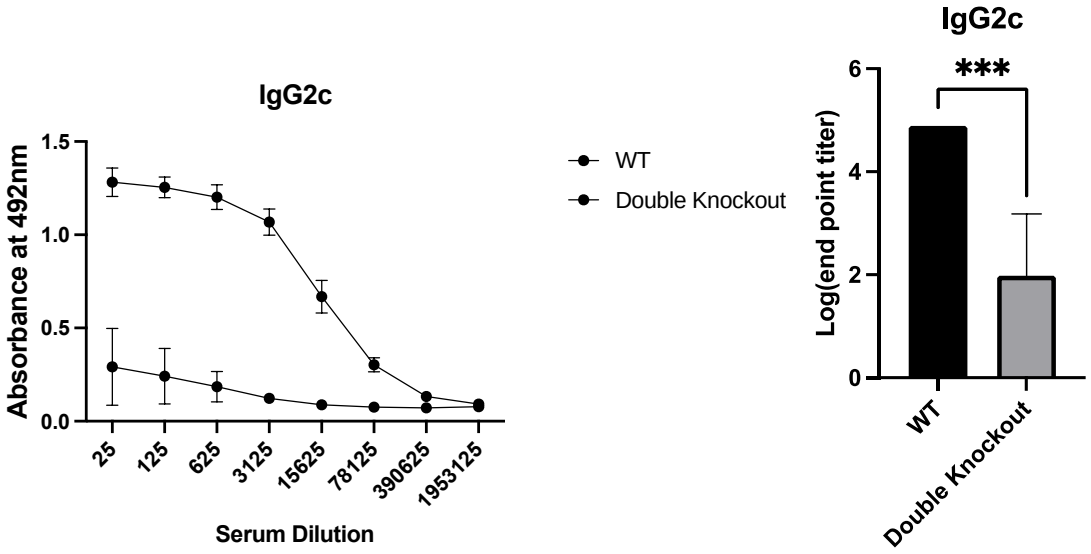


Figure 9: Absorbance and titer graphs for IgG2a for both WT and double knockout mice are shown. Six mice were used for each group.

After looking at the different subtypes of IgG, we began to think about IgG2a and IgG2c. Previous work looked at IgG2a and there would be cross reactivity between IgG2a and IgG2c but in theory, due to the strain of mouse used in our lab, IgG2c would be more specific. We wanted to see how the results came out considering IgG2c is actually the ideal subtype for the strain of mice used in our lab, but this isotype had not been used before. We decided to try this in the context of double knockout mice that have both Tbet and Eomes knocked out to try the IgG2c. Previously, our lab has published the results of the difference between antibody levels in Tbet knockouts and wild type when looking at IgG2a [31]. This way we could look at the results of the IgG2c in a context that can be compared with previously published results in our lab. This also allows potential conclusions to be drawn regarding Eomes and its role in the antibody response. Figure 7 shows that the total IgG levels in the wild type and DKO mice were nearly the

same. Upon first look, this may give the impression that the antibody production in these two strains is very similar. However, when looking at the subtypes of IgG1and IgG2c, there are significant differences when it comes to IgG2c. The DKO IgG2c concentration is far lower than that of the wild type. This indicates that the DKO mice compensate for the lack of IgG2c production with other IgG production, so the overall IgG levels remain about equivalent to the wild type.

Another thing to note is that the graph for IgG1 actually showed higher absorbance values than that of the total IgG. This is problematic and shows potential error because of course the IgG1 should just be a portion of the total IgG. One potential cause of this error would be a failure to acid stop at the proper time. This would lead to an increased background signal for the IgG1 and this could lead to falsely inflated values.

Other Isotypes

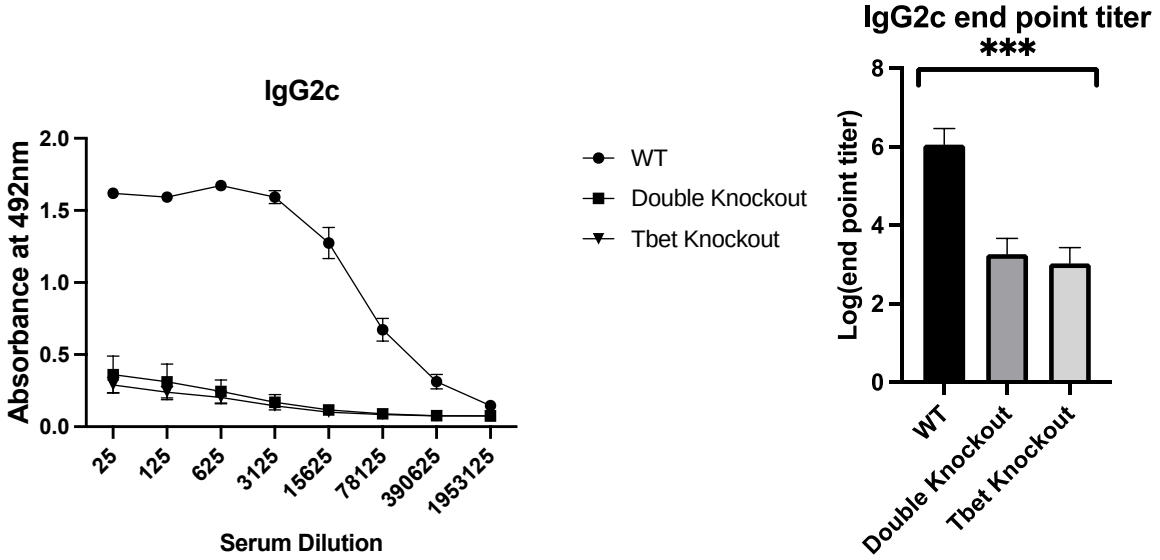


Figure 10: Absorbance and titer graphs for IgG2c for WT, double knockout, as well as Tbet knockout mice are shown. Three mice were used in each group.

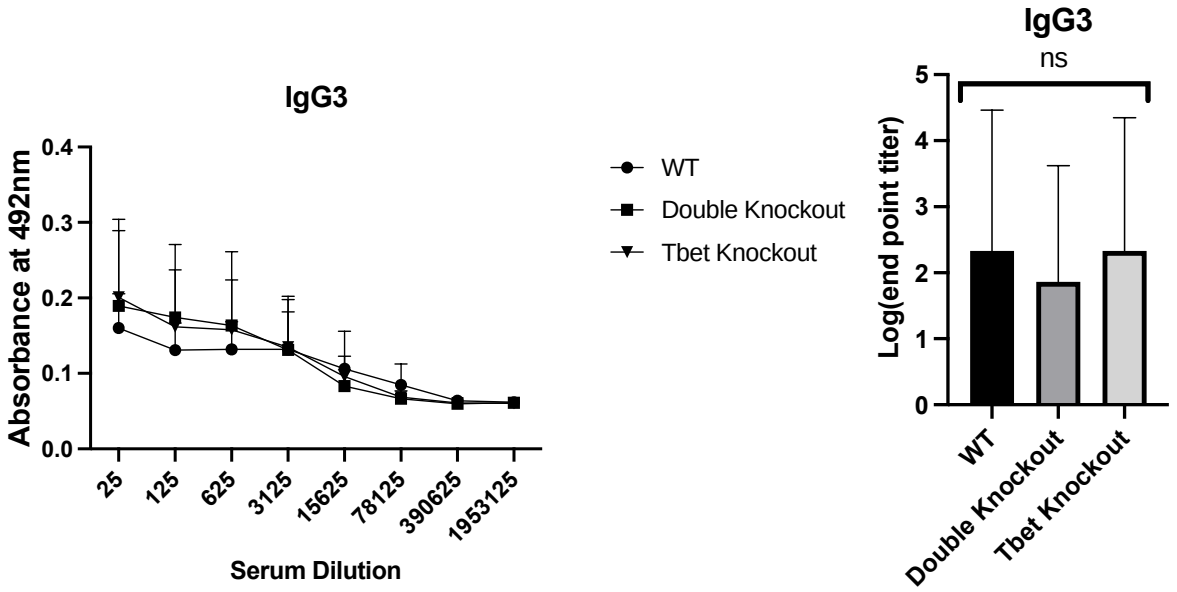


Figure 11: Absorbance and titer graphs for IgG3 for WT, double knockout, as well as Tbet knockout mice are shown. Three mice were used in each group.

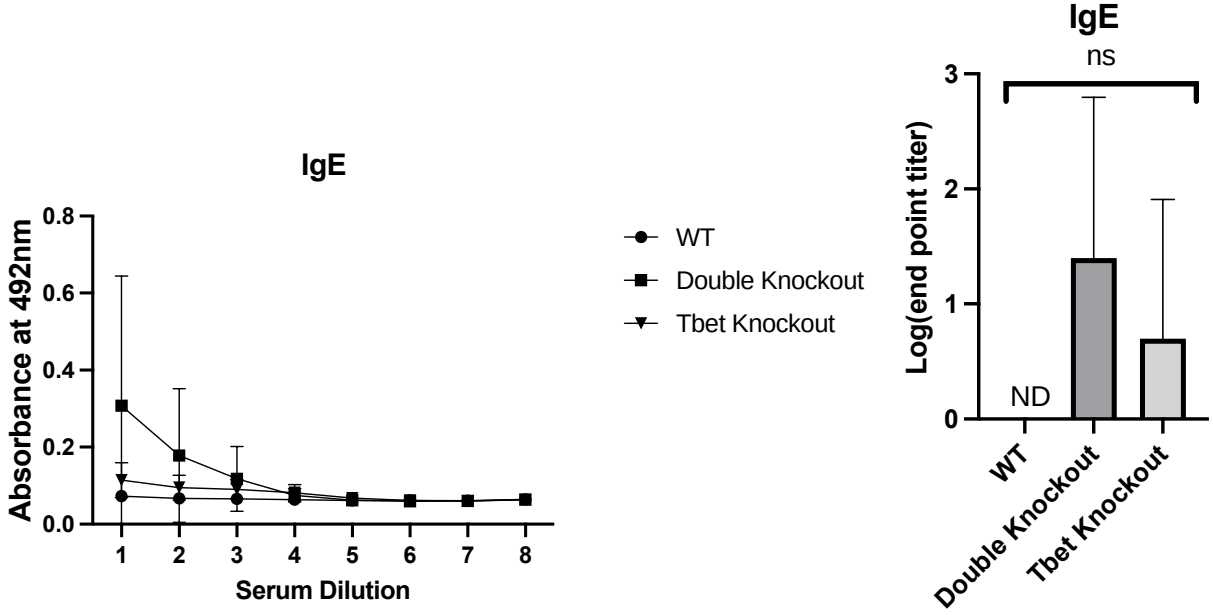


Figure 12: Absorbance and titer graphs for IgE for WT, double knockout, as well as Tbet knockout mice are shown. Three mice were used in each group.

Lastly, we decided to look at a few other isotypes that our lab has not looked at before in this context. We tested the presence of IgG2c once again as shown in Figure 10. Then we looked at IgG3 as shown in Figure 11. We also looked at IgE as shown in Figure 12. We compared the levels of these in several different knockout mice and compared them with wild type mice. We looked at T-bet knockouts and double T-bet and Eomes knockouts. The IgE levels were found to be nearly zero. The IgG3 levels were also extremely low, indicating that this is likely not a place where more IgG3 that is contributing to the overall total IgG is hiding.

For these isotypes, the values were very small due to the lack of a large presence in the serum. These very small values expose the issues with the ELISA. For some of these, it is clear to see that the dilution series used was too broad and missed the end point titer. This is likely due to the fact that the optimization for the dilution series was done using total IgG, IgG1 and IgG2a, which were all present in much higher concentrations than IgG3 and IgE. Potential future work could try to develop an ideal dilution series for IgG3 and IgE in order to study these isotypes more effectively.

DISCUSSION

The ELISA is a widely used and effective way to look at antibody or other protein present in samples. The test has many aspects that can be varied and these things are important to nail down in order to minimize error and yield the most accurate and representative results. ELISA optimization was needed in our lab and had not been done in depth. Optimization can help to create ELISA results that are helpful in the future. In the future, our lab can look at the results achieved through this project in order to help decide on parameters such as virus dilution and sample dilution series. In terms of the virus dilution to coat the ELISA plates, originally 1/100, 1/1000 and 1/10000 dilutions were tested. From those results, shown in Figure 3, the 1/100 dilution resulted in the most ideal absorbance graph, which indicated that the 1/1000 and 1/10000 might not have been enough virus to fully and completely coat the plates. From there, 1/100 dilution was tested along with a 1/500 dilution to see if that would be better. The results of this are shown in Figure 4. Again, the 1/100 dilution showed the ideal absorbance graph. From this it can be concluded that compared with the other dilutions tested, a 1/100 virus dilution to coat the plates may result in the most accurate results. Future testing could be done looking at other dilutions in between 1/100 and 1/500. This could even more narrowly pinpoint an effective virus dilution that is as low as possible in order to conserve the limited coating virus. Next, several sample dilution series were tested. The results seen in Figure 5 show how the absorbance graphs and end point titers compared among the various sample dilution series. Comparing these two, Dilution Series 1 produced the most ideal results which indicated that this series was better able to capture the concentration of antibody in the serum. After this, a few more dilution series were examined to see if those were even better or if not. When looking at these, Dilution Series 1

still produced the best results while still using the least amount of sample possible. Determining this optimal sample dilution series as well as determining the optimal coating virus dilution helps ELISA test results in our lab to be more accurate and not waste resources. Future work to further optimize the test could also potentially test different amounts of washing between steps, different concentrations of antibody, as well as different incubation times.

This experiment also showed a repeated result of decreased IgG production in the IL21r-/- mice. Since Figure 4 showed an overall decreased total IgG concentration in the IL21r-/-, it makes sense that both subtypes IgG1 and IgG2a are decreased in the IL21r-/- when compared to the wild type mice. This shows that IL21r-/- is beneficial to the production of both IgG1 and IgG2a since significantly lower concentrations of both are found in the IL21r-/- mice.

The results shown in Figure 4 showed a decrease in total IgG in IL21r-/- knockout mice which is consistent with previous research indicating IL21's importance in the antibody response [5]. In the literature, there has been some conflicting reports of IL21 antibody response so it was helpful in the grand scheme of the field to repeat this in different circumstances and see the results [30]. Figures 5 and 6 show the results of the IL21r-/- mice' IgG1 and IgG2a compared with that of the wild type mice. The IL21r-/- showed decreased concentrations of both IgG1 and IgG2a. This makes sense because the overall total IgG was lower but it also shows us that it is not just one of the IgG subtypes that is impacted and in turn both IgG1 and IgG2a are decreased in these knockout mice. This is important because gathering information about the components that are needed for the development of antibody, especially T-cell related long lived antibody, is crucial to the development of treatments, vaccines and other things that could potentially help to increase antibody response when needed. In terms of vaccine development in particular,

including IL21 could be essential to helping the body produce long lived antibody against influenza, or other viruses.

Additionally, some experiments were done looking at T-bet and Eomes double knockout mice and comparing with wildtype. T-bet and eomesodermin (Eomes) are related transcription factors that coordinate many immune responses [28]. These two work cooperatively to govern cellular immunity by inducing the effector genes of T cells and NK cells [28]. Our lab has previously published data looking at the IgG2a production in Tbet knockout mice and after looking into the IgG2a isotype, we decided it would be valuable to test the IgG2c isotype in the same context. IgG2a is the TH1 associated isotype, but in the strain of mice used in our lab, it is actually IgG2c. While these two are likely to yield very similar results, we thought it could be useful to look at IgG2c production in these Tbet knockout mice as well as double knockouts in order to see a potential role for Eomes in antibody production and in order to further optimize the ELISA results. Figure 7 displays the results comparing the total IgG levels in wild type and DKO mice. This is to give a basis from which to compare the levels of the subtypes. The total IgG in both the wild type and DKO mice were nearly the same, showing that despite the lack T-bet and Eomes, IgG production does not decrease significantly. However, Figure 9 shows the difference in IgG2c production when comparing the DKO with the wild type mice. Now that IgG2c has shown to work well in this context in our lab and along with the knowledge that it is the preferred isotype to use when working with this B6 mouse strain, future work analyzing the IgG subtypes could use the IgG2c subtype instead of IgG2a. This is helpful in further optimizing this test.

Lastly, the isotypes IgG3 as well as IgE were analyzed to see how much of these were present in the serum of the DKO and T-bet KO when compared to wild type. Figures 11 and 12 show that these two were present in extremely low levels. It was interesting to know that these likely do not play a large role in the processes being examined in this experiment.

In this project, there are many different potential sources of error. This could be exhibited in absorbance graphs that do not show a normal ELISA curve or in end point titers that have high error. One source of error would be that due to the time constraints, antibody was allowed to incubate for varying times for different experiments. This introduces another variable of time that should ideally be kept as consistent as possible. Theoretically, increased time could lead to more bound antibody. However, this likely did not serve as a large source of error because the plates were allowed to incubate for at least 3 days which is ample time for all the antibody to bind. Another potential error comes from washing. Not washing enough could lead to high background signal due to the unbound antibody. An additional source of error would be the variable time of letting the substrate enzyme reaction to proceed before acid stopping. Knowing when to acid stop is a balance between allowing enough signal to develop without allowing background signal to develop.

In the future, there are several things that could be looked at in order to further the information found through this project. The ELISA gives you the total amount of antibody recognizing a particular antigen. However, in reality, different antibodies can be more or less protective and have different affinities. A microneutralization assay may be more accurate. This could be very helpful in looking at IL21 because the level of protectiveness may be quite

significant along with the amount of antibody. A future study using the microneutralization assay could be a great way to further explore IL21.

Other future work related to IL21 could potentially look at the level and effectiveness of Tfh in IL21r-/-. Additionally, looking at the risk of reinfection for WT versus IL21r-/- mice could also be interesting because perhaps the decrease in the antibody response could lead to a higher rate of reinfection.

Overall, the findings of this experiment have implications in growing our knowledge about antibody response in IAV. Knowing the IL21 importance and how the IgG subtypes are involved is important in the development of . This factor has the potential to be manipulated in order to help create the most protective response in the body against IAV. This can even be generalized to other disease processes other than IAV and IL21 may offer help in creating protective antibody responses in other diseases as well.

Looking at the broader applications of antibody responses and isotype classes, it is interesting to consider COVID-19. Severe COVID-19 has been shown to elicit high levels of IgG3 and IgG1 [27]. This is interesting because this contrasts to the data from Figure 11 that shows a very low level of IgG3 in the flu infection. One of the reasons to better study these specific pathways and antibody isotypes involved in influenza is to understand the virus process better and to be better prepared for a pandemic situation like we have experienced with COVID-19.

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