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Therapeutic IgY: Safe, Diverse, And Effective For Use Against Viral Targets

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THERAPEUTIC IGY: SAFE, DIVERSE, AND EFFECTIVE FOR USE AGAINST VIRAL TARGETS

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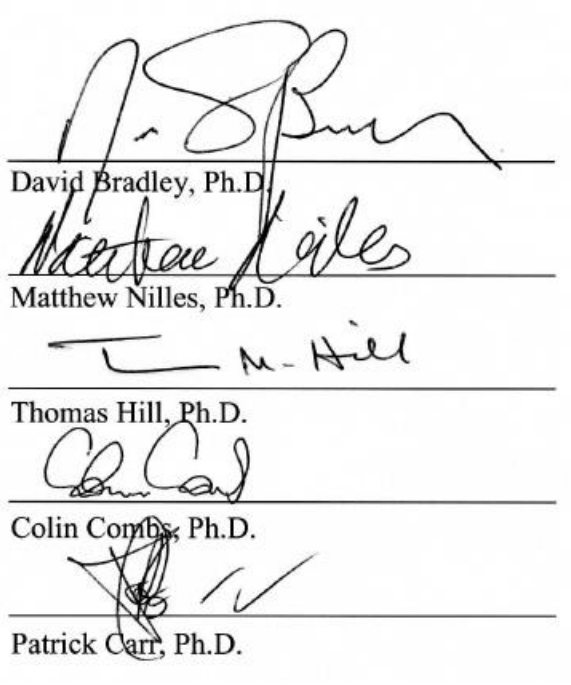

This dissertation is being submitted by the appointed advisory committee as having met all of the requirements of the School of Graduate Studies at the University of North Dakota and is hereby approved.

_____
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PERMISSION

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Nicole N. Haese

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ABSTRACT

Passive antibody treatments are used to target infectious disease, toxins, venoms, and cancer antigens. Recently, there has been an increased interest in the use of avian-derived antibody treatments such as IgY. IgY is the primary serum antibody isotype present in the avian system, and IgY treatments have already been demonstrated to be effective against a variety of bacterial and viral infectious agents. There are two forms of IgY expressed in anseriformes birds, a full length IgY that is functionally similar to mammalian IgG, and an alternatively spliced IgY, IgY(ΔFc), that is comparable to the mammalian $F(ab')_2$ fragment. The difference in structure between IgY and mammalian IgG, prevents IgY from interacting with mammalian Fc receptors, complement, and other inflammatory factors. The phylogenetic distance between mammalian and avian species allows IgY to have a higher avidity for certain mammalian epitopes and a unique antibody repertoire is developed compared to mammals, further enhancing the therapeutic potential of IgY.

Our current research is focused on developing goose IgY anti-viral treatments and ensuring the safety of these treatments in humans. The viral antigens of focus in this research are dengue virus type 2 (DENV-2) and the Andes virus (ANDV). In an attempt to generate monoclonal goose IgY antibodies, using modified mammalian hybridoma techniques, geese were immunized with DENV-2 antigen and blood was collected as a source of immune B-cells and fused with mammalian myeloma cells. Short lasting,

virus- specific IgY producing hyrbidomas were created. To generate ANDV specific goose IgY antibodies geese were vaccinated with a DNA vaccine PWRG/AND-M, containing the full-length M genome segment of ANDV, via a needle-free device at two week intervals up to eight weeks and then at 12 weeks. One year later the same geese were booster vaccinated with either pWRG/AND(opt) or pWRG/AND(opt2) six times over a ten week time period. Average neutralizing titers of sera collected from geese six weeks after the primary vaccination was 10,000. Titers remained at this level for the one year in between vaccinations and then increased to nearly 100,000 after booster vaccination. Epitope mapping confirmed the specificity of the goose- derived antibodies and identified unique highly reactive epitopes. IgY from the initial vaccination recognized 11 epitopes across the M segment, and an additional 9 epitopes after booster vaccination. *In vivo* survival studies in a lethal challenge model of ANDV infection established the post-exposure treatment potential of the ANDV specific IgY.

To test the safety of the anti-viral IgY treatments for use in humans *in vivo* and *in vitro* safety experiments were completed. In a single injection study, mice were injected with a single dose of IgY/IgY(ΔFc) or PBS, and in a multiple injection study, rabbits were injected with multiple doses of IgY/IgY(ΔFc), IgY(ΔFc), human immunoglobulin, or PBS. Organs were collected after injection, hematoxylin and eosin stained, and scored by a blinded pathologist for abnormal pathology and/or inflammation. There were no inflammatory manifestations in the organs from animals in either the single or multiple injection study receiving IgY/IgY(ΔFc) or IgY(ΔFc). PBMCs and neutrophils were isolated from fresh human blood and co-cultured with IgY/IgY(ΔFc), mammalian IgG, and other controls. Culture supernatants were collected at various time points and

analyzed for the presence of IL-1β, TNF-α, IL-10, neutrophil elastase, and nitric oxide using kit-based assays. All assays reported less reactivity of goose IgY/IgY(ΔFc) with human PBMCs and neutrophils compared to mammalian IgG and positive control mitogens. These results further support the lack of reactivity of avian IgY in the mammalian system and the benefits of safely using IgY as a treatment in the mammalian system.

CHAPTER I

INTRODUCTION

Avian Immune System

Though the main objective of the avian immune system remains the same as that of the mammalian immune system, to prevent any foreign antigen from causing an infection, and to keep internal antigens in check, the two systems are structured and function differently. Most research completed to understand, the avian immune systems has utilized the chicken as a model system; consequently the most is known about the immune system of the galliformes (land fowl) order of birds. Despite being closely related to other orders of birds, such as the anseriformes (waterfowl, e.g., geese and ducks), not everything discovered in chickens can be directly applied to the immune systems of all birds. The primary lymphoid organs of the avian immune system are the thymus, bone marrow, and the bursa of Fabricus (BF). The secondary lymphoid organs include the spleen, harderian gland, germinal centers, and other diffuse lymphoid tissues [1]. One major difference between the avian and mammalian immune system is the presence or absence of lymph nodes. Chickens are reported to not have lymph nodes, while researchers have identified what are believed to be lymph nodes in ducks [2]. Lymph nodes are the primary site of antigen presentation in the mammalian immune system and are crucial for the development of a proper immune response. It is unknown for sure where this crucial interaction between antigen and immune cell takes place in

chickens. It is speculated to occur locally at the site of infection, probably in one of the many lymphoid aggregates of the mucosal tissue [3].

The thymus is the primary location for the development of T-lymphocytes (T-cells), including initial development and maturation. There is also a small percentage of B-lymphocytes (B-cells) in the thymus that is dependent on the age of the bird [1]. The BF is an organ unique to the avian immune system and is the sole site of B-cell differentiation and synthesis [4]. Glick and Chang have shown that removal of the BF results in the elimination of the antibody response in the majority of bursectomized chickens [5]. Bursectomized birds, unlike thymectomized birds, lack immunoglonulins, splenic germinal centers, plasma cells, and an antibody response, but were capable of a graft-versus host response, and produced normal numbers of circulating small T-lymphocytes [6]. The bone marrow is the source of precursor stem cells for both B and T-cells that then migrate to the BF and thymus respectively for further differentiation. In the avian system the spleen is the primary site for plasma cell proliferation and memory B-cell maintenance [7,8]. The spleen is not only an important organ for lymphocyte development, but is also the location for granulocyte production, antibody production, and is important for antigen processing [1]. Some important lymphoid organs within these mucosal lymphoid tissues are the Peyer's patches and harderian gland. The Peyer's patches are a location of lymphoid cell accumulations in the gastrointestinal tract and appear to be a major site for IgA responses to pathogenic organisms and undigested antigens [9]. The harderian gland is part of the head associated lymphoid tissue located behind the eyeball within the orbit. Plasma cells make up a large percentage of the cells

in this gland, serving as the major antibody secreting gland of the lacrimal apparatus and are important in development of vaccine immunity [1,10].

The avian immune response can be divided into two major parts, the innate and adaptive immune response. The innate immune response is activated during the acute phase of infection and involves both a non-specific barrier protection and a portion of the response that is specific, not to individual pathogens, but to classes of pathogens. Some examples of non-specific physical barriers that are part of the avian immune system include the skin, mucus membranes, and gastric juices. The more specific portion of the innate response depends on the cellular components of the innate immune system including natural killer cells, monocytes/macrophages, and heterophils. The innate cellular components of the avian immune system are simplified compared to the mammalian system, lacking eosinophils, basophils and neutrophils [3]. Avian heterophils are functionally similar to mammalian neutrophils being the predominant cell responding to innate inflammatory reactions in chickens [11]. Heterophils are highly phagocytic; most of the antimicrobial activities are carried out through phagocytosis and the release of granules within the cell [11]. Some of the innate avian immune cells also have receptors present on their surfaces and endosomes called pattern recognition receptors (PRRs), which can recognize different types of antigens referred to as pathogen associated molecular patterns (PAMPs). One well studied group of PRRs are the Toll-like receptors (TLRs). Research suggests that mammals and chickens have different TLR repertoires, but the basic function is the same [12-14]. The interaction between PRRs and PAMPs can influence the production and release of cytokines, chemokines, and other inflammatory mediators by immune cells that influences all subsequent steps of the

immune response. Cytokines and chemokines are a broad group of secreted intracellular messengers that serve to coordinate both the innate and adaptive immune responses as well as immune cell development and homeostasis. These intracellular messengers can be secreted by many different cell types and can exert their regulatory effects on immune system cells, hematopoietic stem cells, and non-immune tissues to aid in immune host defense and homeostasis. Some of the major groups of cytokines include interferons (IFNs), interleukins (ILs), and colony stimulating factors (CSF). Compared to the mammalian system, fewer cytokines and chemokines have been identified in the avian system [15,16]. The human genome has 129 cytokine and chemokine sequences, while the chicken has only 77 [17].

If the innate immune response is unable to clear the antigen, activation of the adaptive immune response is required. Activation of the adaptive immune response will also result in the formation of immunological memory, leaving the bird with a potential advantage in the future. The innate response is able to influence the adaptive immune response through cytokines, chemokines, and other inflammatory mediators. A key bridging point between the two responses is the presentation of antigen in the context of a major histocompatibility complex (MHC) on the surface of antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages (MOs). There are other cells that act as phagocytes within the avian system: thrombocytes (functional homologs of mammalian platelets, which are absent from birds) [18,19], and heterophils. Depending on the type of antigen, either the cell-mediated or humoral immune response is preferentially developed against the antigen. T- and B-cells are the primary effector cells of the adaptive immune response and are responsible for the cell-mediated and humoral

immunity. The preferential development of either the cell-mediated or humoral responses are driven in part by two subsets of $CD4^+$ helper T-cells: Th1 and Th2. Expansion of one the T-cell subsets over the others are dependent on the cytokines in the milieu during the immune response. The Th1/Th2 $CD4^+$ T-cell subset paradigm has been demonstrated in chickens [20]. What remains unclear however is whether other T-cell subsets (e.g. Treg, Th17 and Th9), exist within the avian immune system. It is thought in the avian immune system typically an endogenous antigen is recognized by the $CD8^+$ T-cell subset, in the context of MHC I, leading to activation of the cell-mediated immune response. $CD8^+$ T-cells become cytotoxic, capable of antigen-specific killing of infected cells. An exogenous antigen activates $CD4^+$ T-cell subset, when presented on the surface of an APC in the context of MHC II. $CD4^+$ T-cell subsets are termed T helper cells, they are responsible for helping with antigen-specific activation of B-cells, aiding in the humoral immune response along with providing help to macrophages, NK cells, and $CD8^+$ T cells [3]. The end result of the immune response is clearance of the pathogen and development of memory.

The effector cells of the humoral immune response are antibody producing B-cells with antibodies carrying out the major functions of the humoral response. Within the avian immune system there are three classes of immunoglobulins (IgM, IgA, and IgY), all of which have been identified immunochemically [21] and genetically as homologs to mammalian IgM, IgA, and IgG [22-24]. Avian IgM is structurally and functionally homologous to mammalian IgM; it is present in the serum as a high molecular weight pentamer and is the first antibody produced during a primary antibody response. IgA is also similar to its mammalian homolog and is able to be found in the

serum of birds, bile, and tears. IgY is generated typically during a secondary antibody response and behaves in part like mammalian IgG and mammalian IgE. In anserifomes birds there is an additional structurally different isoform of IgY that is termed IgY(ΔFc). During egg formation, IgY in the serum is selectively transferred to the yolk via a receptor on the surface of the yolk membrane specific for IgY translocation, transferring immunity from the hen to the developing embryo [25-28]. The amount of IgY transferred is directly proportional the IgY serum concentration [27]. Both avian IgA and IgM can be found in the egg white [29]. So far there have not been any identified avian homologs of mammalian IgD or IgE [23].

As mentioned previously the organs in the avian immune system that are responsible for antibody production differ from those utilized in the mammalian system. The primary lymphoid organ responsible for the differentiation of stem cells into B-cells, as well as diversification, is the BF [4]. The spleen is where plasma cells and memory B-cells can be found. There are various mechanisms used by the immune system to generate the necessary antibody diversity, the primary mechanisms used do differ between the mammalian and avian systems. In the avian system the primary antibody repertoire is generated primarily by a process of somatic gene conversion and by somatic point mutations. Committed precursor B-cells enter the BF with fully rearranged immunoglobulin genes and rely on upstream pseudogenes to create diversity for both light and heavy chains [30-32]. Antibody gene rearrangement in birds takes place in the developing embryo during a 10 day window prior to hatching. The antibody light (L) chains are mostly of one type (λ) [33], meaning the genomic L chain locus is made up of one constant region (C_L or C_λ). The rest of the L chain locus is made up of a single

unique junctional region (J_L), which undergoes rearrangement to the adjacent function variable region gene (V_L) [33]. The same V_L and J_L segments are used in all B-cells during VJ_L rearrangement, limiting the diversity that can be gained within the L chain from the process of gene rearrangement. A similar organization has been characterized for the heavy (H) chain locus. There is one V region (V_H), a single J segment (J_H), used in all B-cells, and approximately 15 function diversity (D_H) segments [24,34]. The H chain immunoglobulin gene loci also contain constant region genes μ, ν, and which allow for the production of IgM, IgY and IgA respectively. Because there are multiple D_H segments there is a minimal amount of junctional diversity generated from VDJ_H rearrangement. There is potential for rearrangements with fused D-D elements, due to the sequence homology between the D_H segment sequences [23]. Unlike in the bone marrow of the mammalian system there is no specific order in which the rearrangements need to take place; rearrangement of one chain is not dependent on the other. In fact VDJ_H and VJ_L rearrangements have been detected at the same time in the developing embryo [35].

After successful gene rearrangement primary antibody diversity is generated through the use of pseudogenes and somatic gene conversion. Both the immunoglobulin L and H chain gene loci have pseudogenes upstream from the V_L and V_H regions. Upstream from V_L is a group of appxomimately 25 psdeudogenes (ΨV_L), and upstream from V_H there are approximately 80 pseudogenes (ΨV_H) [36]. These gene segments are defined as pseudogenes because they typically lack the promoter sequences required for transcription, have a truncated 5' or 3' coding region, or lack the necessary recombination signal sequence (RSS) [33]. The process of somatic gene conversion

starts approximately 15-17 days after the immature B-cells migrate to the BF [37]. Diversity is ultimately generated when sequences derived from the upstream pseudogenes replace homologous sequences within the V region of rearranged VJ_L or VDJ_H genes and undergo gene conversion [38]. After gene conversion is complete, the B-cells have reached the end of their time in the BF and migrate into the periphery. B-cells usually start to migrate from the BF in the last few days before the bird hatches. At first the percentage of cells migrating from the BF is low. After hatching about five percent of bursal B-cell migrate each day into the periphery through the blood and then into the spleen, thymus and caecal tonsils, where they will eventually produce antibodies and be active in the humoral immune response [36,39]. The remaining percentages of bursal B-cells end up dying in the bursa during various steps in development due to autoreactivity, or nonproductive H and L chain rearrangements. However, B-cells deaths are rarely due to the process of gene conversion, that process is ~98% successful [40,41]. It has been observed that by the time baby chickens reach the age of four weeks a sufficient number of B-cells have migrated out of the BF. At that point the B-cell immune system is considered mature in the periphery, resulting in involution of the BF [42]. Once the B-cells are in the periphery, the spleen is responsible for maintenance and proliferation of plasma cells and memory B-cells.

Function and Structure of IgY Antibodies

Structural and functional research shows avian IgY is similar to mammalian IgG. Avian IgY is the functional equivalent of mammalian IgG within the avian immune system, acting as the major system antibody responsible for defense against infectious

diseases. Unlike IgG, full length IgY is also able to sensitize tissues to anaphylactic reactions [43]. Structurally avian IgY is similar to mammalian IgE based on size and number of exons. IgY is classified as a low-molecular weight (LWM) immunoglobulin, thus it is made up of two H chains and L chains with a molecular mass of ~180kDa [44]. Each of IgY's L chains consists of one variable and one constant domain with a molecular weight of ~18kDa. The H chains of IgY have one variable domain and four constant domains denoted Cν1-Cν4, with a molecular weight of ~65kDa [44]. As was mentioned previously, IgY is structurally similar to mammalian IgE. Both of these antibody's H chains have four constant domains and are lacking a hinge region. It is also the H chains that structurally differentiates mammalian IgG from IgY. The H chains of mammalian IgG have only three constant domains (Cγ1-Cγ3) with molecular weights of ~59kDa each, causing the molecular weight of IgG to be only ~159kDa, less than that of IgY. Sequence comparison between IgG and IgY has shown that the Cγ2 and Cγ3 domains of IgG are closely related to the Cν3 and Cν4 domains of IgY, while the equivalent Cν2 domain is absent in the IgG H chain [45,46]. The Cν2 domain that is present in IgY is thought to have been replaced by the hinge region in IgG [23]. This is supported by the observation that the hinge region of IgY is much less developed compared to IgG, and the flexibility of IgY is also decreased compared to IgG [45]. The lack of a developed hinge region in IgY may account for some of the differences in function between the two types of antibody.

There is an additional isoform of IgY that can be found in anseriformes birds, which lacks two C-terminal constant domains (Cν3-Cν4) of the H chain. This isoform of IgY is referred to as alternatively spliced IgY or IgY(ΔFc) and has a molecular weight of

~120kDa. IgY(ΔFc) and full length IgY may coexist in an individual organism as they do in anseriformes birds, or one form may be produced exclusively. For example, chickens only produce full length IgY. Both full length IgY and IgY(ΔFc) are products of a single gene generated by alternate pathways of mRNA processing [47,48]. Despite being from the same genetic source IgY and IgY(ΔFc) do have some functional distinctions. During an immune response the IgY(ΔFc) isoform is found later in the immune response than full length IgY [49]. In the avian immune system, full length IgY is able to fix complement and sensitize tissues to anaphylaxis, but both of these immune functions are not carried out by IgY(ΔFc) [49]. These functional differences between the isoforms are most likely due to the alternatively spliced isoform lacking the two terminal Fc domains. This limits IgY(ΔFc) to functions that do not require secondary effector functions such as virus neutralization. It is even possible that IgY(ΔFc) was tolerated evolutionarily because of its inability to sensitize the host birds to IgE-like reactions leading to less host damage.

Physiochemical Properties of IgY and IgY(ΔFc) Antibodies

Other properties of IgY that differ from mammalian IgG include β-sheet content, hydrophobicity and isoelectric point. The β-sheet content of IgY, especially in the constant domain, is lower than that of IgG [50]. Decreased β-sheet content has the potential to cause a more disordered conformation in IgY leading to a less stable antibody than IgG. IgY is also more hydrophobic than IgG. This is due in part to the fact that the Fc fragment of IgY is larger than that of IgG, and the fact that the Fc portion of the antibody is the most hydrophobic part of the antibody molecule. Experiments completed

comparing the kinetics of either IgG or chicken IgY interacting with latex particles under varying conditions further support the increased hydrophobicity of IgY versus mammalian IgG [51]. The isoelectric point of IgY is in the range of 5.7 to 7.6, lower than that of IgG [52].

The use of IgY as a potential passive therapeutic is dependent on the ability of the antibody to remain stable and active when exposed to a variety of environmental conditions including heat, pH, and proteolytic enzymes. When compared to rabbit IgG, IgY is much less stable during acid denaturation. IgY has been found to be stable in a range between pH 4 to pH 11, but above or below this range IgY activity decreases [53,54]. IgY activity is decreased at a pH of 3.5 and completely lost at pH 3, while rabbit IgG activity is not lost until pH 2 [50,55]. The stability at harmful pH's can be improved by adding different carbohydrates of sugars to act as stabilizers [56,57]. Temperature is also a threat to the activity of IgY. Researchers have reported that as temperature increases, IgY activity decreases. Heating IgY to a temperature of 60-65ºC will result in a minimal loss of activity, but the major decrease in activity is reported at temperatures of 70ºC or higher, when heated for more than 15 minutes [50,53,55]. The addition of sugars can help IgY to maintain activity at higher termpatures in the range of 75-80ºC [57].

IgY is also susceptible to proteolytic cleavage by enzymes such as pepsin, trypsin, and chymotrypsin. In general, IgY has been found to be more resistant to the activity of trypsin and chymotrypsin than that of pepsin. Almost all IgY activity is lost after digestion with pepsin while, on the other hand IgY remains active even after eight hours of incubation with trypsin or chymotrypsin [53]. The sensitivity of IgY to pepsin seems to be dependent on the pH and enzyme to substrate ratio [53]. Different variations of pH

and enzyme to substrate ratio will yield different effects on IgY activity. Overall, IgY is more susceptible to all three of these proteolytic enzymes that IgG. These differences in susceptibility could be related to the structural differences between the two antibodies, including the lower β-sheet content of IgY along with the lack of a hinge region [50]. Both of these structural characteristics have an impact on the overall properties and stability of the IgY molecule.

Advantages of Passive IgY Antibody Immunotherapy

Avian IgY antibody treatments have many advantages over traditionally used passive antibody treatments, some of which stem from the phylogenetic distance between the mammalian and avian species. The phylogenetic distance leads to an increased immune response in birds when compared to mammalian antibodies. The IgY produced has a higher avidity for mammalian antigens, and recognizes different epitopes compared to mammalian antibodies, creating a unique antibody repertoire [58,59]. Even when mammals and birds are identically immunized often times different antibody specificities will develop in birds than mammals. Di Lonardo *et al*. showed through epitope mapping that when rabbits and chickens were immunized with the same antigen, in this case human papilloma virus type 16E7 oncoprotein (HPV16E7), the resulting antibodies recognized different peptides[58]. Eight HPV16E7 peptides were used for the epitope mapping; the chicken antibodies reacted to all eight peptides, while the rabbit antibodies only reacted with two peptides [58]. In another example, eggs from immunized chickens were used as a source of IgY antibodies against the insulin receptor of rats, as a means to gain more information about the function and structure of the insulin receptor. Previous

results utilizing rabbit antibodies against the α-subunit of rat insulin receptors did not entirely mimic the insulin resistance response seen in patients that naturally produce antibodies against insulin receptors. In order to better mimic the natural human syndrome, chicken IgY antibodies against insulin receptors were generated. The properties of the IgY antibodies were similar to those of the antibodies found in the human disease and caused the inhibition of insulin binding, stimulated glucose oxidation, and autophosphorylation of the insulin receptor [59]. Rabbit antibodies of the same specificity failed to mimic all of the same effects as the IgY antibodies, mainly insulin binding was not prevented [59]. These are just two examples that clearly demonstrate the avian IgY antibodies ability to recognize different epitopes than mammalian antibodies, regardless of the origin of the antigen. For some mammalian antigens IgY antibodies have increased binding specificity and sensitivity compared to mammalian antibodies. IgY can be used to make antibodies against conserved antigens within the mammalian system, in some situations there may be no other option if the antigen is too highly conserved [60]. Using IgY antibodies from the eggs of immunized birds offers researchers access to a different antibody repertoire than what can be achieved with the mammalian techniques currently used.

A key advantage to the use of avian IgY antibodies is being able to utilize the egg as the source of the antibodies. Eggs can be easily harvested from laying birds, eliminating the need for bleeding animals representing a non-invasive and more humane method of obtaining antibodies that meets current regulations regarding animal use in research. Not only is it less invasive, but an equivalent or greater amount of antibodies can be isolated from the egg yolks of a chicken, duck, or goose egg compared to what can

be isolated from the serum of rabbits or other animal sources of antibody. The amount of IgY purified from a chicken egg can vary depending on age and breed of the hen, along with the antigen used for injection, from 60-150mg per egg [61,62]. Based on the laying habits of chickens the average monthly antibody production is in the range of 1000 to 2800 mg [37]. Goose eggs are larger than chicken eggs increasing the amount of IgY that can regularly be purified from a single goose egg to 500mg. In comparison, to isolate 200mg of mammalian IgG from rabbit serum it would take approximately 40mL of blood which is total amount of IgG available for the month [37]. Because IgY levels in eggs can be influenced by bird age, studies have been completed comparing laying patterns to IgY content of eggs from laying hens over an extended period of time. When laying hens were monitored for a two year period, there was a decline in the laying capacity of the hens in the second year. However, there was also an increase in the total IgY per egg, balancing out the decrease in egg numbers [62]. Antigen specific IgY titers remained stable during the two year time period and the IgY was functional [62]. These findings are supported by the observations of Trot *et al*. that older hens have higher IgY titers compared to younger hens. When taking into consideration both egg mass and laying frequency, young hens in the study had a 1.3 fold increase in total egg mass compared to older hens, but this difference was offset by a 1.5 fold increase in IgY titers in older chickens [61]. Not only can eggs easily be collected from their avian sources, IgY can also easily be isolated from the egg yolk. Throughout literature various methods have been described to isolated IgY from the egg yolk, but all utilize one or more the following basic lab procedures: precipitation, chromatography, and/or ultracentrifugation.

Some labs are extracting IgY from eggs on an industrial scale, supporting the potential large-scale production of IgY therapeutics [63,64].

Most biological effector functions of immunoglobulins are activated by the Fc-region. As described above, the Fc region is where the major structural differences between IgG and IgY are located; which leads to a difference in Fc dependent functions between the mammalian IgG and avian IgY molecules. This difference can be used to prevent interferences in immunological assays as well as adverse reactions within the body caused by rheumatoid factor (RF), complement activation, human anti-murine antibodies (HAMA), or binding to human and bacterial Fc receptors. There are antibodies within the body that react with the Fc portion of mammalian IgG antibodies, one example is RF. Most of the time RF is an autoantibody associated with rheumatoid arthritis (RA), but it can be found in the patients with other diseases and in the blood of healthy individuals [65]. HAMAs form often when antibodies are used for treatment or imaging. More specifically, the murine antibody is seen as a foreign protein by the human body, which triggers an immune response resulting in the formation of HAMAs. In some cases HAMAs have also been found in the serum of patients who have not been treated with antibodies [66]. The HAMA response can persist in the blood for several months, and can be easily reactivated if memory B-cells are formed. This response can interfere with assays used in diagnosis and/or surveillance of disease and cause potentially painful symptoms for the patients. Attempts have been made to prevent the HAMA response by making humanized antibodies through replacement of the Fc portion of murine antibodies with the constant region of human antibodies. Some of these engineered antibodies are only 10% murine or even "fully human." These engineered

antibodies have protein epitopes that can be seen as immunogenic by the human immune system and ultimately cause an antibody response by the body. This response is referred to as an anti-drug antibody response (ADA). The HAMA/ADA responses within the body can result in a variety of symptoms from more mild allergic reactions, generalized pain, hponatremia, fever, rigors, chills, rash, paresthesia, weakness, chronic refractory postural hypotension [67,68], to hypersensitivity reactions that range from serum sickness with urticarial and bronchosapsms [68-72], to anaphylactic shock [73-75].

HAMA/ADAs can be classified as either binding antibodies (Bab) or neutralizing antibodies (Nabs) depending on if they alter the pharmacokinetics of the antibody treatment and/or lead to symptomatic reactions in the body [76-79]. Babs bind to sites on the antibody that do not directly interfere with the ability of the antibody to interact with the intended target of the antibody. They can affect the pharmacokinetics, specifically the clearance of the antibody treatment from the body before it can function properly [79-81]. Nabs bind to sites on the antibody that are important for ligand-receptor interaction thereby inhibiting antibody function; this can cause diminished efficacy and lead to failure of the treatment [82-84]. Nabs are primarily of the IgG isotype and more commonly cause life-threatening conditions that Babs [73]. Using IgY antibodies as an alternative to current IgG antibody treatments on the market comes with a decreased risk of adverse reactions because IgY antibody does not interact with human RF or cause HAMA/ADA responses [84-86]. IgY can also be used to replace mammalian IgG in immunoassays to avoid interference from RF or HAMA/ADAs produced during treatment with mammalian IgG [86,87]. Reduced interference in immunoassays will also

decrease the number of false positive results and unnecessary medical procedures done because of inaccurate test results.

The complement pathway is another example of an Fc- dependent function that IgY antibodies are unable to activate because of the distinctive structure of their constant region. Within the avian immune system IgY antibodies binding to antigens are able to bind to activate the complement system. The same is not true when avian IgY antibodies are placed in human immune conditions. Avian IgY antibodies do not activate the human complement system [88]. In the mammalian system, under normal conditions antibodies react to antigen to form an antibody-antigen complex. The Fc portion of the antibody can then activate the complement system and lead to recruitment of inflammatory cells, opsonization of pathogens, and killing of pathogens. When the complement pathway is inappropriately activated it can lead to unnecessary inflammatory reactions. The anaphylatoxins C4a and C5a products of the complement pathway are often responsible for initiating parts of these inflammatory responses. The anaphylatoxins are able to stimulate mast cells to trigger IgE-independent histamine release as well as TNF-α release [89]. This can mimic allergic shock depending on the number of mast cells involved [90,91].

A serum sickness-like illness can also develop depending on the number of complement immune complexes formed during the course of treatment. Serum sickness can also result from complement-binding immune complexes, which can lead to activation of leukocytes and then to widespread tissue damage. Though the disease is usually self- limiting, after a second dose of antigen the illness will follow the same kinetics as a secondary immune response. C5a also acts directly on neutrophils and

monocytes to increase their adherence to vessel walls, their migration towards sites of antigen depositions, and their ability to ingest particles [89] further contributing to the progression of serum sickness.

Complement activation is dependent on the carbohydrate determinants in the Fc region of antibodies; the composition of antibody subclasses IgG1 and IgG3 make them most effective at complement activation [92,93]. IgY is unable to initiate the complement cascade in mammals due the difference in the Fc portion of the antibodies. IgY is thought to be lacking the necessary carbohydrate determinants to activate the complement pathway. This prevents both complement-mediated and antibody dependent cell-mediated lysis from taking place in the presence of IgY [37]. IgY therapeutics have the potential to avoid many of the adverse reactions, including unnecessary inflammatory reactions, in patients caused by mammalian IgG activation of the complement pathway. The activation of the complement pathway by IgG can also interfere in immunological assays, causing problems with background and test result errors. The lack of complement pathway activation by IgY also makes it a useful reagent in immunological assays, especially those that utlize serum, decreasing the background and reporting errors [88,94].

There is evidence that IgY, depending on the source, does not interact with bacterial proteins that are immunoglobulin Fc-binding. The most well-known of these proteins are *Staphylococcal* protein A and *Streptococcal* protein G. These proteins are able to bind to the Fc portion of IgG from many mammalian species [95,96]. The interaction between the bacterial proteins and IgG is utilized in purification assays and immunoassays. Problems can arise from this interaction depending on the assay and

specimen leading to false positives. Results of initial research using chicken antibodies reported that IgY does not react with *Staphylococcal* protein A *Streptococcal* G [95-98]. There have been conflicting results regarding whether or not IgY from anseriformes birds interacst with bacterial *Staphylococcal* protein A and *Streptococal* protein G. Research by Kronvall *et al*., using various methods, report that IgY from anseriformes birds does not interact with *Staphylococcal* protein A or *Streptococcal* protein G [97]. In some studies duck IgY has been reported to bind strongly to *Staphylococcal* protein A, but poorly to *Streptococcal* protein G in Sepharose affinity columns [2]. A more recent study completed by Justi-Valliant *et al*. compared the reactivity of immunoglobulins from various avian and mammalian species with *Staphylococcal* protein A and *Streptococcal* protein G, and other bacterial proteins. In this study, using a direct ELISA, duck IgY was found to react with *Staphylococcal* protein A while chicken IgY was unreactive. Overall, direct use of ELISA demonstrated that duck IgY reactivity was less than that of many mammalian species [99]. When tested via sandwich ELISA, duck serum did not react with *Streptococcal* protein G, and chicken IgY was also unreactive. By contrast all mammalian species were reactive with *Sreptococcal* protein G.

The interaction of avian IgY antibodies with bacterial *Staphylococcal* protein A and *Streptococcal* G is most likely not Fc mediated, as it is in mammals, because of the difference in the Fc portion of the antibodies between avian IgY antibodies and mammalian IgG antibodies. This is especially true for IgY(ΔFc) produced by anseriformes birds, which is lacking both C_H3 and C_H4 domains. It is more likely that IgY interacts with the bacterial proteins through the C_H1 and C_H2 domains of the immunoglobulin, although some involvement of the other domains cannot be ruled out.

Differences in reactivity between chicken IgY, duck IgY, and potentially other anseriformes birds could be due to differences in the histidine (His) content of H chains of the two avian species. Research with human IgG suggests that His residues in the Fc region are important in binding to protein A [2]. Duck IgY H chains have eight unique His residues compared to chicken IgY: four in C_H1, one in C_H2, two in C_H3, and one in C_H4. Most likely to be involved in binding to *Staphylococcal* protein A are those in C_H1 and C_H2 [2]. Using avian IgY antibodies in assays is advantageous because of the lack of interaction between avian IgY antibodies and bacterial proteins that are Fc binding. This lack of interaction can lead to a reduction in the interference problems in assays seen with mammalian IgG.

Passive Immunization

Passive immunization makes use of the ability of artificial passive immunity to treat or prevent a foreign attack- often infectious agents. In this type of immunity, antigen- specific antibodies or serum from another source, typically an immune human or animal, is given to another individual for protection. The protection conferred is immediate and will last as long as the transferred antibodies remain in the recipient's body. Passive antibody therapeutics have been around since the late 19^{th}-20^{th} centuries with pioneering research done by Robert Koch, Emil von Behring, and Paul Ehrlich. This research started with the observation that blood from rats who were resistant to anthrax was able to kill the anthrax bacterium in later experiments [100]. These researchers worked to develop products termed antitoxins, antibodies, and magic bullets, all the predecessors of today's monoclonal antibody therapeutics [100,101]. Advancement in

technology led to the design of intravenous immune globulin (IVIG). In this process, antibodies from patient's serum were isolated and then used to treat immune and infectious diseases [102-104].

A major discovery was made in 1975, with Kohler and Milstein's development of a method for the isolation of monoclonal antibodies from hybridoma cells. Kohler identified how to obtain antibodies from mortal B-cells in culture, while his colleague Milstein found a way to transform myeloma cell lines and promote cell fusion. Joining together they generated a method for fusion of immortal myeloma cells and antibody producing B-cells that secrete a single monoclonal antibody recognizing a specific antigen [105-107]. The first therapeutic monoclonal antibody approved by the Federal Drug Administration for human use in 1986, was murononab, a murine derived antibody used for acute organ rejection [107]. Since then the field has accelerated quickly with 25 therapeutic antibodies currently in clinical use and many more in development.

Most therapeutic antibodies used today are of murine origin, most likely due to the early development of mouse hybridoma technology. But the use of murine antibodies has its limitations. The efficacy of murine- derived antibody therapeutics is challenged by their potential for high immunogenicity in humans. The HAMA response after murine monoclonal antibody treatment in severe cases can result in anaphylaxis. Not only does the HAMA response adversely affect the patients, but the antibody can also be inactivated and eliminated from the body before it can function properly. Murine antibodies can generate a less than ideal protection in humans, reacting with epitopes that are not protective [101]. There has been an increasing interest in the use of avian

antibodies as passive immune therapeutics. Many of the weaknesses of current therapeutics can be resolved by using avian IgY antibodies.

Passive IgY AntibodyTreatments

Current research using polyclonal avian IgY antibodies as a potential passive antibody treatment is ongoing in both the veterinary and human fields. The goal of a majority of these treatments is to establish protective immunity in the host by delivering pathogen- specific avian IgY antibodies to the host. Polyclonal antibodies have been made against a variety of infectious disease agents including bacteria, viruses, fungi, toxins, and venoms. Their effectiveness has also been tested in a number of disease models and human trials. A few representative examples are reviewed below.

Pseudomonas aeruginosa

The most successful clinical application of avian IgY antibodies has been the prevention of *Pseudomonas aeruginosa* (*P. aeruginosa*) colonization in cystic fibrosis (CF) patients. CF is a hereditary, life threatening disorder with repeated respiratory infections and malnutrition as main clinical manifestations [108]. Chronic lung infections with *P. aeruginosa* are a major cause of morbidity and mortality in CF patients, and are accompanied by a more rapid deterioration in lung function. Usually these *P. aeruginosa* infections are treated with antibiotics, which can help to reduce, and sometimes clear the infection. However, the infection often continues to reappear until the patient is chronically infected and the *P. aeruginosa* becomes impossible to eradicate [109-115]. Chronically infected patients need to be continually treated with antibiotics; this continual treatment can lead to secondary infections, bacterial resistance, allergic and

toxicity reactions, and negative effects on commensal flora [116-119]. Anti- *P. aeruginosa* IgY has been and is continuing to be researched as a complement and stand-alone treatment for *P. aeruginosa* in CF patients. It is impossible to eradicate *P. aeruginosa* once a person becomes chronically infected. As a short term treatment anti-*P. aeruginosa* IgY could be used to prolong the amount of time between infections. As a long-term treatment avian IgY antibodies could be used to decrease the number of *P. aeruginosa* infections and prolong the onset of chronic *P. aeruginosa* infection.

In vitro studies have already established the potential of the anti- *P. aeruginosa* IgY antibodies to prevent *P. aeruginosa* infections by blocking the ability of the bacteria to adhere to the epithelium in the oropharynx and thereby prevent the bacteria from entering the lower airways [66,120]. For *in vivo* research Kolberg *et al.* developed *P. aeruginosa* specific avian IgY antibody by immunizing white leghorn hens with formaldehyde-fixed *P. aeruginosa* [120]. Eggs were collected from immunized hens and IgY was isolated from the egg yolks. The anti- *P. aeruginosa* IgY was then prepared in such a way that CF patients could use it as a mouth rinse. Experimental group CF patients were instructed to gargle with the IgY mouth rinse for two minutes and then swallow it every evening after their final meal and brushing teeth. The goals of this *in vivo* study were two-fold. First, to see if daily use of anti- *P. aeruginosa* IgY could prolong the length of time between the first and second *P. aeruginosa* colonization in patients that are not yet chronically infections. The second goal of this research was centered on long-term treatment and if continuous use of the anti- *P. aeruginosa* IgY mouth rinse could decrease the intermittent colonization rate and prevent chronic infection, ultimately decreasing the need for antibiotics. Based on results from sputum

samples collected from CF patients throughout the study, the amount of time between the start of treatment and the next *P. aeruginosa* positive culture was longer in patients treated with anti- *P. aeruginosa* IgY than the control treatment. Though the trend was obvious, the difference was not significant. In long-term study, the 10 CF patients in the experimental group participated for an average of 57 months and the 21 control group CF patients participated for an average of 36 months. There was a total of only 14 positive cultures in the anti- *P. aeruginosa* IgY treatment group out of a total 315 (4.4%) cultured that were positive for *P. aeruginosa.* In comparison, the control group patients had 105 positive cultures out of the 315 collected during the study (18.7%) [120]. Also, none of the 13 patients in the experimental group became chronically colonized with *P. aeruginosa*, while 5 of the patients in the control group did become chronically colonized [120].

A similar experimental design was followed in *in vivo* studies completed by Nilsson *et al*. in 2008 to determine the efficacy and adverse reactions of long-term treatment with anti- *P. aeruginosa* IgY [108]. Results were similar, chronic colonization occurred in only 2 of the 17 CF patients treated with anti-*P. aeruginosa* IgY, but in 7 of the 23 CF control treatment CF patients [108]. The median time to reinfection after treatment in the control treatment group was 19 months and in the anti- *P. aeruginosa* IgY treated group it was 25 months [108]. These results support the use of anti- *P. aeruginosa* IgY as a means to decrease the number of *P. aeruginosa* infections over a given length of time and to prevent chronic *P. aeruginosa* infections.

Additional studies were completed to gain more insight into the mechanism that leads to the decrease in colonization reported after treatment with anti-*P. aeruginosa* IgY.

It was determined that the anti - *P. aeruginosa* specific IgY binds to flagellin present on the surface of the bacteria, which may prevent *P. aeruginosa* in CF patients by hindering host infections due to reduced adherence. The avian IgY antibodies may be able to directly affect adherence or indirectly reduce the bacteria's motility [121].

Streptococcus mutans

The mircobiota within the oral cavity is important in host defense, acting as a barrier by creating unfavorable conditions for exogenous organisms that might be pathogenic to the host. Under the right conditions, some of the bacteria present in the mouth can cause infections, including dental caries, which are most often caused by *Streptococcus mutans* (*S. mutans*). Dental caries result from interactions between the host, diet, and the microflora on tooth surface [122]. Virulence factors of *S. mutans* include adherence to the enamel surfaces of the tooth, production of acidic metabolites, and the ability to synthesize extracellular polysaccharides [123,124]. Specifically, glucosyltransferases (gtf) have been shown to be one of the major virulence factors in the pathogenesis of dental caries [125,126].

Passive treatments administered topically [127,128], by local injection [129-131], intranasally [132,133], and orally [134-138], specifc for S. *mutans* have all been previously tried with varying degrees of success in both animal models and human trials. The most direct evidence supporting the potential for *S. mutans* specific avian IgY antibodies derives from the research of Michalek *et al*. and Filler *et al* [134,136]. Michalek *et al.* was able to show in an experimental animal model of dental caries that oral administration of *S. mutans*- specific bovine milk antibodies was able to reduce caries. In humans it was reported that those who used a mouth rinse with *S. mutans*

bovine milk antibodies had an initial decrease in the number of recoverable *S.mutans* [134].

To test the potential success of an avian IgY antibody passive treatment for dental carries caused by *S. mutans*, Hatta *et al.* isolated avian IgY antibodies specific for the whole bacteria. The *S. mutans* specific IgY was formulated into a mouth rinse to be used by volunteers as a way to prevent *S. mutans* colonization in the mouth. Levels of *S. mutans* decreased in volunteers who gargled with the *S. mutans* specific IgY mouth rinse [139]. The presence of antibodies in the volunteers correlated with protection against caries formation. It is likely that the polyclonal *S. mutans* specific IgY in the mouth rinse had antibodies specific to the insoluble glucans covering the surface of the bacteria, and less to the serotype-specific antigenic sites on the surface of *S. mutans*, Thus allowing the potential to interact with the various seroptyes of the mutans streptococci that can exist in human saliva and plaque [139].

Kruger *et al.* targeted the gtf virulence factor of *S. mutans* by developing anti-cell-associated (CA) gtf IgY antibodies from the egg yolk of chickens immunized with gtf. Gtf is essential for the production of glucans by *S. mutans* and an important virulence factor. *S. mutans* with defective glucan function induce markedly reduced levels of smooth and sulcal surface carious lesions in rats [122]. These *S. mutans*- specific chicken-derived anti-gtf antibodies were tested in desalivated rats to mimic clinical caries situations in patients with reduced salivation (dry mouth) due to medication, Sjogren's syndrome, or other causes. Desalivated rats given drinking water containing the anti-CA-Gtf-IgY had significantly lower development of smooth surface and sulcal caries lesions compared to rats given IgY control water and sterile water.

Helicobacter pylori

Helicobacter pylori (*H. pylori)* infects over 50% of the population worldwide and is the most important etiological agent of gastroduodenal ulcers and malignancies [140]. After oral ingestion of the bacteria, once in the gastrointestinal tract, *H. pylori* has numerous virulence factors that help it to survive and cause disease, including the ability to move via flagella. The bacteria are able to produce a cytotoxin, and have adhesins on their surface to aid in attachment to epithelial cells. The urease enzyme produced by the bacteria is one of the most important virulence factors that aids in the organism's ability to colonize gastroduodenal mucosa [141]. Urease is able to hydrolyze urea into carbon dioxide and ammonia, permitting *H. pylori* to survive in the acidic environment of the gastrointestinal tract [142,143]. *H. pylori* disruption of the epithelial layer in the gastrointestinal tract causes various types of gastritis that can be indicative of further clinical outcomes, including ulcers. *H.pylori* is responsible for the majority of duodenal gastric ulcers. The lifetime risk of peptic ulcer development in persons infected with *H.pylori* ranges from 3% in the United States, to 25% in Japan [144,145]. Eradication of *H. pylori* drastically lowers the recurrence rates of *H. pylori*- associated ulcers [146]. The goal of current *H. pylori* treatment is to eliminate the organism. But, there are several cases where anti-microbial agents do not work efficiently enough to clear the infection and there is a potential for the development of resistant organisms. This leaves the infected individual at risk for the recurrence of ulcers and continued pain. There is still the need for *H. pylori* treatments that can be used alone or in conjunction with current anti-microbial agents.

A novel passive immunization treatment for the prevention and reduction of *H. pylori* infections was created by Horie *et al.,* utilizing current knowledge on the urease virulence factor of the bacteria [140]. The cross-reactivity between the anti *H. pylori* whole cell lysate antibodies, and other bacteria in the human gastrointestinal tract, can decrease the efficacy of the anti IgY *H. pylori* treatment. This fact makes it necessary to develop antibodies that target specific antigens of the bacteria. A *H. pylori* urease-specific polyclonal chicken IgY antibody (IgY-urease) was isolated from the yolks of eggs collected from hens immunized with an aqueous extraction of purified *H. pylori* urease enzyme. A drinking yogurt was formulated containing 1% IgY-urease along with *Lactobacillus acidophilus* and *Bifidobacterium spp.* [140]. The effectiveness of the IgY-urease containing yogurt was tested in human volunteers who were positive for the presence of *H. pylori* as determined by a urease breath test (UBT). The volunteers who drank the yogurt with the IgY-urease had a significant reduction in UBT values compared to controls, meaning the IgY-urease yogurt was effective at suppressing *H. pylori* infection in individuals previously colonized with *H. pylori* [140].

Another avain IgY antibody treatment specific for *H. pylori* has been tested in BALB/c mice. This treatment targets a highly immunoreactive and bacteria specific 58-kDa antigen (Hp58) that was originally identified in the whole cell lysates of *H. pylori* [147]. Polyclonal IgY was generated against the target by the immunization of White Leghorn hens with purified Hp58. Eggs were collected and Hp58- specific IgY was isolated from the egg yolk. IgY from non-immunized hens was used as a control. The reactivity of IgY-HP58 antibody was tested by western blotting. The IgY-Hp58 antibody identified the 58-kDa immunoreactive band in whole cell lysates of *H. pylori* using

western blotting. There was no band present when egg yolk antibodies from non-immunized hens (IgY-N) reacted with cell lysates [147]. To test the effectiveness of the IgY-HP58 antibody against *H.pylori* infection, a mouse model of infection was used. Mice were passively immunized with IgY-Hp58 antibodies at 1 day, 1 week, 4 weeks, or 12 weeks post infection with *H. pylor*i. The highest effect of passive immunization was achieved when the IgY- Hp58 was given 1 week after infection. There was a significant difference in the degree of gastritis at this point, and a higher recovery rate when compared to the IgY-N treated group [147]. Together, these results suggest that the IgY-Hp58 antibody is able to prevent *H. pylori* infection in the mouse model.

Salmonella

Salmonella enterica serovar Enteritidis (*Salmonella* ser. Enteritidis) and *Salmonella enterica* serovar Typhimurium (*Salmonella* ser. Typhimurium) are the most frequent serotypes of *Salmonella* to cause infection in humans and other animal species [148-150]. *Salmonella* is a foodborne pathogen that can cause a variety of clinical manifestations including minor gastroenteritis to a more serious bacteremia that appears in about 8% of untreated cases [151]. *Salmonella* can also persist in the gastrointestinal tract after symptoms have resolved, causing some individuals to become chronic carriers, who are able to continue to spread bacteria unknowingly.

The first reported case of *Salmonella* resistant to a single antibiotic was in the early 1960s [152]. Since then, the chances of isolating a resistant strain has continued to increase. *Salmonella* has many surface components, which are important for the bacteria's virulence and which are ideal targets for passive antibody treatments. Passive protection by egg yolk antibodies targeted against the many *Salmonella* surface

antibodies serve as a potential method for the control of intestinal colonization by *Salmonella*. One such surface antigen on *Salmonella ser*. Enteritidis, involved in the pathogenesis of the bacteria, is the fimbria. *Salmonella* ser. Enteritidis has been shown to produce three types of fimbria, referred to as SEF 14, 21, and 17 [153-155], which are correlated with bacterial virulence [156-159], and bacterial adherence to mucosal epithelium.

To better understand the role of fimbriae in *Salmonella* ser. Enteritidis pathogenicity, and the potential for protection of egg yolk IgY antibodies, SEF-14 specific IgY antibodies were isolated from the egg yolk of chickens immunized with purified SEF-14. Western blotting confirmed the specificity of the SEF-14 IgY antibodies by the presence of a single immunoreactive band at 14-kDa, after the membrane-bound surface proteins of *Salmonella* ser. Enteritidis were electrophoretically transferred to a nitrocellulose membrane [160]. To evaluate the clinical potential of SEF-14 IgY antibodies, mice were challenged with *Salmonella* ser. Enteritidis and treated with the SEF-14 IgY antibody. The SEF-14 IgY antibody dose dependently increased the survival rate of mice treated after challenge with *Salmonella* ser. Enteritidis, compared to control mice treated with avain IgY antibody from non-immunized hens. Mice treated with the highest titer of SEF-14 IgY antibody had the greatest survival rate at 77%. The lower antibody titer treatment group had a 59.3% survival rate, and the control group had a 32% survival rate [160]. The difference in survival rate between the high antibody titer treatment group and the control group was significant [160]. The *Salmonella* ser. Enteritidis infected mice did not develop any overt symptoms of diarrheal disease, but in the mice that had the infection, the challenge bacterial strain was isolated from the liver,

spleen, kidney, and small intestines. In mice that survived infection because of the antibody treatment, only a few harbored any bacteria in the organs listed above.

In vitro, the SEF-14 IgY antibodies were able to reduce the adherence of *Salmonella* ser. Enteritidis to isolated murine small intestinal cells, compared to control antibodies. The exact mechanism that allows the SEF-14 IgY antibody to increase survival is unknown; the *in vitro* results and lack of bacteria in the organs after antibody treatment, point towards the SEF-14 IgY antibodies being able to inhibit the initial attachment of the bacterial to cell surfaces, and, potentially, the next steps of tissue invasion. The prevention of bacterial adhesion is a phenomenon that has been observed by others studying the effects of *Salmonella* specific IgY antibodies. *In vitro*, IgY antibodies specific for the LPS of *Salmonella* ser. Typhimurium and *Salmonella* ser. Enteritidis have been reported to inhibit *Salmonella* adhesion and to prevent disease [161]. Chalgohoumi *et al.* created IgY antibodies specific for the outer membrane (OMP) of *Salmonella* ser. Typhimurium and *Salmonella* ser. Enteritidis. The OMP-specific IgY was able to prevent *Salmonella* adhesion in a concentration dependent manner, along with inhibiting bacterial growth [162].

Yokoyama *et al.* determined the treatment potential of avian IgY antibodies specific for the OMP, lipopolysaccharide (LPS), and flagella (fla) of *Salmonella* ser. Enteritidis and *Salmonella* ser. Typhimurium[150]. The OMPs are exposed on the surface of the bacterial cells where they can easily interact with antibodies [163]. There is increasing evidence that motility facilitated by the flagella increases the probability the bacteria will reach a suitable site for invasion, an important part of causing an infection for the bacteria [164,165]. The importance of these three cellular structures in bacterial

pathogenesis and their surface exposure makes them ideal targets for antibody treatments. Avian IgY antibodies specific to the OMP, LPS, and Fla of both *Salmonella* ser. Enteritidis and *Salmonella* ser. Tyhimurium were made by immunization of five month old White leghorn chickens with OMP, LPS, or FLA from the two bacteria strains. Avian IgY antibodies specific to the six antigen targets were then isolated from the eggs of immunized chickens. Control antibodies were isolated from the eggs of non-immunized chickens to test the efficacy of the *Salmonella* specific IgY. Mice were challenged with either *Salmonella* ser. Enteritidis or *Salmonella* ser. Typhimurium and then orally treated with either OMP-, LPS-, or Fla- IgY specific to the bacteria they were challgenged with. The antibody treatment continued three times a day for three consecutive days. For mice challenged with *Salmonella* ser. Enteritidis treatment with OMP-IgY resulted in 80% surivial, 47% survival with LPS-IgY, and 60% survival with Fla-IgY; survival for the control IgY treated mice was only 20% [150]. The difference in survival between the OMP-IgY treated group and the control group was significant. There was a similar trend in results for the mice challenged with *Salmonella* ser. Typhimurium. The group of mice treated with OMP-IgY had 40% survival, LPS- IgY 30% survival, and Fla- IgY 20% survival; all of the mice in the control group died [150].

Lee *et al*. developed avian IgY antibodies specific for *Salmonella* ser. Enteriditis and *Salmonella* ser. Typhimurium, but the aim of their research was to gain a better understanding of IgY antibody specificity and how the IgY antibody interacts with the bacteria. The IgY antibodies used in their research was specific for whole cell bacteria. There was cross reactivity between the two antibodies, but ultimately both types of *Salmonella*-specific IgY antibodies were found to inhibit growth of homologous

Salmonella in liquid medium [54]. They were unable to find out the exact mechanism of growth inhibition, but there were structural changes to the surface of *Salmonella* where IgY bound [56]. The above mentioned research in the area of avian IgY antibody treatments for *Salmonella* has the potential to be transferred into humans to be used clinically to treat salmonellosis caused by food poisoning.

Escherichia coli

Enterotoxigenic *Escherichia coli* (ETEC) causes enteric colibacillosis, severe watery diarrhea, in newborn (suckling) calves, and suckling as well as weaned pigs, but is rare and essentially non-existent as a cause of illness in other farm animals. In humans ETEC is recognized as one of the most frequent causes of childhood diarrhea in developing countries and an important causative agent of traveler's diarrhea [166,167]. The similarities in prevalence and severity of disease between humans and animal cases of ETEC provide researchers a chance to understand human ETEC infection through the use of animal models. ETEC bacteria are known to adhere to the small intestinal epithelium without inducing significant morphological changes and secrete enterotoxins that alter the functions of enterocytes by increasing secretion and reducing absorption [168]. Because of the importance of adhesion in the pathogenic process of ETEC, adhesins on the surface of fimbriae or pili are essential virulence factors. The fimbriae of animal ETEC are K88, K99, K987P, Fy.Att25, F41, and F18 [169]. Potentially pathogenic *E. coli* are found in the intestinal tract and feces of many healthy animals. Continuous birthing and poor sanitary conditions often lead to ETEC infections in offspring. Infectious ETEC on the skin and mammary glands of mothers can easily be ingested by offspring.

Treatment of ETEC infections can start with prevention of the infection all together, by building up the immunity of adult female cows and pigs through vaccination. If it is necessary to treat ETEC- infected offspring, antimicrobial treatments are often used. Usually, if one offspring in the litter is infected all in the litter will have to be treated. Because of which, the chances of the infection spreading through the herd are high.

Avian IgY antibody treatments of ETEC infections in animals are an attractive option because it would reduce the use of antibiotic treatments in livestock. An avian IgY antibody treatment would only persist in the animal long enough to help clear the infection, and would not have any long term effects in pigs or calves used as a food source. Oral administration of antibodies derived from serum and colostrum and even monoclonal antibodies have been very successful treatments; however, it is expensive to obtain the large amounts of antibodies required [170]. A cost effective source of antibodies to treat ETEC is avian IgY antibodies, specifically egg yolk antibodies from immunized chickens. There are several researchers that have used animal models of ETEC infection to evaluate the potential of ETEC- specific IgY antibodies. A main target for these IgY antibodies are the bacteria's adhesins most likely due to the role they play in bacterial pathogenesis. Yokoyama *et al.* used a porcine model of ETEC infection to assess the treatment potential of avian IgY antibodies specific for fimbriae adhesins K88, K99, and K987P. The adhesion- specific antibodies were isolated from the egg yolks of *E.coli* frimbrial immunized chickens. *In vitro* the presence of anti-adhesin IgY prevented the adhererence of K88+, K99+, and K987P+ *E.coli* strains to isolated procine small intestine epithelial cells [171]. There was no cross-inhibition between the three

types of antibodies used. Similar results were obtained *in vivo* in control piglets. In control animals, adhering *E.coli* could be observed along with whole length of the small intestines, whereas there was no adherent *E.coli* along the intestinal epithelial surface of piglets treated with *E.coli* fimbriae- specific IgY [171].

In another experimental design, Marquardt *et al.* isolated polyclonal IgY antibodies from the egg yolks of chickens immunized with purified K88 fimbrial antigen from a local strain of ETEC (*E.coli* K88+ MB, Manitboa, Canada)[172]. The egg yolk antibodies were freeze dried and made into a powder to be tested in a porcine model of ETEC. Piglets or neonatal pigs were challenged with K88+ MB orally and then orally treated with K88 MB- specific IgY antibodies or control non-specific IgY antibody powder at various time points post-challenge. K88 MB- specific IgY antibody treatment was able to protect both neonatal and 21 day old weaned piglets from diarrhea induced by the challenge. In piglets challenged and treated with K88 MB specific IgY antibodies, diarrhea was cured within 24 hours after treatment, whereas the piglets treated with the control IgY antibodies continued to have diarrhea during the entire duration of the experiment, resulting in 62.5% death [172]. Not only was the occurrence of diarrhea decreased in the neonates and piglets treated with K88 MB- specific IgY antibodies, but mortality was also significantly decreased.

Further research using the K88 MB specific IgY antibody was completed, studying the potential of the antibody to inhibit *in vitro* adhesion of K88+ *E.coli* to piglet intestinal mucus. The effectiveness of the chicken IgY antibodies for inhibiting adhesion of ETEC to mucus in this study was influenced by two factors: dose of antibody, and concentration of ETEC. K88 MB- specific IgY antibody, when diluted 50-100 fold, was

able to strongly inhibit adhesion of K88+ *E.coli* at a concetnraion of 10^9 CFU mL^{-1} (adhesion was <6%) [173]. However, if the antibody was diluted 100 times it was unable to prevent adhesion of *E.coli*. These results correspond with the previous dose-dependent results of Marquardt *et al.* and others. There were limits to what the K88 MB- specific IgY antibodies were capable of; the antibodies were unable to displace K88+ MB *E.coli* once it was already bound to the receptor in the mucus. Also, when the IgY antibodies and K88+ MB *E.coli* were incubated together, prior to exposure to immobilized mucus, there was no difference in inhibition of adherence between K88 MB- specific IgY antibodies and control IgY antibodies [173]. For this specific application of the IgY antibody a prophylactic treatment approach would probably be more advantageous.

As was previously mentioned, ETEC is a major cause of diarrhea and death in neonatal calves [174-176]. The K99 pilus is one of the adherence factors found on ETEC isolated from neonatal calves [177-180]. As a potential alternative to current treatment methods for ETEC in calves, Ikemori *et al*. developed a pilus- specific IgY antibody powder[180]. Newborn colostrum-fed calves were used as the model of ETEC infection [180]. Calves were challenged with ETEC and then treated with either pilus- specific IgY antibody powder in milk, or non-specific IgY antibody powder in milk at varying titers. Calves in the control group developed severe diarrhea and died on day three post challenge. The opposite was true of calves treated with pilus IgY antibody powder. The calves in the higher titer groups (1:800 and 1:1600) all recovered from disease; diarrhea was temporary, and was not accompanied by dehydration or weight loss [180]. Calves in the lowest antibody titer group (1:200) did experience diarrhea and weight loss. These results again support the dose-dependent effects of ETEC specific IgY antibodies.

Rotavirus

Rotavirus is a key enteric pathogen in infants and young children. Each year rotavirus causes millions of cases of diarrhea in children. Almost 2 million cases will end up in the hospital [181], and an estimated 453,000 will result in the death of children under the age of 5 [182], 85% of whom live in a developing country [183]. Recently two vaccines have been approved for use against rotavirus. One downfall is the vaccines' low protection efficacy in children from African and Asian countries [184,185], the two countries that contain areas where children are at high risk for infection. Vaccination is an effective method of controlling disease in developed countries because of well-established health systems. But, in developing countries, the outcome is much different. Approximately 1.76 million children die each year from gastroenteritis in developing countries [186], many of which are due to rotavirus [183], as indicated above. In addition to vaccines against rotavirus there needs to be an easy-to-administer and cost-effective treatment for rotavirus. An antibody-based passive treatment is an ideal alternative approach to prevent and treat rotavirus infections.

Several *in vitro* studies have proven the potential use of rotavirus specific IgY antibodies for treatment of rotavirus infections [187-190]. In the second human trial, children with proven rotavirus diarrhea were treated with anti-rotavirus IgY antibiodies isolated from the eggs of human rotavirus immunized hens. There was a trend towards lower daily and cumulative stool output, compared to children treated with control IgY antibodies, however the daily and cumulative stool output difference was not significant. Probability of clearance of rotavirus from stool of children treated with anti-rotavirus IgY antibodies was significantly higher compared to the control treated children. At day four

post-treatment, 74% of children treated with anti-rotavirus IgY antibodies no longer had detectable rotavirus in their stool, while more than 50% of children in the control IgY antibody treatment group still had rotavirus in their stool at this point [191]. One explanation for only one significant set of data in this research could be the relatively low dose of anti-rotavirus IgY antibodies that was used.

To gain insight into the dose of the efficacy of anti-rotavirus IgY antibodies, an animal model of rotavirus infection was used, which allowed for higher doses of anti-rotavirus IgY antibodies to be given. The same anti-rotavirus IgY antibodies that were used in the clinical trials by Sarker *et al.* were also used in these experiments [191]. Four day old mouse pups were challenged with Rhesus rotavirus. Twenty-four hours after challenge, mice were treated with one of the four following doses of anti-rotavirus IgY antibodies: 0.01 mg/mL 0.1mg/mL, 1.0 mg/mL, or 10.0 mg/mL. A significant difference in prevalence of diarrhea was reported for mice treated with doses of 1.0 or 10.0 mg/mL of anti-rotavirus IgY antibodies compared to control, 33% vs. 67% and 6% vs 67%, respectively [192]. At lower doses there was no significant difference for this parameter. The duration of diarrhea was reduced with anti-rotavirus IgY antibody treatment, and, again, larger doses caused the strongest reduction compared to the control, with a significant difference [192]. These results support the results of other studies: treatment with anti-rotavirus IgY antibody is dose dependent. It is also possible, based on these results, that using a higher dose of anti-rotavirus IgY antibody in the previous clinical trial would have given a better outcome, especially considering the highest dose provided the best protection.

Venoms

Envenomation, due to snakebites and other venomous animals, is an often a neglected public health issue affecting many individuals in underdeveloped countries, specifically rural and tropical Africa, Asia, and Latin America [193]. While true, individuals in developed countries are most at risk for envenomation during recreational activities. A recent study estimates that at least 421,000 envenomings, and 20,000 deaths occur worldwide from snakebites each year [193], and an estimated 1.2 million scorpion stings occur each year, leading to 3,250 deaths [194]. Scorpion and snake venoms are a complex mixture of peptides, enzymes, and other factors that cause a wide variety of physiological reactions. Hyaluronidase, for example, is an enzyme present in all snake venoms that aids in the spread of other venom components throughout the prey's tissue [195]. Snake and scorpion anti-venoms are most often derived from horse sera. Purified anti-venoms often contain large amounts of non-specific serum proteins that can cause side effects, some of which can be severe [196-198]. Side effects can range from serum sickness, occurring in up to 80% of all patients treated with the current North American rattlesnake anti-venoms [199,200], to the more severe anaphylactic reactions. These reactions are in part due to the impurity of the anti-venoms and the reactivity of the anti-venom immunoglobulins with human immune cells [199,201]. Treatment is further complicated by the many unknowns involved, such as the amount of venom injected by each poisonous animal. The lack of standardization in venom causes the amount of active ingredients in commercially available North American anti-venom treatments to vary. In an attempt to circumvent the unwanted reactions between anti-venom immunoglobulins and components of the human immune system, avian IgY- specific antibodies for snake venom were developed.

Some of the first anti-venom IgY antibodies were made by immunizing chickens with either a mixture of Cortalid snake venom or *Leiurus quinquestiratus hebraeus* scorpion venom. Anti-venom IgY antibody was isolated from the egg yolks of the immunized hens with each respective venom and affinity purified. The neutralizing potential of the anti-venom IgY antibodies against both types of venoms was tested by mixing the anti-venom IgY antibodies with the minimum lethal dose of each venom, respectively, and injected into mice; the mice were then monitored for death. All ten mice survived the 24 hour observation period when treated with the anti-Cortalid IgY antibodies and only one out of nine mice survived without the anti-venom IgY antibodies [202]. Results were similar for the anti- *Leiurus quinquestiratus hebraeus* IgY antibodies. When mice were injected with *Leiurus quinquestiratus hebraeus* venom pre-incubated with anti-venom IgY antibodies, seven out of eight mice survived, and only one out of six mice survived injection without anti-venom IgY antibodies [202]. Both the snake and scorpion anti-venom IgY antibodies were able to neutralize the toxins present in their respective venoms.

Similarly, IgY antibodies specific for the venom of snakes within the *Bothrops* and *Crotalus* genera were developed through immunization of chickens with mixtures of venoms from snakes within each genus. Venom- specific IgY was present in the serum of hens just two weeks after immunization. Eggs were collected from the immunized hens and IgY isolated from the yolks of the eggs that was snake venom specific. One snake venom component is phospholipase A_2 (PLA_2), which causes inflammation. This is caused by liberation of archadonic acid, a precursor of prostaglandins and leukotrienes from the cell membranes [203]. *In vitro*, anti-venom IgY specific for venom from both

genera of snakes was able to inhibit the activity of PLA_2 in a dose- dependent manner [204]. Both venom- specific IgY were also able to neutralize the lethal toxicity of the venom as assessed by an *in vitro-in vivo* assay [204].

In two independent studies, Meenatchisundaram *et al*. studied the neutralizing ability of venom- specific IgY antibodies against the venom from four different snakes that are a common cause of human envenomation; the *Naja naja* (cobra), *Bungarus caeruleus* (krait), *Vipera ruseeli* (Russell's viper), and *Echis carinatus* (Saw-scaled viper) [205,206]. The neutralizing ability of the venom- specific IgY antibody was assessed through comparison of PLA_2 activity, edema formation, and procoagulant activity in anti-venom IgY antibody treated and control treated groups of mice. Anti-venom IgY antibodies made against all four venoms inhibited PLA_2 activity, decreased edema formation, and neutralized procoagulant activity compared to controls [205,206]. These results support the possible neutralizing potential that these anti-venom IgY antibodies have in future *in vivo* studies and in humans.

Others

Avian IgY antibodies have been developed against other targets such as the cytokines IL-1β and TNF. Allergic asthma has been shown to be successfully treated with mammalian antibodies specific for the cytokine TNF-α [207-209]. However, further studies with the anti-TNF-α antibodies identified the unfavorable risks associated with long-term use of these treatments [210]. Another cytokine, IL-1β, is an important proinflammatory cytokine involved in the progression of allergic asthma, contributing to the formation of inflammatory responses within the airway [211]. Targeting of IL-1β by anti-IL-1β IgY represents an alternative treatment option for allergic asthma.

Wei-xu *et al*. used a hamster model of allergic asthma induced by ovalbumin (OVA) to test anti-IL-1β IgY antibodies isolated from the eggs of laying hens immunized with human recombinant IL-1β [212]. Allergic asthma hamsters were treated with either saline alone as a control, anti-IL-1β IgY antibodies, or budesonide as a positive control. There was decreased tissue damage in the lungs of hamsters treated with anti-IL-1β IgY antibodies compared to control. There was no significant difference in the pathological damage between the animals treated with budesonide and those treated with anti-IL-1β IgY antibodies [212]. Along with preventing tissue damage, there was a significant decrease in the numbers of eosinophils, neutrophils, and lymphocytes along with a reduction in IL-1β, IL-4, IL-8, IL-13, TNF-α, TGF-β, and IgE in the peripheral blood as well as bronchial alveolar lavage fluid [212].

IgY specific TNF-α antibodies have been developed for treatment of Chron's disease and ulcerative colitis as a potential alternative to current treatments. Current treatments include immunosuppressants, corticosteroids, and antibiotics, all of which come with limitations and side effects that can limit the efficacy of the treatments [213]. TNF-α is a key early mediator in these two diseases along with many others [214]. Other drugs have been made against TNF-α, but they can be costly, result in multiple trips to the medical provider for patients, and have adverse side effects. Anti-TNF IgY antibodies were developed and tested in a rodent model of inflammatory bowel (IBD) as a way to better treat TNF-α influenced diseases. The anti-TNF IgY was able to effectively treat IBD in mice when administered both prophylactically and therapeutically, and was effective against both the acute and chronic phase of colitis in the animal model [215].

West Nile Virus

Previous research in the Bradley lab at the University of North Dakota has been done with the immune serum from geese and purified goose IgY antibodies. Initial studies were done to determine if serum from West Nile Virus (WNV) immune geese could prevent or treat infection. Geese were treated with immune serum either prior to WNV infection or after WNV Infection. There were approximately 10,000 geese in each study, which showed a marked decrease in the mortality for both groups. More specifically, there was approximately a 65% reduction in mortality when used as a pretreatment and an approximate 62% reduction in mortality when used therapeutically (unpublished results Schiltz, Petell, and Bradley). Similar results were seen with purified WNV- specific goose IgY antibodies.

The effectiveness of the WNV-specific IgY antibodies were then tested during WNV infection in WNV-infected Golden hamsters. WNV-specific IgY antibody treatment resulted in both mortality and viral titers of zero. Comparatively, sham treated hamsters had a mortality rate of 100% and 65% (unpublished results Schiltz, Petell, and Bradley). This research demonstrated that the goose antibodies were able to protect mammalian subjects against infection.

Overview of IgY

In summary, avian IgY antibodies are functionally similar to mammalian IgG homologs, but have sufficiently distinct structural characteristics. This difference is what makes the use of avian IgY antibodies an attractive alternative for passive immunizations for mammals, specifically, in various diseases and infections in humans. Discussed below is our research focused on investigating the ability of goose IgY antibodies to

ameliorate two human pathogens: Dengue virus type 2 and Andes virus. Andes virus-specific goose IgY antibody treatments that were developed include antibodies that recognize unique epitopes in comparison to virus specific mammalian IgG antibodies. The structural differences in IgY antibodies, compared to mammalian IgG, results in IgY antibodies inducing less of an inflammatory response than what can be detected with mammalian IgG antibody therapies. Discussed next are the general safety studies demonstrating the potential to utilize avian IgY antibodies in therapeutic application for mammalian disease.

CHAPTER II

DEVELOPMENT OF GOOSE MONOCLONAL IGY ANTIBODIES FOR TREATMENT OF DENGUE VIRUS TYPE 2 INFECTIONS

Introduction

History of Dengue Virus in Humans

The World Health Organization estimates that 50 to 200 million dengue virus (DENV) infections occur each year [216]. However, newer research estimates these numbers to be closer to 390 million cases per year, 96 million of which will lead to clinical manifestations of varying severity [217]. DENV is currently regarded as the most important arboviral disease internationally because over 50% of the world's population live in areas where they are at risk of infection, and approximately 50% of people live in DENV endemic countries [218-222].

DENV is a member of the *Flaviviridae* family of RNA viruses. There are four antigentically distinct serotypes of the DENV (DENV-1, DENV-2, DENV-3, and DENV-4) differing at the amino acid level by 25-40% [223]. Genetic studies of the four DENV have provided evidence that the four viruses evolved from a common ancestor that circulated between non-human primates and mosquitoes, approximately 500 years ago, with all four virus serotypes emerging separately into a human urban transmission cycle [224]. DENV is primarily transmitted to humans by mosquitoes from the genus *Aedes*, primarily the *Aedes aegypti* (*A. aegypti*) species, and also the *Aedes albopictus* species in

some geographical areas. Mosquitoes become infected after taking a blood meal from an infected human. After the blood meal, the virus travels into the mosquitos' midgut and continues on to infect other tissues of the mosquito. Ultimately, to infect humans, the virus needs to travel to the salivary glands of the mosquito and be shed in the saliva of the mosquito while taking a blood meal [225]. The *A. aegypti* mosquito is a very efficient vector; it has a high affinity for human blood, is adapted to urban living, and it likes to breed in and around houses in water containing vessels [226].

The geographical spread of the virus is primarily in tropical and subtropical countries currenlty. DENV disease awareness increased after the end of World War II, but the disease has a much longer history of interaction with humans dating back to China between 265-420 A.D. [227]. The first two modern cases of DENV infection led to the isolation of DENV-1 in 1943, from Japan and DENV-2 in 1945, from Hawaii [228]. After these two cases, epidemics of DENV began to spread to more of the tropical regions of the world. This was facilitated by the spread of the mosquito at the end of World War II and was accelerated by urbanization and globalization; including increasing international travel not only of people, but also goods in which mosquitoes harboring the virus can survive [218]. The increased prevalence of DENV was further facilitated by inadequate housing, poor water systems, and improper sewer and waste management systems causing *A. Aegypti* levels to reach increasingly high densities.

In the 1960s, there was a dramatic surge in DENV activity in many tropical locations throughout the world [229-232]. Increased disease severity was associated with these epidemics due to the overlapping spread of the four DENV serotypes, referred to as hyperendemicity [233-235]. Research in hyperendemic areas looking at secondary

heterotypic DENV infections led to the development of the antibody dependent enhancement (ADE) theory of pathogenesis [236]. In the 1970s, only nine countries had reported severe DENV cases; that number has since quadrupled [221]. This is, in part, due to multiple DENV serotypes circulating in the same geographic area. Until the 1980s, a majority of the endemic areas had only reported one or two types of DENV. Now all four DENV serotypes are circulating in Asia, Africa, and the Americas [227,237].

As this brief history of DENV demonstrates, the spread of the virus is ultimately connected to the spread of its mosquito vector, which is in turn influenced by changes in the climate, globalization, and travel. Moving into the future these variables will continue to change and so will the geographic spread of the virus into non-endemic areas and, as the four DENV serotypes continue to circulate, more hyperendemic areas will be created. All of which will contine to increase the chances of secondary heterotypic infections increasing patient's chances of severe DENV infection.

Spread of DENV to the Americas was delayed by an *A. aegypti* mosquito control campaign initiated by the Pan American Health Organization. The primary goal of the program was to control the spread of yellow fever, but also happened to control the spread of DENV. This prevented the spread of DENV to the Americas for 20 years, until the program was discontinued in the late 1970s [218,226,238]. In the 1980s, the number of DENV cases began to rebound and by 1995 the *A. aegypti* mosquito was reintroduced into the Americas, and its geographic distribution spread to include Asia, and continues to spread to this day [222,226]. The first indigenous case of DENV in the United States since 1945, was reported in Brownsville, Texas in 1980. DENV-1 was isolated from a 5-

year-old girl that did not have a history of traveling outside of Brownsville. An additional 63 DENV-1 cases were confirmed in Texas in 1980, 52 of these cases were in counties located near the Mexico-Texas boarder [239]. More recently in 2005, autochthonous DHF was reported in a woman in Texas, and 25 additional cases of DENV infections were reported in Brownsville, Texas, of which at least three were locally acquired [240]. Between 2009 and 2012, a total of 103 autochthonous DENV cases were reported in Florida [241,242]. Testing of mosquitoes in the area detected the presence of DENV-1 in *A. aegypti* mosquitoes [243]. Previously, people living in the United States only have to be concerned about DENV infections, when traveling to areas where the virus was endemic, but now the mosquito vector has spread such that infections of DENV can be acquired within the continental United States.

DENV Structure

DENV is a small, enveloped virus with a single-stranded, positive-polarity RNA genome of 10.7kb [244]. The 10.7kb genome is translated as a single polyprotein and then cleaved into three structural proteins (capsid [C], premembrane/membrane [prM/M], and envelope [E]) and seven nonstructural (NS) proteins, (NS1, 2A, 2B, 3, 4A, 4B and 5) by host and viral proteases [245-247]. DENV primarily infects cells of the monocyte-macrophage lineage, liver, and endothelial cell linings of blood vessels [248-250]. The first cells to be infected with DENV are typically tissue resident Langerhans cells in the skin, due to their close proximity to the mosquito bite and virus entering the host. E protein on the surface of the virus interacts with receptors on the host cell surface. It is unknown which receptor(s) on host cells facilitate the entry of DENV; one possible receptor is DC-SIGN [251]. Once in the cell, the acidic environment of the endosome

triggers a structural change in the E protein, which results in fusion of the viral, and cell membranes [252,253]. The virus nucleocapsid is released into the cell cytoplasm, the viral RNA and the capsid protein can then dissociate. Initially the viral RNA genome is translated into a single polyprotein that is then co- and post-translationally cleaved into the appropriate proteins. During translation, structural proteins are translocated and anchored in the ER by various signal sequences and membrane anchor domains. After appropriate cleavages the capsid protein and viral RNA localize in the cytoplasm, the capsid protein remains associated with ER, and the prM and E proteins form heterodimers on the lumenal side of the ER [254-256]. After the initial round of translation, viral replication begins.

Replication is carried out by the NS5 protein, which functions as a RNA dependent, RNA polymerase along with help from a viral helicase and other unidentified proteins [257]. At the start of replication the positive stranded RNA genome is first transcribed into a complementary negative-strand RNA, this results in a double-stranded RNA (dsRNA) intermediate [258]. The negative strand of the dsRNA continues on to serve as the template for any positive-strand RNA that is made. Viral RNA synthesis is asymmetric, meaning ten times more positive-strand RNA is made than negative-strand. Positive-strand RNA can serve as the viral genome or enter another translation cycle, while all negative-strand RNA exists only as part of the dsRNA intermediate [259]. Viral components then come together for assembly of viral particles. Initially, immature virus particles are assembled in the lumen of the ER. These viral particles are immature because they are unable to facilitate host-cell membrane fusion due to the fact that the surface of the virus has an icosahedral arrangement with 60 trimeric spikes, each made up

of prM heterodimer with the prM protein positioned at the tip of each E protein trimer [246,260-262]. The immature virus particles are transported through the trans-Golgi network where they will mature into infectious virions. In the low pH of the trans-Golgi network the pr peptide is cleaved from the M protein by the host protease furin. The 60 timers of prM-E then dissociate to reform as 90 anti-parallel E protein homodimers which lie flat on the surface of the mature virion [245,252,263,264]. The mature virion is then released from the host cell by exocytosis [252].

DENV Immunopathogenesis

The pathogenesis of DENV relies on the interplay between the virus and host factors that result in a variety of clinical manifestations. Clinical disease caused by DENV infections can range from an asymptomatic infection in some individuals, to mild dengue fever (DF), or the more severe dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS). Most primary DENV infections in patients over the age of one year old manifest as either asymptomatic or DF. During the primary DENV infection, lifelong immunity is established in the individual against the infecting DENV serotype [265,266]. During subsequent infections with heterotypic DENV serotypes individuals are at a 15-80 times greater risk for developing the more severe DHF; 90% of all cases of DHF occur during secondary heterotypic DENV infections [267]. The increase in severity of the secondary infection is dependent, in part, on ADE [227,268-270].

ADE is caused by cross-reactive or sub-neutralizing antibodies binding to the virus, forming immune complexes. The complexes utilize their Fcγ receptor (FcγR) binding to bind cells, facilitating virus entry into cells and triggering inflammation and associated shock-like symptoms [267,269,271,272]. There are a small number of severe

DHF cases in primary DENV infections, most of which are often transmitted to infants born to DENV-immune mothers [273]. It is thought that children born to DENV-immune mothers are protected against DENV by antibodies transferred from the mother during the first three to four months after birth. Following those first few months, the neutralizing ability of the antibodies declines and become non-neutralizing, which actually enhancing the chances of infection until about the age of one. At age one, the maternal antibody levels are cleared from the child and no longer pose a risk [227,274-276].

The majority of DENV infections are asymptomatic, but for those who develop a symptomatic infection, the incubation period can range from three to fourteen days, and typically span four to seven days [265,277-280]. The majority of individuals with symptomatic DENV infections develop DF, which most commonly presents as a sudden onset of fever lasting two to seven days. Accompanying the fever is a severe headache, pain behind the eyes, generalized myalgia and arthralgia, rash, anorexia, abdominal pain, and nausea [281,282]. DF is typically milder in younger children compared with older children and adults [268,273,280]. Common laboratory abnormalities seen in DF patients are leukopenia, thrombocytopenia, and elevation in serum transaminases [273,283-285]. Most patients recover from DF fully without any lasting chronic symptoms or complications within a week after the onset of disease [286]. Some atypical clinical features of DENV infections include encephalitis, myocarditis, hepatitis, pancreatitis, and acute respiratory distress syndrome. The atypical manifestations are most likely due to disease pathology at different endothelial surfaces [287-291]. The more severe forms of DENV infection, DHF and DSS, begin with the same clinical symptoms as DF, but then

progress to include hemorrhaging and increased vascular permeability due to the viral infection. Viremia in DHF/DSS is generally 10-to-100 fold higher than in DF [292]. As was noted above, DHF/DSS is most often seen in secondary heterotypic DENV infections and is therefore more common in areas where all four DENV serotypes are circulating [221]. Four to five days after the onset of DF-like symptoms, DHF patients usually show a decline in their fever and shortly thereafter the patient's condition begins to deteriorate, starting with a narrowing of the pulse pressure. Patients then typically become hypotensive due to plasma leakage, and there may hemorrhagic manifestation [266]. The DSS stage is entered at the onset of increased vascular permeability, resulting in a loss of plasma fluids into the interstitial spaces, so much so that cardiac output is compromised resulting in hypovolemic shock [293]. The shock stage is short in duration but life threatening and within 24 hours patients will either survive or succumb to the infection [294].

The World Health Organization (WHO) categorizes DHF/DSS into four grades based on severity, from the less severe (grade 1) to the more severe (grade 4). If patients progress to DSS they are categorize in either grades 3 or 4 [216]. Unfortunately, only supportive treatment is available for DF, DHF and DSS patients. Currently there are no antiviral treatments available for DENV in general, and vaccine development has been challenged by the requirement to protect against the four serotypes to prevent ADE. Presently, the only way to prevent DENV infections is to control to the mosquito vector.

Immune Response to DENV

Humoral and cellular immunity both play a role in the protection and clearance of DENV infections. During a primary DENV infections, these two branches of the

immune response are able limit most infections of milder DF, and allow for the development of immunological memory that can protect the individual against subsequent infections with that DENV serotype. In contrast, during secondary heterotypic DENV infections the memory immune cells from the previous infection generate a less than ideal immune response against the virus. This enhances the infection and often times causes a more severe DHF/DSS reaction.

During a primary infection, DENV enters the skin and is processed by Langerhans cells and immature dendritic cells (iDC) present in the dermis. The Langerhans cells, and other immune cells in the area, are activated to release IFN-γ and other cytokines. The iDCs then travel to the lymph node where they mature and play a part in initiating the cellular and humoral immune responses through interactions with $CD4^+$ and $CD8^+$ T-cells. During a primary DENV infection, cytolytic effects of $CD8^+$ T-cells are important in the host's defense against the virus [295]. The T-cells also produce IFN-γ and TNF-α, which augment the anti-viral response. $CD4^+$ T-cells can go on to activate B-cells to produce IgM followed by IgG, predominantly subclasses IgG1 and IgG3 [296]. At this stage of infection, the humoral response may also play a role in controlling the infection. IgM antibodies are typically the first antibody isotype present after infection, detectable three to five days after illness in 50% of patients, increasing to 93-99% of patients after six to ten days of illness [227]. The DENV-specific IgM levels usually peak two weeks after the onset of fever and then decline over the next two to three months [221,297-299]; virus specific IgG is detectable in patients after the first week of illness and then continues to increase [227]. After the virus leaves the skin it can

travel through the blood to infect other tissues and tissue macrophages, especially those in the liver and spleen [250].

In a secondary heterotypic DENV infection, patients are at an increased risk of developing DHF/DSS; pre-existing immunity from a previous DENV infection, age, and time between infections, are each primary risk factors. The exact pathogenesis of DHF/DSS is still poorly understood, but there are two known contributing immune factors: ADE [227,268-270,300] and original antigenic sin (OAS) [301,302].

In ADE antibodies from the primary DENV infection remain active, but are non- or sub-neutralizing against the DENV serotype causing the current infection. These antibodies bind to the virus, forming antibody-virus complexes that gain access into FcγR bearing cells via the Fc portion of the antibody [266,267,269,271,272]. This increases the number of DCs and monocytes/macrophages that are infected in the blood of DHF/DSS patients increasing the viral load in patients, immune activation of the cells, and cytokine release [292]. Antibody-antigen complexes also have the potential to activate the complement pathway, leading to temporal plasma leakage [303,304]. There is a 10- to 100-fold greater level of virus in the blood of DHF/DSS patients compared to DF patients [292].

OAS is the second proposed contributing factor to DHF/DSS. During a secondary heterotypic DENV infection, memory T-cells from the previous DENV infection are re-activated. As a result the T-cell response is directed against the DENV serotype from the primary infection rather than developing a response specific to the current DENV serotype causing the infection, further increasing the viral load due to decreased viral clearance. There is also an increased production of cytokines by these T-

cells and other uninfected bystander cells along with an increase in the apoptosis of bystander cells, which can lead to heptatic injury. Additionally, there are autoimmune factors involved in the pathogenesis of DENV. Antibodies to NS1 have been reported to be cross-reactive with human platelets and endothelial cells. Antibodies against the E protein are also cross-reactive with human plasminogen [305]. The interaction of these cross-reactive antibodies with their targets within the host can further augment the symptoms of DHF/DSS [304,306].

Overall in DHF/DSS there is an increase in the number of virally infected cells due to both ADE and OAS, as well as an increased production of cytokines, some of which are proinflammatory. These proinflammatory cytokines, especially TNF-α [307-309], IL-6 [250,308], IL-8 [250] and IL-12 [250], interact with endothelial cells creating leaky junctions, increasing the vascular permeability, and deficiencies in coagulation, leading to the associated symptoms of DHF/DSS [250,309-311].

The pivotal role that antibodies play in either clearing or enhancing DENV infections makes identifying neutralizing epitopes of the virus a key area of research. Just six days after the bite from a DENV-infected mosquito, the body can begin to develop a humoral immune response against the virus [266,296,312-315]. Most protective antibodies, typical of this humoral immune response, are directed at the three domains (DI, DII, and DIII) of the structural E protein [316-319]. The central domain, DI, contains nonneturalizing epitopes and is thought to be involved in the low-pH-triggered conformation changes [320]. Neutralizing epitopes are found within the highly conserved fusion loop in DII, which interacts with the host cell membrane during fusion [320-323], and the lateral ride or fusion loop in DIII [247,271,319,324-326]. It has been

found that for other flaviviruses, protective antibodies also recognize some of the NS proteins (NS3 and NS5), and the prM protein [316,318,327]. However, the antibody response against the NS proteins is weak, especially in primary DENV infections [318,328].

Research completed with a common experimental strain of DENV-2 has identified three major epitopes that are recognized by mammalian neutralizing antibodies: the lateral ridge in DIII, the fusion loop in DII, and the less common A strand in DII of the E protein [247,329]. Despite the protective potential of passive antibody treatments for DENV infection, their use is hindered by the threat of ADE. It has previously been shown that genetically modified mammalian IgG antibodies that bind DENV-2, but lack the ability to bind FcγRs, work as prophylactic and therapeutic treatments against DENV-2 infections without interference from ADE [270]. The difference in the Fc portion of avian antibodies compared to mammalian antibodies makes avian monoclonal antibodies a possible treatment for DENV infections. Avian antibodies have the potential to avoid ADE and allow successful passive antibody treatment of DENV infections.

Goose Monoclonal IgY Antibodies as a Treatment Against DENV-2 Infections

The aim of this project was to develop a goose monoclonal IgY antibody that could be used to as a treatment against DENV-2 infections. Avian monoclonal IgY antibodies are a logical treatment option for DENV-2 infections because of their potential to overcome the shortcomings of current therapeutic antibodies, including the HAMA response and other adverse reactions because of the structural difference in the Fc portion of mammalian IgG and avian IgY antibodies [44,46]. This is a favorable treatment option over polyclonal antibodies because of the homogenous and consistent response

that is generated. Once the hybridomas are developed, the monoclonal IgY antibodies become a renewable resource. Using monoclonal IgY antibodies would also provide the protection while preventing ADE and other complications caused by DENV-antibody complex uptake.

In this study we show for the first time attempts to use traditional hybridoma techniques to develop a goose monoclonal IgY antibody.

Materials and Methods

Geese

Geese were vaccinated against *Pasteurella multocida* types 1, 4, and 3x4 (the causative agent of fowl cholera) at 12-18 weeks of age, and again four to six weeks later using the AviPro 108 FC4 vaccine (Lohmann Animal Health, Winslow, Maine, USA). Blood was collected from the vaccinated geese and used as a source of immune B-cells. Geese were immunized subcutaneously with DENV-2 antigen (Microbix, Mississauga, ON, Canada) derived from the parental DENV-2 strain PLO46 Taiwanese isolate [330]. Vaccinations took place on day zero with a 1:1 mixture of DENV-2 antigen with complete Freund's adjuvant. A booster vaccination was administered two and four weeks later with a 1:1 mixture of the DENV-2 antigen with incomplete Freund's adjuvant. Six weeks later, blood was collected from the immunized geese. Geese were housed in either an isolation unit (ISO) or specific pathogen free unit (SPF) for the duration of the experiment.

Hybridoma Techniques

Blood from vaccinated geese was separated using a ficoll-paque gradient. The lymphocyte layer was isolated from the gradient and used as a source of immune B-cells. Cells were then washed, and viability was determined in a hemocytometer using 0.2% trypan blue. The goose immune B-cells were mixed with the Sp2/0-Ag14 mouse myeloma cell line under fusion conditions with 50% polyethylene glycol (Polysciences, Inc., Warringto, PA, USA).

To select for B-cell fusions, B-cells were cultured at $2.5x10^6$ cells/mL in 96-well plates in complete DMEM-20/HEPES/Pyruvate/HAT media (advanced DMEM), supplemented with 2mM L-glutamine, 50μM 2-ME (Sigma, MO, USA), 100 U/mL penicillin 100μg/mL, streptomycin sulfate, 20% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA), 10mM HEPES 1mM sodium pyruvate (Cellgro, Mediatech Inc., VA, USA), and 10x HAT (Cellgro, Meditech Inc, Va, USA) at 37º C in 5% humidified CO_2 for two weeks. Before switching to DMEM/HEPES/Pyruvate/HT containing media (advanced DMEM, supplemented with 2mM L-glutamine, 50μM 2-ME (Sigma, MO, USA), 100U/mL penicillin 100μg/mL, streptomycin sulfate, 20% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA), 10mM HEPES 1mM sodium pyruvate (Cellgro, Mediatech Inc., VA, USA, and 1x HT (Gibco, Grand Island, NY, USA)), and then on day 15 and for the remainder of the experiment, cells were cultured in DMEM/HEPES/Pyruvate media (advanced DMEM, supplemented with 2mM L-glutamine, 50μM 2-ME (Sigma, MO, USA), 100U/mL penicillin 100μg/mL, streptomycin sulfate, 20% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA), 10mM HEPES 1mM sodium pyruvate (Cellgro, Mediatech Inc., VA, USA).

After approximately three weeks, culture supernatants were screened for fowl cholera- or DENV-2- specific goose antibodies. Hybridomas were ready to screen when: 1) there was 10-25% confluence in wells with cell growth, and 2) when the wells with dense growth turned yellow within two days after feeding due to waste production.

Hybridoma Screening

Culture supernatants from wells containing potential hybridomas were screened for either fowl cholera- or DENV-2- specific goose antibodies using an ELISA. Briefly, one hundred microliters of DENV-2 antigen (Microbix, Mississauga, ON, Canada), or fowl cholera vaccine was used as a capture antigen to coat Costar EIA/RIA96-well plates (Corning, New York, NY, USA) and then incubated overnight at 4ºC. Plates were washed five times with PBS/0.5% Tween-20 (PBST), and then blocked by adding 200µL 1% BSA in 1xPBS to each well after incubation for two hours at room temperature (RT). Plates were washed five times with PBST before samples were added. Culture supernatants samples were diluted 1:3. Serum from fowl cholera or DENV-2 immunized geese was used as a positive control, diluted 1:50. One hundred microliters of samples and controls were loaded into wells in triplicate. Samples were incubated overnight at 4ºC and washed five times with PBST. DENV-2 specific IgY was detected by incubation with either an HRP-conjugated donkey anti-chicken IgY (Gallus Immunotech, Fergus, ON, Canada) or AP-conjugated rabbit anti-chicken IgY (Sigma, MO, USA), for one hour at 37ºC. The plates were washed five times with PBST and the reaction was visualized with TMB substrate (Thermo Scientific, Rockford, IL, USA) or pNPP substrate (Thermo Scientific, Rockford, IL, USA). The colorimetric change of each well was determined at

an optical density of 450nm or 405nm on a Thermomax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Cloning by Limiting Dilution

Candidate hybridomas were prepared at a concentration of 50 viable cells/well and five viable cells/well in 10mL of cloning and expansion medium, DMEM/HEPES/Pyruvate media supplemented with cloning media (advanced DMEM), supplemented with 2mM L-glutamine, 50μM 2-ME (Sigma, MO, USA), 100 U/mL penicillin 100μg/mL, streptomycin sulfate, 20% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA), 10mM HEPES 1mM sodium pyruvate (Cellgro, Mediatech Inc., VA, USA), 5% BriClone (QED Bioscience Inc. San Diego, CA, USA). 96-well plates were then seeded with the cell suspensions at 200μL per well and cells were cultured at 37º C in 5% humidified CO_2. Wells containing hybridoma clones were screened for production of fowl cholera- or DENV-2- specific IgY antibodies using the same ELISA screening method.

Results

Modification of Mammalian Hybridoma Techniques using Fowl Cholera

Preliminary work was done to first demonstrate the modification of mammalian techniques to the avian system using geese vaccinated against fowl cholera caused by the bacteria *Pasturella multocida* (*P. multocida*). Fowl cholera can infect both wild and domestic birds causing either an acute or chronic infection, resulting in high morbidity and mortality that spreads easily from bird-to-to bird within flocks. Asymptomatic and chronic carriers play a key role in disease transmission. Disease prevention is important

especially for commercial farmers to prevent fowl cholera from wiping out their flocks. Blood was used as an alternative source of immune B-cells. Blood was collected from geese vaccinated against fowl cholera. The lymphocyte layer was isolated from goose blood separated using a ficoll-paque gradient. The purified goose B-cells were fused with mouse myeloma cells (Sp2/0-Ag14) at a 1:1 ratio in the presence of 50% PEG and grown in HAT containing media to select for the growth of only B-cell-myeloma cell fusions. After three weeks of growth, culture supernatants were screened for the production of anti-cholera IgY antibodies using an ELISA.

Supernatant from sixty potential hydridoma containing tissue culture plate wells was screened. Anti-fowl cholera IgY antibody levels in culture supernatant were compared to the anti-cholera IgY antibody levels found in serum from fowl cholera vaccinated geese; antibodies found in the serum were used as a positive control. Results for 41 a hybdridoma that did not produce any fowl cholera- specific IgY antibodies are inducled for comparison. Of the sixty wells screened, six contained high responding hydridomas, 6, 16, 18, 21, 22, and 24 (Fig 1). These hybridomas produced higher amounts of anti-cholera IgY antibodies than the three low responder hybdriomas 52, 59, and 60 (Fig 1). These results demonstrated that it is possible to make goose B-cell hybdridoma using mammalian techniques and supports the feasibility of the development of a goose monoclonal IgY antibody. Focus was then shifted to making goose monoclonal IgY antibodies against the relevant human pathogen DENV-2.

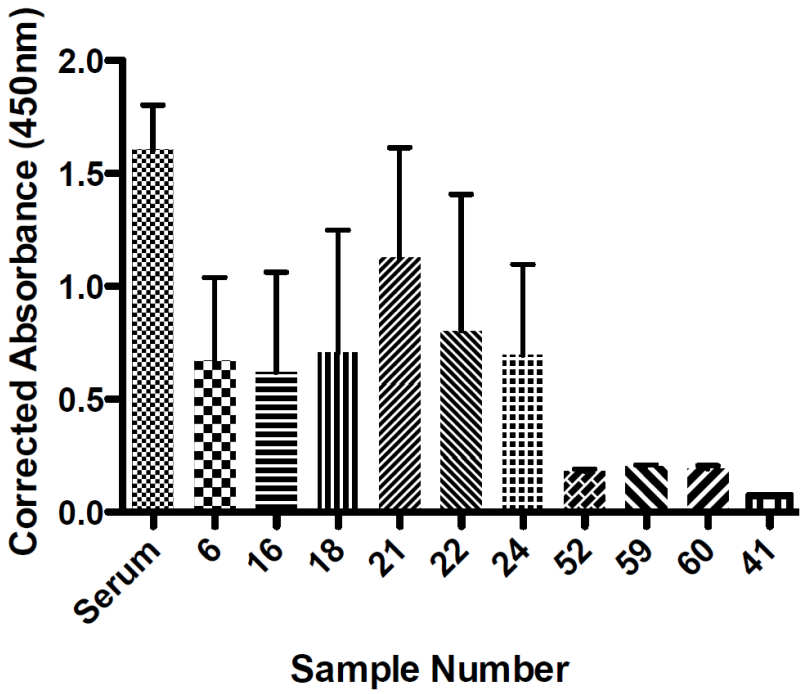

Figure 1. Presence of fowl cholera specific IgY antibodies as determined by ELISA. Culture supernatants from potential hybridoma containing tissue culture wells were collected approximately three weeks after fusion and analyzed for the presence of fowl cholera specific IgY antibodies by ELISA. Serum from vaccinated geese was used for comparison. Readings were taken at an optical density (OD) of 450nm. Each sample was run in triplicate and the bars represent standard error.

Development of DENV-2 Specific Goose Monoclonal IgY Antibodies

This study focused on the development of a goose monoclonal IgY antibody using the modified mammalian hybridoma technique developed using fowl cholera. Female geese housed in either an ISO or SPF unit were vaccinated with a DENV-2 antigen in adjuvant on experiment day zero, and then booster vaccinated two and four weeks later. Eggs and blood were collected from the vaccinated geese weekly starting two weeks into the experiment. The lymphocyte layer containing immune B-cells was isolated from blood collected from DENV-2 antigen vaccinated geese using a ficoll-paque gradient. The immune goose B-cells were fused with the Sp2/0-Ag14 mouse myeloma cell line at a 1:1 ratio to form hybridomas. This process was repeated several times as new blood samples were received. After approximately three weeks of incubation in various types of media, culture supernatants from wells containing potential hybridomas were screened using an ELISA.

Twenty-nine potential hybridoma containing wells were screened for the presence of DENV-2 specific IgY antibodies. Serum from the immunized geese showed detectable amounts of DENV-2 specific IgY antibodies and was used as a positive control in the ELISAs (Fig 2). Of the 29 wells screened, six were producing high levels of DENV-2 specific IgY antibodies: those in wells C1, E6, E8, F2, H3, and D6. Results for E1, a hybridoma that did not produce any DENV-2 specific IgY antibodies, are included for comparison. The DENV-2 specific IgY antibody levels produced from the hybdridoma present in well C1 were higher than that of the positive control goose sera (Fig 2). The hybdriomas present in wells C1, E6, E8, F2, H3, and D6 were subcloned by limiting dilution in cloning/expansion medium containing a subcloning media

supplement. After limiting dilution, culture supernatants were again screened for the presence of DENV-2 specific IgY antibodies. There were no DENV-2 specific IgY antibodies detected in any of the culture supernatants from wells containing the subcloned hybdriomas or the parent hybdriomas compared to controls (data not shown). This suggests the fusion between the goose B-cells and mouse myeloma cells cannot be maintained over a long period of time.

IgY antibodies were also isolated from the yolks of eggs collected from vaccinated geese and an ELISA was completed to confirm the presence of DENV-2 specific IgY antibodies and to determine the titers of the antibodies present in the eggs. Because IgY is transferred from the serum of the goose to the egg yolk to protect the developing embryo, egg yolk IgY antibody titers are also indicative of serum titers. The average titer of IgY antibody from the eggs of geese housed specifically in the SPF facility was 1:732,459, with the highest being 1:6,400,000 (Fig 3). For the eggs of geese from the ISO unit, the average titers was 1:877,465, with the highest titer being 1:3, 200,000 (Fig 3). There results indicate that an active immune response was formed against the DENV-2 antigen by the geese, and that DENV-2 specific immune B-cells are present in the blood of vaccinated geese.

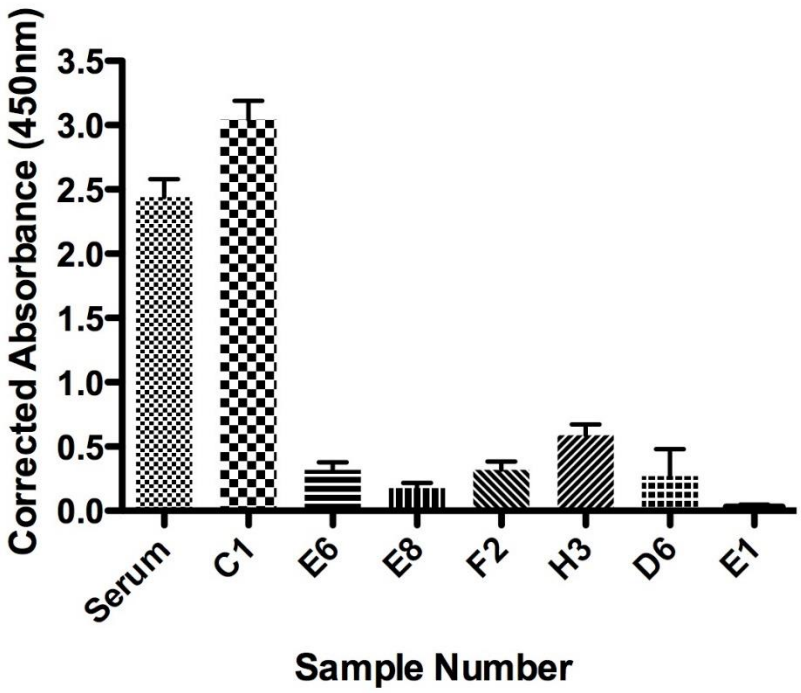

Figure 2. DENV-2 specific IgY antibodies were initially present in culture supernatants from candidate hybridomas. Culture supernatant samples were collected from candidate hybridoma containing wells approximately three weeks after fusion with myeloma cells. The presence of antigen specific DENV-2 antibodies was detected using an ELISA read at an OD of 450nm. Serum from vaccinated geese was used as a positive control for comparison. Each sample was run in triplicate, and bars represent standard deviation.

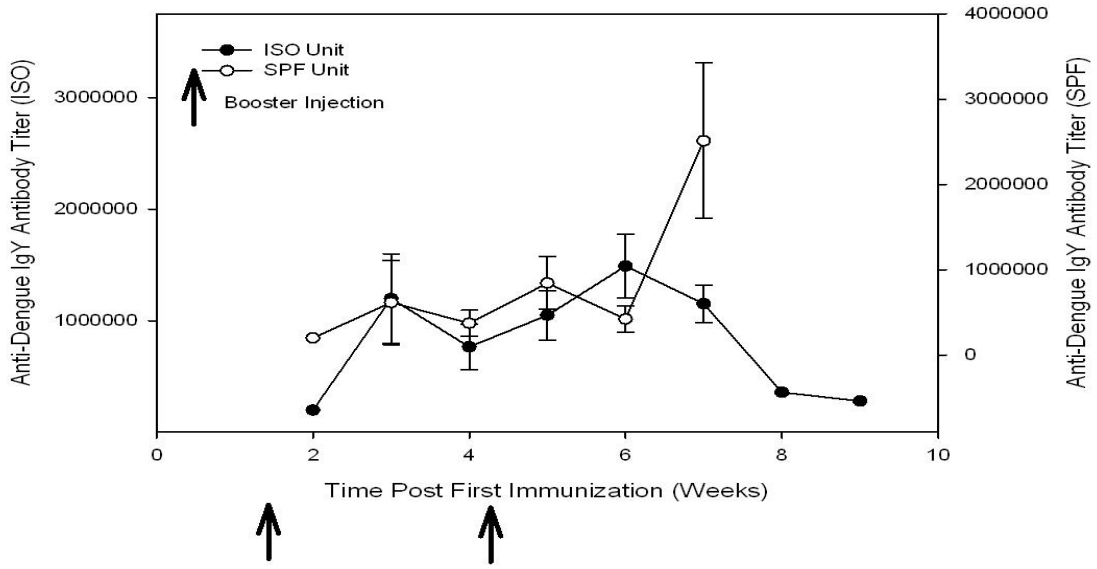

Figure 3. DENV-2 specific IgY antibody is present in the yolks of eggs collected from immunized geese. Eggs were collected from vaccinated geese housed in both the ISO unit (closed circle) (n=71) and SPF unit (open circle) (n=61), respectively. A sample was collected from each egg after processing for IgY antibody isolation. DENV-2 specific IgY antibody titers present in the eggs were determined using an ELISA. Arrows indicate booster immunizations. Data presented is a representative mean and bars indicate standard error.

Discussion

We show in this study, for the first time, that is possible to develop short-lasting hybdridoma fusions between goose B-cells and mouse myeloma Sp2/0-Ag14 cells capable of producing antigen- specific monoclonal avian IgY antibodies. Previously, monoclonal IgY antibodies have been developed using murine/avian chimeric constructs and phage display techniques [331,332]. However. chimeric antibodies utilizing the constant region of an avian IgY antibody, and the variable region of mammalian IgG, are still limited to the antigens they can recognize by the mammalian variable region, which is responsible for binding the antigen [59]. These antibodies would not be useful against targets that are highly conserved in the mammalian system, which is why the ability to make completely avian monoclonal antibodies is advantageous. In the present study, it has been shown that, at least the initial stages of hybridoma, development is possible using traditional mammalian hybdridoma techniques, fusing goose B-cells and mouse myeloma cells.

Vaccination against fowl cholera and immunization with DENV-2 antigens induced strong humoral responses in the geese, as shown by the amount of antigen specific IgY antibody detected in the serum of immunized geese (Fig 1 and Fig 2). This coincides with research that emphasizes the role of antibodies in the immune response to DENV-2 infections [296]. Dengue specific IgG antibody is present in the serum of DENV-2 infected patients after just a week of illness [227,296]. Similarly, DENV-2 antigen specific titers were maintained within eggs at least six weeks into the experiment, and peaked with titers well into the millions (Fig 3). The high antibody titers in the eggs and the positive response by the serum from DENV-2 geese are both evidence for the

development of a humoral response by these geese against the DENV-2 antigen. The development of a humoral response also means antigen- specific antibody producing B-cells are present in the blood of the vaccinated geese. Therefore, a lack of immune B-cells was not the main contributing factor to the problems with hybridoma development. These results also support the general assumption that birds develop a robust immune responses against a diverse array of antigens.

The fowl cholera vaccine used in these experiments, was an inactivated vaccine formulation containing inactivated *P. multocida* in an oil-based emulsion. We report here that vaccination induced high serum anti-fowl cholera IgY antibody production. This is in agreement with studies completed in turkeys infected with an avirulent fowl cholera vaccine, in which systemic antibody titers were induced [333,334]. The efficacy of such vaccine formulations have also been studied in chickens and have been shown to be effective in protection against bacteria challenge [335,336]. These results, along with those of others, support the presence of fowl cholera specific immune B-cells in the blood of vaccinated geese and confirms the successful development of antibody producing hybdriomas between goose immune B-cells and mouse myeloma cells.

There was a reasonable amount of variability in the amount of fowl cholera-specific goose monoclonal IgY antibodies detected in the cultured supernatants from the various hybdriomas. This varability in results could have been due to an uneven coating of the ELISA plates. ELISA plates used to detect the fowl cholera specific IgY antibodies were coated with a vaccine in an oil-based emulsion, the extra material present in this preparation could have caused an uneven coating of the plate with the target antigen and led to variability in results.

Chicken monoclonal antibodies have been produced using hydbridoma technology: through fusion of immune B-cells with either a chicken B-cell line, or chicken non-secreting mouse myeloma cell line, both of which are not commercially available [337-343]. These chicken monoclonal antibodies were developed against various antigens, including prions and protozoa. Antigen specific IgY antibody production by chickens was induced using a similar method as in these currently described experiments [339,341,342]. Chickens were immunized with a given antigen and then fusion experiments were completed with the chicken cell lines. One difference between their protocol and that of this study was the use of immune spleen cells from the immunized chickens as a source of antigen specific B-cells for fusion with the chicken cell lines. In this study immune B-cells from the blood were fused with the mouse myeloma cells. Monoclonal IgY antibodies made by fusing immune spleen cells with chicken non-secreting myeloma cells lines were reported to continue to produce high levels of antigen- specific IgY antibody for over six months even after subcloning [340]. It is possible that the chicken myeloma cells would work as a cell fusion partner for the goose B-cells and lead to successful hybdriomas, but the difference in antibodies produced by anseriformes birds (geese) and galiformes birds (chickens), mainly the lack of IgY(ΔFc) production by chickens when compared to geese, could impact fusion viability. Using an alternative source of goose immune B-cells could have been one way to increase the fusion strength between the goose B-cells and mouse myeloma cells.

CHAPTER III

CHARACTERIZATION OF ANDES VIRUS SPECIFIC IGY ANTIBODIES FROM DNA VACCINATED GEESE FUR USE AS AN ANTI-VIRAL TREATMENT

Introduction

History of Andes Virus in Humans

The Andes virus (ANDV) is a New World Hantavirus from the genus *Hantavirus* within the *Bunyavirdae* family of viruses. The 21 hantaviruses that cause disease in humans are distributed worldwide, and can be differentiated based on phylogenetic origins into Old World and New World hantaviruses. Old World hantaviruses can be found in Asia and Europe, and New world hantaviruses can be found in North, Central and South America. Along with differences in location, New and Old World hantaviruses also differ in the type of syndrome they cause. Old World hantaviruses cause a hemorrhagic fever with renal syndrome (HFRS) while New World Hantaviruses cause hantavirus pulmonary syndrome (HPS).

In the past century there have been two major disease outbreaks that led to the discovery of both the Old and New World hantaviruses. The first outbreak occurred during the Korean War in the early 1950s, during which over 3,000 military troops fell ill with a hemorrhagic fever, later determined to be HFRS [344,345]. In 1978, the Hantaan virus, an Old World hantavirus, was identified as the causative agent of the troops cases of HFRS [346]. The second outbreak occurred in the Four Corners region of the United

States in 1993. Adults in this region were afflicted with a severe respiratory illness caused by a virus, later identified as HPS caused by the Sin Nombre virus (SNV), a New World hantavirus [347]. In 1995, a hantavirus similar to SNV was identified in South America, the ANDV. ANDV was first reported and identified as the causative agent of a HPS outbreak in Argentina [348-350]. Since then additional outbreaks of HPS caused by ANDV have occurred throughout South and Central American, including Brazil, Chile, and Uruguay [351,352]. ANDV continues to be the only hantavirus known to cause HPS in Chile.

Within the *Bunyaviridae* family hantaviruses are unique in that they are mainly transmitted to humans by the inhalation of virus particles that become aerosolized from the excrement or bodily fluids (urine, feces, or saliva) of chronically infected rodents [353]. Infection of the reservoir rodents is primarily subclinical. In nature, transmission of the virus between reservoir and non-reservoir rodents is thought to occur primarily through aggressive behavior and exposure to saliva and excreta [354,355]. It has also been shown that rodents can transmit virus through infected excrement within rodent nests [356-358]. Hantaviruses follow the single virus/single host rule of natural reservoirs, meaning each hantavirus has a specific rodent species as a natural host [345,359,360]. As a result, the geographical distribution of different hantavirus strains is determined by the distribution of their rodent reservoir. The primary natural reservoir for the ANDV in Chile is the *Oligoryzomys longicaudatus*, the long- tailed pygmy rat [361].

Along with normal rodent transmission, ANDV is the only hantavirus that is known to be transmitted person-to-person [352,362,363]. Person-to-person transmission occurs primarily within families or between sexual partners due to the likelihood of close

contact with the infected person [364]. This type of transmission is more likely to occur during the prodromic phase of disease. This is an early stage of disease that can range from 12 to 27 days [363,365]. Other examples of transmission are by bites from rodents and blood transfusions [351,366]. In Chile, more than half of the HPS cases are associated with outdoor occupations such as forestry or agriculture [367,368]. Seasonal HPS cases in Chile during November to April are typically a result of outdoor recreational activities (20-35% of reported cases) during which time individuals are more likely to come in contact with infected rodents [367,368]. HPS outbreaks can be unpredictable, localized, and sporadic due to fluctuations in the rodent reservoirs and changes in the prevalence of the hantaviruses in the reservoirs. In the Americas, the emergence of hantaviruses has been linked to precipitation [369,370]. Studies suggest that precipitation can indirectly increase reservoir species populations and therefore hantavirus prevalence [369,371,372].

ANDV Structure and Life Cycle

ANDV is an enveloped virus with a negative sense trisegmented single-stranded RNA genome. The three genome segments S, M, and L encode for three different proteins: the nucleocapsid (N) protein, two glycoproteins Gn and Gc, and an RNA-dependent RNA-polymerase (RdRp), respectively [373]. Within the virion the three genome segments are individually encapsidated by N proteins to form ribonucloproteins (RNPs) [373-375]. Hantaviruses are spherical in shape with a grid-like pattern on their outer surface due to G_1 and G_2 heterodimers projecting from the lipid bilyaer surface of the virus.

ANDV and other hantaviruses primarily target vascular endothelial cells of the lung and macrophages for replication. Entry is most often facilitated by attachment of the virus to β_3 integrins on the surface of the cell, followed by endocytosis into the cell [376-378]. These may not be the only receptors for the virus because cells without β_3 integrin also allow hantavirus infection [379,380]. After internalization the virions are trafficked to either an early or late endosome. The low pH of the endosome triggers a conformational change in the G_2 glycoprotein allowing fusion of the viral and cellular membranes [381-383]. The viral genome, in the form of RNPs, is then released into the cytoplasm and transported to the site of viral replication within the cell [384,385]. This allows the viral genome to be used as a template in two different processes: transcription and replication. Both transcription and replication are facilitated by the viral RdRp [386]. Initially transcription is started by the viral RdRp to give rise to the S, M, and, L mRNAs. The S and L mRNA transcripts are then translated on free ribosomes, and the M segment transcript is translated on membrane-bound ribosomes, which is cotranslated on the rough endoplasmic reticulumn (RER) [351]. The glycoprotein precursor that results from translation of the M segment transcript is proetolytically processed into the G_1 and G_2 glycoproteins during import into the ER [386,387]. The resulting G_1 and G_2 glycoproteins are also glycosylated in the ER and then transported to the Golgi complex [387,388]. After the initial burst of transcription, the viral RdRp switches to replication of the viral S, M, and L genomic RNAs [351]. The first step in replication is transcribing the vRNA into complementary RNA (cRNA), which is then used as a template [383]. Replication can then proceed using the cRNA *de novo* without the need for primers [386]. After the viral genome has been replicated it is then encaspidated by the N protein

to form RNPs [389]. When enough viral components have been made assembly can take place. For New World hantaviruses it is most likely that assembly takes place at the Golgi complex where the G_1 and G_2 proteins accumulate, but evidence suggests that this step might also take place at the plasma membrane [390-392]. During assembly the RNPs for each genome segment along with L proteins come together and the virion buds inside of the Golgi, through the host membrane with G_1 and G_2 embedded in it, and is subsequently release into the mileu from the Golgi, probably by exocytosis [393]. Alternatively the virion could bud directly through the plasma membrane, with G_1 and G_2 embedded in it, entering the cellular mileu [392]. Infected cells are not lysed during infection with hantaviruses [394,395].

HPS caused by ANDV

Following infection of target cells, ANDV leads to HPS by indirect effects of the virus on endothelial cell barrier function. Activation of the innate and adaptive immune responses leads to the production of proinflammatory cytokines and disease specific immunopathology. The most damage caused by ANDV is focused on the lungs and typically presents as a quickly developing diffuse non-cardiogenic pulmonary edema [396]. The average incubation period for ANDV is 18-19 days with a possible range of 11-32 days [397,398]. Current hantavirus diagnostic tests are capture ELISAs that detect the IgG/IgM response to the viral N protein [399,400]. For HPS infections the initial prodromal period lasts two to five days with symptoms of fever, headache, back pain, myalgia, and gastrointestinal symptoms [401]. Thrombocytopenia is one of the earliest laboratory findings of HPS, seen in more than 80% of patients, and can be detected as early as the prodromal phase of illness [402,403]. As the condition continues to worsen,

patients will begin to experience HPS specific respiratory symptoms such as cough, shortness of breath, tachycardia, and hypotension [402,404]. More severe cardiopulmonary symptoms arise from an increase in pulmonary edema and low cardiac output [405]. This can quickly lead to respiratory failure, often requiring treatment with supplemental oxygen, intubation, or intubation with mechanical ventilation [406]. The final stage of illness is the development of non-cardiogenic shock from the increased capillary permeability caused by inflammatory damage to the pulmonary vascular endothelium [404]. After the cardiopulmonary stage of illness, thrombocytopenia continues to be a detectable laboratory finding along with circulating immunoblasts, and elevated hematocrit values [407,408]. Gross pathological findings show that the lungs of patients with HPS are dense, rubbery, and heavy, usually weighing twice as much as the average lung [399]. Almost all deaths due to HPS occur 24-48 hours after the onset of cardiopulmonary symptoms and are a result of shock [351]. The mortality rate for HPS is approximately 50%, but it can vary depending on the specific hantavirus causing the infection [394,409]. If patients survive the acute phase of HPS, the convalescent period can be long; often patients experience weakness and fatigue with abnormal lung capacity. It may take months to years to fully return to normal [397,410].

Despite the relatively high mortality rate there continues to be no virus- specific treatment or vaccine for HPS caused by ANDV. This makes treatment for HPS only supportive in nature. Patients often receive intravenous fluids and supplemental oxygen. Another setback to the development of a HPS treatment has been the lack of a quality rodent model of infection. It was not until recently that a quality lethal rodent model of infection was developed. ANDV causes a lethal disease in adult Syrian hamsters that

closely resembles disease in humans. The Syrian hamster model has been a useful tool to study potential treatments and gain a better understanding of both the disease pathogenesis and the immune response to the virus [411].

Immune Response to ANDV

The immune response is a necessary evil during a hantavirus infection: it is necessary to clear the infection, and develop immunological memory to prevent future infections, but it can also exacerbate the symptoms of infection. Initially when a hantavirus infects a host cell it is recognized by various PRRs that will lead to the release of proinflammatory and anti-viral cytokines including type 1 IFNs and IFN-γ [412]. These two groups of IFNs will work together as the first line of defense to restrict viral replication and spread of the virus.

Hantaviruses have developed several ways to evade the host IFN response including changing the look viral transcripts. The viral G_1 and G_2 glycoproteins are able to down regulate the IFN pathways by inhibiting IRF-3 phosphorylation, dimerization, and nuclear transport, along with impairing the function of IKKe and TBK-1 kinases [413,414].

Infected macrophages are activated to produce cytokines such as TNF-α, IL-6, and IL-1 [351]. Those same cytokines along with IL-2 and IFN-γ can be detected in the lung tissues of patients with HPS during early stages of infection. These cytokines are most likely being produced by infected macrophages and monocytes within the tissue [415]. *In vitro,* hantavirus infection of endothelium results in the induction of MCP-1, RANTES, and IP-10, all of which can recruit immune cell infiltrates to the lungs and other organs [395,416,417]. Furthermore, the excess of proinflammatory cytokine

production by immune cells is probably critical for HPS pathogenesis. TNF-α is known to affect vascular permeability and results from clinical patients suggests that the IL-6 plays a role in inhibiting cardiac function and inducing hypotension in HPS [351].

A robust $CD4^+$ and $CD8^+$ T-cell response is also activated against hantaviruses. $CD8^+$ T-cells play a major role in the induction of HPS, whereas $CD4^+$ T-cells play a more protective role by activating B-cells to produce protective neutralizing antibodies. Patients with severe HPS have higher amounts of virus- specific CD8+ T-cells present in their blood compared to patients with moderate disease, and those infected with other viruses [418]. T-cells obtained from patient's blood primarily recognize epitopes within the N, G_1, and G_2 proteins [418-420], with the most immunodominant epitopes being part of the G_1 protein [421]. Other studies indicate that G_1- specific effector memory T-cells may contribute to protective immunity in ANDV infected patients [421].

Activation of the humoral immune response and production of neutralizing antibodies are essential to suppress hantavirus dissemination and to avoid fatal disease. Both IgM and IgG hantavirus- specific antibodies can be detected at or shortly after the onset of the prodromal period. Initially, antibodies are derived against the N protein, and then later after the development of clinical symptoms almost all patients will develop IgM and IgG antibodies specific for the G_1 protein [422]. It was shown that HPS patients with mild HPS had higher neutralizing antibodies titers to SNV or ANDV than those that with severe HPS [350,423]. Also, higher hantavirus specific IgG levels early in disease have been associated with survival [424]. After convalescence from hantaviral infection, humans are considered to be protected for life from reinfection [405].

The importance of the humoral response in protection against HPS infections caused by the ANDV is further supported by the ability to passively transfer protection through the transfer of neutralizing antibodies. Research has shown that plasma from HPS survivors is protective in the ANDV hamster model of infection [425]. Vaccine induced neutralizing antibodies collected from the serum of rhesus macaques or rabbits vaccinated with a DNA vaccine also protected against lethal disease development in the ANDV hamster model when given before or up to five days post-challenge [425,426]. As a means to prevent the risks of reactionicity associated with the use of mammalian antibodies, duck IgY anti-ANDV antibodies were generated from the egg yolks of ducks vaccinated with a DNA. The anti-ANDV IgY and IgY(ΔFc) antibodies were able to be used as a treatment in the ANDV Syrian hamster model of infection when administered after challenge [427]. These results provide direct evidence that goose- derived anti-ANDV IgY antibodies can be used a passive antibody treatment against HPS caused by the ANDV.

Despite the encouraging studies that support the use of a passive treatment against HPS, current options are not fulfilling the need. Human convalescent serum is in short supply, has risks of reactogenicity in humans, and is often contaminated with other infectious pathogens. There are currently no neutralizing monoclonal antibodies available. To fill this gap. this study demonstrates post-exposure treatment potential of goose-derived anti-ANDV antibodies both *in vitro* and *in vivo*. In addition, the study identified epitopes within the ANDV glycoproteins recognized by IgY/IgY(ΔFc) antibodies purified from egg yolks of DNA vaccinated geese. It is also shown that

neutralizing titers were maintained during the time period between initial and booster vaccination, and the neutralizing titers were increased after booster vaccination.

Materials and Methods

Virus and Cells

ANDV strain Chile-9717869 was propagated in Vero E6 cells (Vero c1008 ATCC CRL 1586, Manassas, VA). Cells were maintained in Eagle's minimal essential medium with Earle's salts (EMEM) (EMEM, supplemented with 10% FBS, 10mM HEPES (pH 7.4), 200U/mL penicillin, 200ug/mL streptomycin, 1x nonessential amino acids, 1.5µg/mL amphotericin B, and 50ug/mL gentamicin sulfate), at 37ºC humdified in a 5% CO_2 incubator.

Vaccination of Geese

Naïve geese were vaccinated with the ANDV DNA vaccine pWRG/AND-M(opt) (1mg) at two week intervals starting at day zero for ten weeks. Injections were given intramuscularly (i.m.) using a needle free device (NFD). One year later the same geese were booster vaccinated with either the same pWRG/AND-M(opt) or an optimized version of the same vaccine pWRG/AND-M(opt2). Six 1mg injections were administered over a ten week time period. Goose eggs were collected after initial vaccination and booster vaccination at time point indicated in figure 4. Sera was also collected from the vaccinated geese at five different time points both after the initial and booster vaccination series as indicated in figure 4.

Epitope Determination

Linear IgY epitopes were identified using JPT PepStar microarrays (JPT, Berlin, Germany). The entire glycoprotein precursor sequence of the ANDV strain Chile-9717869 was synthesized into 13 amino acid overlapping peptides. The resulting 376, 13-mer peptides were covalently attached to a microarray slide, with a 10 amino acid overlap between adjacent sample peptides. Peptides were analyzed for their reactivity with IgY antibodies isolated from goose eggs, following protocols recommended by JPT. Briefly, slides were incubated with the primary antibody diluted to 30μg/mL at 4°C overnight in a moist environment. The slide was washed and incubated with a fluorescently labeled secondary antibody, goat anti-chicken IgY conjugated to Cy5 (1μg/mL) (Abcam, Cambridge, England) for 1 hour at 30°C. After washing and drying the slide, bound antibodies were detected using a microarray reader (Genepix 4000). Fluorescence was measured at a 10μm pixel size and mean values with the background corrected were calculated and used for analysis. The reactivity was classified based on a spectrum ranging from no activity in black, mild reactivity in gray, to strong reactivity in red.

PsVNA

Pseudovirion assays (PsVNA) utilized a replication deficient vesicular somatic virus (VSV) coated with the ANDV glycoproteins and the VSV surface glycoprotein gene replaced with a Renilla luciferase reporter gene to be used for quantification. The assay was completed as follows. Vero cells were seeded at $1x10^4$ cells/well in a 96-well plate and grown overnight. Pseudovirions were pre-incubated with antibody treatments at 37ºC for 30 minutes before being added to cells. The pseudovirion/antibody mixtures were incubated in wells containing cells for one hour at 37ºC, after one hour the

pseudovirion/antibody mixtures were removed and replaced with 200μL of fresh DMEM supplemented with 10% FBS. Luciferace levels were measured 24 hours after infection using a luciferase assay system (Promega, Madison, WI).

ANDV challenge of hamsters and passive treatment

Adult female Syrian hamsters (*Mesocricetus auratus)* were injected i.m. in the caudal thigh, using a 25-gauge 5/8-in. needle, with ANDV diluted in 0.2mL of sterile PBS (pH 7.4). The challenge dose for ANDV was 2,000 PFU, which is 250 LD_{50} doses. Work involving ANDV infected hamsters was completed in a biosafety level 4 laboratory.

Five and eight days post-challenge hamsters were anesthetized by i.m injection with approximately 0.1mL/100g of body weight of a ketamine-acepromazine-xylazine mixture, and then subcutaneously (s.c.) injected with, one of the six following treatments: (1) anti-ANDV rabbit sera, (2) anti-ANDV goose sera, (3) anti-ANDV purified IgY, (4) normal goose sera, (5) normal purified IgY, or (6) no treatment. The effects of challenge and subsequent antibody treatment on the survival outcome of the hamsters was assessed.

Results

Experimental Design

Previous work has demonstrated that anti-ANDV polyclonal IgY/IgY(ΔFc) antibodies from the eggs of vaccinated ducks limited the infection in the ANDV/Syrian hamster model when administered five to eight days after intranasal (i.n.) ANDV challenge [427]. This provides evidence for the use of anti-ANDV IgY antibodies as a post-exposure treatment for ANDV. To further investigate the treatment potential of IgY

antibodies against ANDV infection and identify other sources for IgY, polyclonal IgY and IgY(ΔFc) antibodies were isolated from goose egg yolks. Geese were vaccinated with an ANDV DNA vaccine, pWRG/AND-M (Fig 4A), that contains the full-length M genome segment of ANDV strain Chile- 9717869, at two-week intervals up until 8 weeks and then at 12 weeks using a needle-free device. One year later the immune geese were booster vaccinated with either pWRG/AND-M or pWRG/AND-M(opt) at week 53, 54, 56, 60, 61, and 62 (Fig 4B). Eggs were collected from the vaccinated geese after the initial vaccination series and immediately after the long-range booster vaccination series.

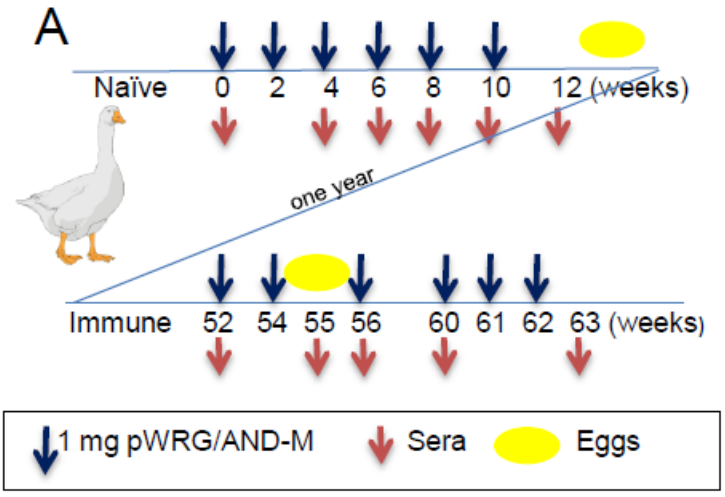

Figure 4. Experimental design. Geese were vaccinated i.m. with 1mg ANDV DNA vaccine pWRG/AND-M at 2 week intervals for 10 weeks, using a needle free device. One year later the same, geese were booster vaccinated again with 1mg ANDV DNA pWRG/AND-M(opt) or pWRG/AND-M(opt2) vaccine on weeks 52, 54, 56, 60, 61, and 62. Sera was collected from the vaccinated geese during the times indicated by red arrows. Eggs were collected, after the initial vaccination series and after the booster vaccination as indicated by yellow ovals.

IgY Antibodies Isolated from Eggs of ANDV Vaccinated Geese Recognize Unique Epitopes On ANDV Glycoproteins G_1 and G_2

To determine the specificity of the IgY/IgY(ΔFc) antibodies purified from the egg yolks of vaccinated geese, epitope mapping was completed using microarray slides. The M genome segment is the primary viral component of the ANDV DNA vaccine; two glycoproteins G_1 and G_2 are synthesized from the M segment and are present in dimers on the outside surface of mature ANDV virions. Based on vaccine composition and virus structure it was hypothesized that antibodies produced by vaccinated geese would bind to epitopes present on the ANDV glycoproteins. To identify potential epitopes on ANDV glycoproteins G_1 and G_2 recognized by anti-ANDV goose IgY and IgY(ΔFc) antibodies. Microarray slides were covalently linked with 13-mer peptides with a 10 amino acid overlap, for a total of 376 peptides, spanning the entire sequence of the glycoproteins from ANDV strain CHIL 9717869 (GenBank accession number AF291703). Total IgY/IgY(ΔFc) antibodies from the egg yolks of geese receiving the initial vaccination series was separated into full length IgY antibody and the alternatively spliced IgY(ΔFc) antibody fractions for testing with microarray slides; unseparated IgY/IgY(ΔFc) combination was also tested. IgY/IgY(ΔFc), IgY(ΔFc), or IgY/IgY(ΔFc) antibodies purified from the eggs of geese vaccinated at both initial and booster vaccination time points were incubated with microarray slides. Reactivity was compared to negative control features on the slide that did not contain any protein; the average of the negative controls was taken and used for comparison.

Eleven IgY/IgY(ΔFc) antibody reactive epitopes were identified across glycoproteins G_1 and G_2 (Fig. 3A and 3B). Seven of the epitopes were specific for the G_1 glycoprotein, peptides starting with aa 121-130, 166-169, 205-211, 241-247, 511, 589,

and 613-625 (Fig 5), and the remaining four epitopes were within the G_2 glycoprotein, reactive peptides started with aa 679-685, 727-745, 787-799, and 889-892 (Fig 6). When comparing the epitopes recognized by IgY/IgY(ΔFc) antibodies to the separated IgY or IgY(ΔFc) antibodies, some of the epitopes were recognized exclusively by IgY(ΔFc) antibodies, (e.g., peptides aa 1024), while others were recognized by both even when separated and incubated with the microarray slides independently. There were also some regions that elicited a higher reactivity from IgY antibodies versus IgY(ΔFc) antibodies (e.g., peptides starting with aa 121-130 and 166-169). There was no reactivity detected with all negative control slide features (Fig 5 and Fig 6).

Because geese receiving the booster vaccination showed increased neutralizing IgY antibody titers compared to the initial vaccination, we wanted to see if there was any change to the epitopes recognized by IgY/IgY(ΔFc) antibodies from the booster vaccinated geese. Figures 5 and 6 (column 4) show that IgY/IgY(ΔFc) antibodies from the egg yolks of the booster vaccinated geese reacted with all of the same epitopes as the IgY/IgY(ΔFc) antibodies from the initial vaccination. Reactivity of the booster IgY/IgY(ΔFc) antibodies with these epitopes was either at the same reactivity level or increased. Additionally there were nine new regions of reactivity with the booster IgY/IgY(ΔFc) antibodies compared to all other IgY treatments, corresponding to peptides starting with aa 31-40, 82-85, 259, 355, 475, 484-493, 640, 691-697, and 940-955 (Fig 5 and Fig 6). One region of high reactivity was extended from seven peptides being recognized, start aa of 727-745, to 10 peptides 707-754. This could be due to an additional epitope being recognized or an increase in the area of being recognized, after booster vaccination.

Sera from rabbits that were previously vaccinated with pWRG/AND-M were also incubated with microarray slides to determine potential epitopes recognized by mammalian antibodies generated after vaccination. In general, the rabbit sera IgG antibodies recognized similar ANDV glycoprotein epitope regions as all IgY treatments. But, for a majority of the epitopes that were recognized by both rabbit sera IgG antibodies and goose egg IgY antibodies there was an obvious difference in reactivity. The rabbit IgG antibodies were less reactive than the IgY antibodies. There was increased binding by the rabbit IgG antibodies to only one region, peptides starting with aa 313 and 358 in G_1, when compared just to all IgY treatments (Fig 5). Areas of notably decreased reactivity compared to all IgY treatments were found in both G_1 and G_2, specifically the peptides starting with aa 511, 589, and 685-697 (Fig 5 and Fig 6). When comparing the reactivity of rabbit IgG antibodies to IgY/IgY(ΔFc) antibodies from the eggs of booster vaccinated geese, there was one obvious region of decreased reactivity in G_1 corresponding to the peptide starting with aa 34 (Fig 5). There were two unique epitope recognized strongly by the rabbit IgG antibodies compared to IgY/IgY(ΔFc) antibodies from the eggs of vaccinated geese comprised of peptides starting with aa 223-229 in G_1 along with peptide 760 in G_2 (Fig 5 and Fig 6).

Of the reactive epitopes identified, those with the highest reactivity were in the regions of peptides starting with aa 613-625 in G_1 and peptides starting with aa 727-745 in G_2 (Fig 5 and Fig 6). These two regions were recognized by all IgY treatments to the highest reactivity level. These two regions were also recognized by the rabbit sera, but to a lesser reactivity level than all of the isolated IgY treatments. Both of these highly reactive regions are outside of the regions identified as reactive by convalesecent human

sera from ANDV patients, and sera from naturally infected rodents [428]. Taken together these results show that IgY isolated from eggs yolks of vaccinated geese was ANDV specific and recognized unique epitopes compared to human and rodent serum.

Figure 5. Identification of ANDV glycoprotein G_1 epitopes. Amino acid sequence for glycoproteins of ANDV strain Chile 9717869 used to make 13-mer peptides for microarray slide. G_1 microarray slides were incubated with IgY antibodies, IgY(ΔFc) antibodies, or IgY/IgY(Fc) antibodies isolated from egg yolks of vaccinated geese either

after the initial or booster vaccination series, or serum from vaccinated rabbits (*first column*- amino acid peptide starts with, *second column*- IgY antibodies from egg yolks after initial vaccination series, *third column*- IgY(ΔFc) antibodies from egg yolks after initial vaccination series, *fourth column*- IgY/IgY(ΔFc) antibodies from egg yolks after initial vaccination series, *fifth column*- IgY/IgY(ΔFc) antibodies from egg yolks after booster vaccination, *sixth column*- sera from DNA vaccinated rabbit). Reactivity was measured based on a spectrum ranging from no activity in black, mild reactivity in gray, to strong reactivity in red (E-Empty features).

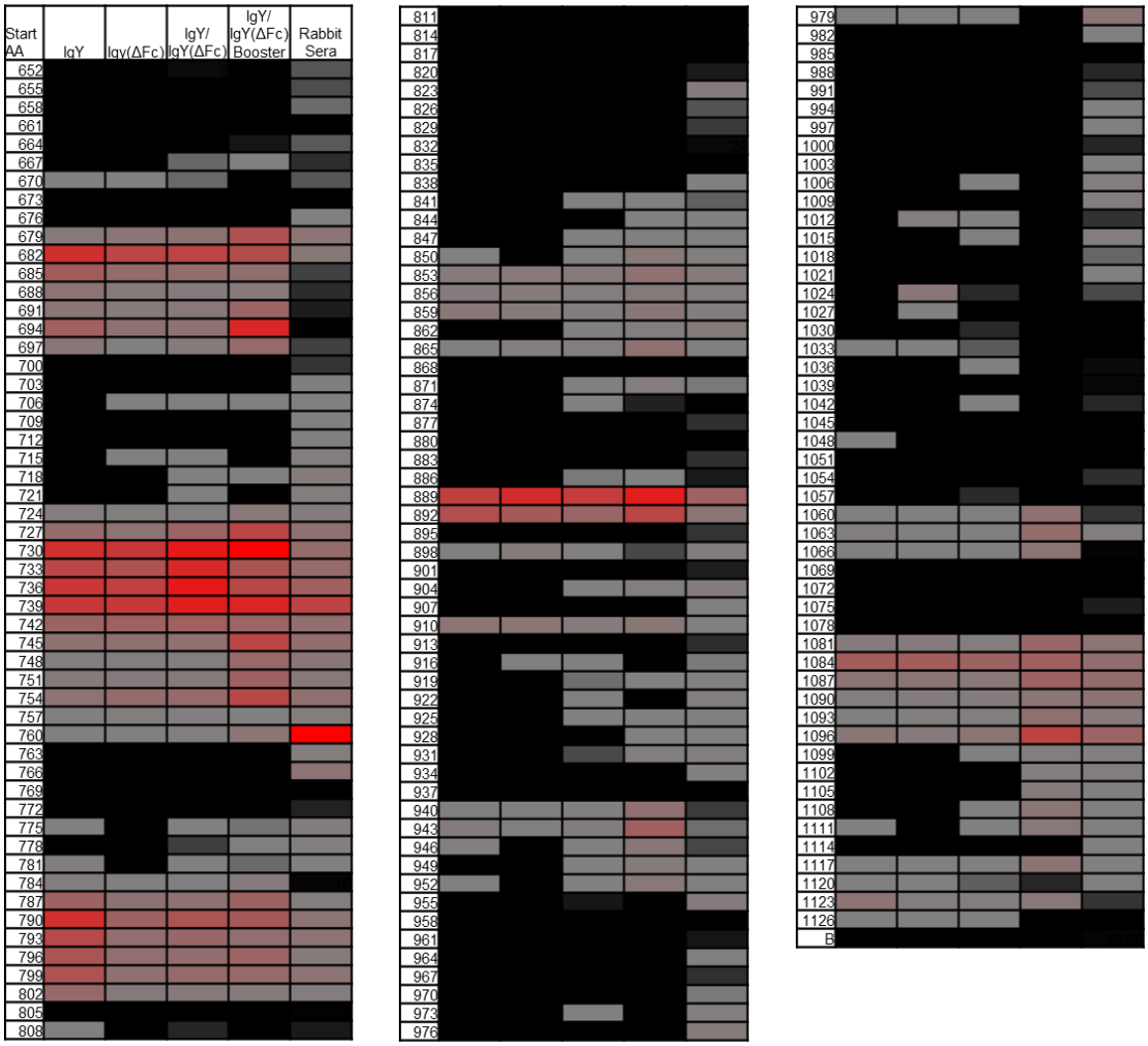

Figure 6. Identification of ANDV glycoprotein G_2 epitopes. Amino acid sequence for glycoproteins of ANDV strain Chile 9717869 used to make 13-mer peptides for microarray slide. G_2 microarray slides were incubated with IgY antibodies, IgY(ΔFc) antibodies, or IgY/IgY(Fc) antibodies isolated from egg yolks of vaccinated geese either after the initial or booster vaccination series, or serum from vaccinated rabbits (*first column*- amino acid peptide starts with, *second column*- IgYantibodies from egg yolks after initial vaccination series, *third column*- IgY(ΔFc) antibodies from egg yolks after initial vaccination series, *fourth column*- IgY/IgY(ΔFc) antibodies from egg yolks after

initial vaccination series, *fifth column*- IgY/IgY(ΔFc) antibodies from egg yolks after booster vaccination, *sixth column*- sera from DNA vaccinated rabbit). Reactivity was measured based on a spectrum ranging from no activity in black, mild reactivity in gray, to strong reactivity in red (E-Empty features).

IgY/IgY(ΔFc) Antibodies From ANDV Vaccinated Geese are Capable of Virus Neutralization *In Vitro*

Based on results from previous experiments showing the ability of IgY/IgY(ΔFc) antibodies from goose eggs to recognize epitopes on the ANDV glycoproteins, we next looked at the neutralizing capabilities of the antibodies. Initially the ANDV-neutralizing abilities of anti-ANDV IgY/IgY(ΔFc) antibodies were determined for serum samples taken from vaccinated geese on weeks 0, 4, 6, 8, 10, and 12 using the *in vitro* PsVNA assay. $PsVNA_{80}$ results show that after the initial vaccination anti-ANDV IgY/IgY(ΔFc) antibodies were highly neutralizing with $PsVNA_{80}$ titers ranging between 1,000 and 10,000 just four weeks into the experiment (Fig. 7). Neutralizing titer levels remained stable during all of the experimental time points, and titers were still detectable at week 12 (Fig 7, right side week 0). The neutralizing titers were also maintained during the year in between immunizations. $PsVNA_{80}$ titers of serum samples taken just prior to long-range booster vaccination were similar to those of the vaccinated naïve birds. Surprisingly, when geese were given a booster vaccination, neutralizing antibody titers of the sera were increased to nearly 100,000 (Fig. 7).

It has already been well established that in geese IgY is transferred from the sera to the egg yolk; because of this we predicted IgY/IgY(ΔFc) antibodies from the egg yolks of vaccinated geese would have similar ANDV neutralizing abilities as sera from vaccinated geese. To test this $PsVNA_{80}$ neutralizing titers were determined for IgY/IgY(ΔFc) antibodies isolated from eggs collected after the initial vaccination series and the long-range booster vaccination. $PsVNA_{80}$ titers for IgY/IgY(ΔFc) antibodies isolated from eggs collected after the initial series of vaccinations were within the same range as the sera from DNA vaccinated geese collected 1,000-10,000 (Fig 8). After long-

range booster vaccination series eggs were collected and IgY/IgY(ΔFc) from these egg yolks reached $PsVNA_{80}$ titers of 100,000 for both geese boosted with pWRG/AND-M(opt) and pWRG/AND-M(opt2) (Fig 8). IgY/IgY(ΔFc) egg yolk $PsVNA_{80}$ titers also correlate with the epitope mapping data that shows increased reactivity to nearly all of the epitopes recognized after the initial immunization, and additional regions of IgY/IgY(ΔFc) antibody binding after booster vaccination. This data suggests that better neutralizing antibodies are made after the booster vaccination series.

ANDV Specific Goose IgY/IgY(ΔFc) Antibodies Treat ANDV Challenged Syrian Hamsters *In Vivo*

Based on the neutralizing capabilities of the goose anti-ANDV IgY/IgY(ΔFc) antibodies *in vitro* and previous success with passive treatment using duck IgY [427], we were interested in testing the goose anti-ANDV IgY/IgY(ΔFc) *in vivo.* To test the *in vivo* therapeutic treatment potential of ANDV specific goose IgY/IgY(ΔFc) antibodies, groups of eight Syrian hamsters were lethally challenged i.m. with 250 LD_{50} doses of ANDV. Five and eight days later the hamsters were passively treated with one of the six following treatments: (1) anti-ANDV rabbit sera from DNA vaccinated rabbits (positive control), (2) anti-ANDV goose sera (from DNA vaccinated geese), (3) anti-ANDV purified IgY/IgY/(ΔFc) antibodies (from the eggs of DNA vaccinated geese), (4) normal goose sera, (5) normal purified IgY/IgY(ΔFc) antibodies, (6) no treatment (Fig 9A). Hamsters that receive normal goose sera, normal purified goose IgY, or no treatment started to die at day 12 with most of them dying by day 14. In contrast, there was 100% survival in the groups of hamsters receiving either the anti-ANDV goose sera or anti-ANDV purified IgY/IgY(ΔFc) antibodies. The 100% survival in the ANDV- specific IgY treatment groups was just slightly better than the 90% survival for the anti-ANDV

rabbit sera, the difference was not statistically significant (Fig 9B). These results demonstrate that anti- ANDV goose IgY/IgY(ΔFc) antibodies are capable of being used as a treatment for ANDV lethal challenge *in vivo* when administered five and eight days post-challenge in Syrian hamsters.

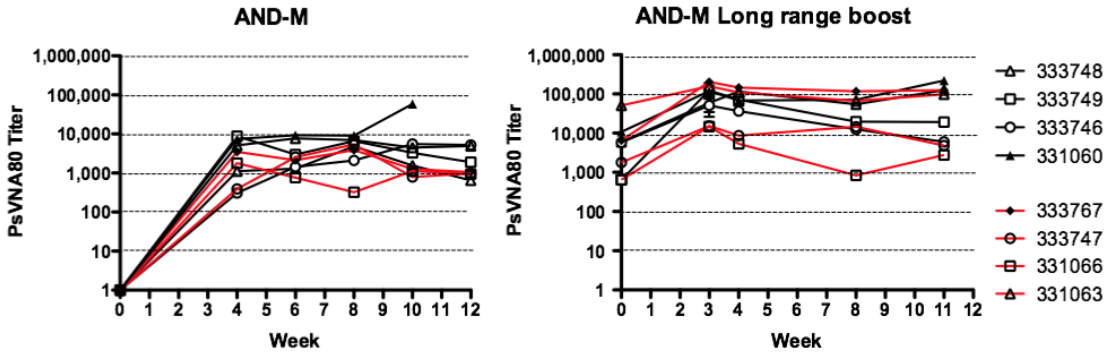

Figure 7. Neutralizing activity of anti-ANDV IgY/IgY(ΔFc) antibodies from serum of ANDV DNA vaccinated geese analyzed *in vitro*. Neutralizing abilities of anti-ANDV IgY/IgY(ΔFc) antibodies in goose sera was measured by PsVNA. On the left, PsVNA were run on sera collected during the initial vaccination series on weeks 0, 4, 6, 8, 10, and 12. On the right, PsVNA were run on sera collected before and after the booster vaccination series on weeks 52, 55, 56, 60, and 63. ID and results for individual animals are shown where Group A has black lines and Group B has red lines. Group A was boosted with pWRG/AND-M(opt) and Group B was boosted with pWRG/AND-M(opt2).

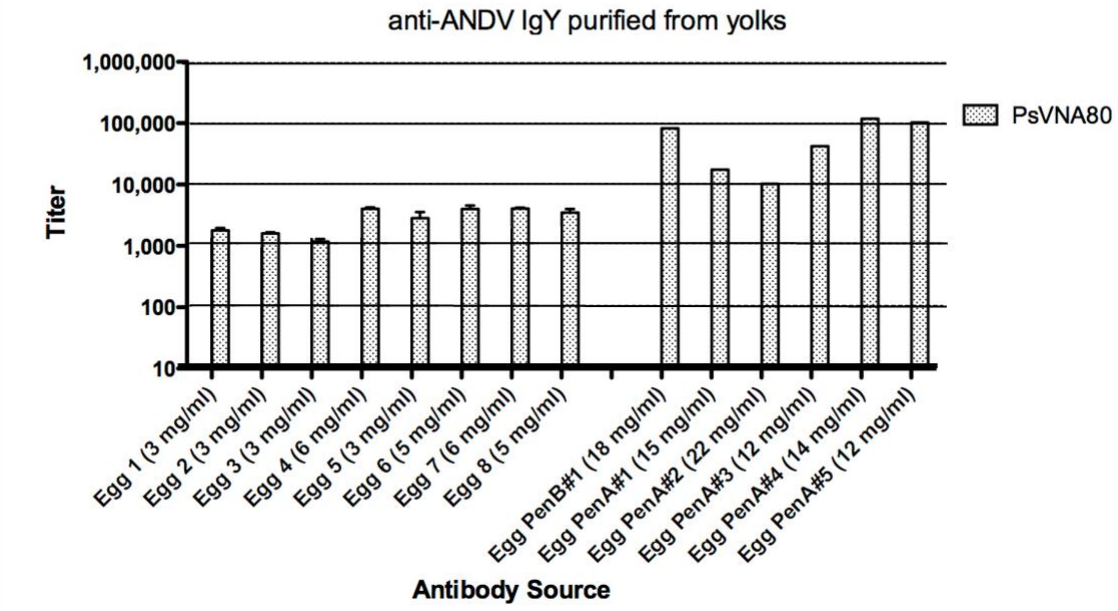

Figure 8. *In vitro* neutralizing activity of anti-ANDV IgY/IgY(ΔFc) antibodies isolated from the eggs of ANDV DNA vaccinated geese. Neutralizing activity of IgY/IgY(Fc) isolated from the individual eggs of geese after initial and booster DNA vaccination was measured by PsVNA. PsVNA values for IgY/IgY(ΔFc) isolated from eggs collected from geese after initial vaccination are to the left of the center and to the right are PsVNA values for IgY/IgY(ΔFc) from eggs collected after booster vaccination. Each sample was run in triplicate and bars represent standard deviation.

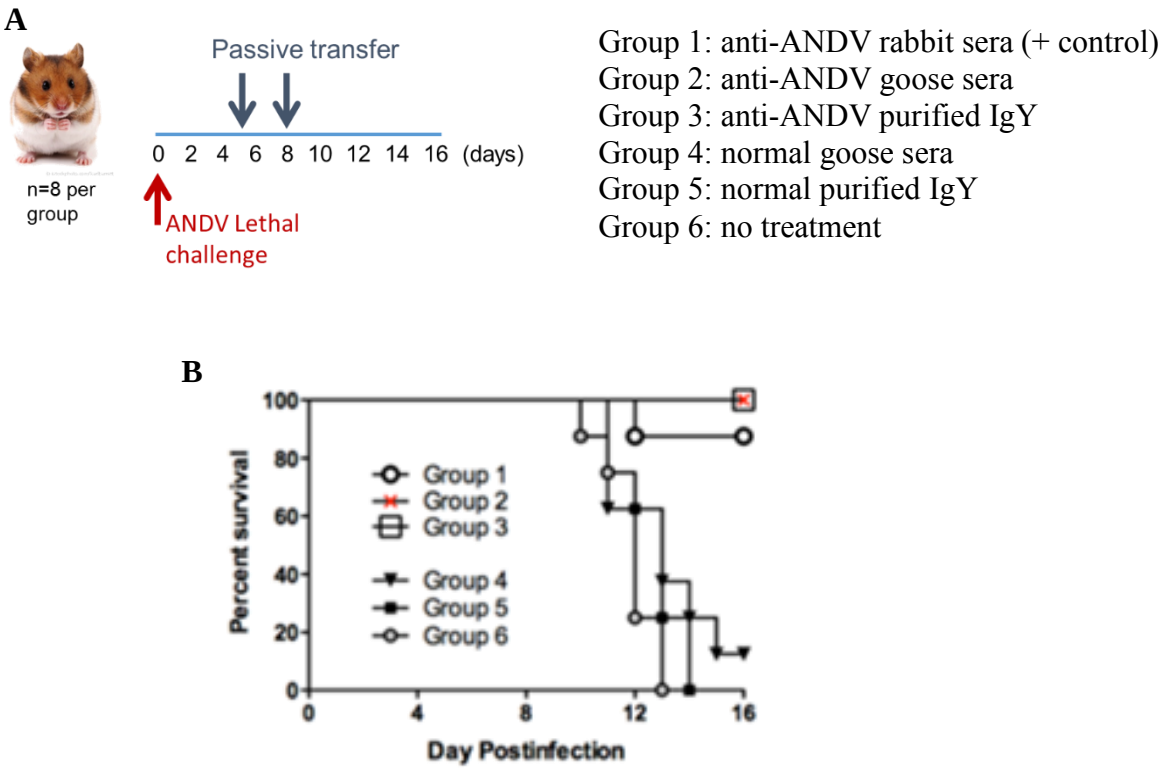

Figure 9. Anti-ANDV IgY/IgY(ΔFc) antibodies purified from goose eggs treats hamsters against lethal ANDV challenge when administered after challenge. A) Experimental design. Six groups of eight hamsters were challenge with 250 LD_{50} of ANDV by i.m. route on day 0. Groups of eight hamsters were then passively treated with the indicated material on days five and eight post-challenge. Sera from a previously ANDV vaccinated rabbit served as a positive control. B) Survival curve of hamsters that were challenged and then passively treated.

Discussion

ANDV has been associated with most HPS cases [363] and continues to be the only hantavirus capable of human-to-human transmission. In spite of the continuing number of HPS cases and the staggering mortality rate of approximately 40%, there are no available treatments or preventative vaccines. The potential for the use of passive treatments to protect against HPS has already been established by previous studies utilizing immune serum from infected patients and isolated antibodies from vaccinated animals in the ANDV/hamster model [425-427]. In addition, research using HPS patients has highlighted the importance of neutralizing antibody production in recovery from infection [423,424]. A clear source of antibodies for passive treatment would be immune plasma from recovered patients, but immune plasma is in short supply, can pose problems of reactogenicity when given to other patients, and can be contaminated with other infectious pathogns. Protective monoclonal antibodies are another option for treatment and have been used with other viral infections [429], but thus far there have been no identified ANDV-neutralizing monoclonal antibodies.

As an alternative source, polyclonal antibodies have been generated against toxins and venoms in vaccinated sheep and horses, but this method has yet to be successful in generating any anti-viral treatments. Avian antibodies are again a logical alternative source of passive therapeutics with the potential to overcome the shortcomings of current therapeutic antibodies be it limitations in source, reactivity to Fc portions of mammalian antibodies, or lack of reactivity with neutralizing epitopes. IgY is the primary serum antibody of birds and is transferred to the egg yolk via receptors on the surface of the yolk membrane, that is specific for IgY antibody translocation, causing the yolk to have

high IgY antibody concentrations [21,25-27,44]. IgY can then be purified from egg yolks and in large quantities for use as therapeutics. Current and previous research using polyclonal avian IgY antibodies has already established a baseline for its therapeutic potential against infectious agents e.g. *Pseudomonas aeruginosa* [66,108,120,430] and *Candida albicans* [431]. IgY antibodies have also been developed against different venoms and antitoxins [202,205,206,432-434]. Most important for our research, DNA vaccinated birds have also been used to produce virus- specific IgY antibodies [427,435,436].

There was a direct relationship between the ANDV neutralizing titers of IgY/IgY(ΔFc) antibodies from both the serum and eggs of vaccinated geese after the initial and booster vaccination series. $PsVNA_{80}$ titers for sera from DNA vaccinated geese increased from a range of 1,000-10,000 just four weeks into the initial vaccination series, to nearly 100,000 three weeks into booster vaccination (Fig 7). In this current study, it has also been shown that neutralizing titers were maintained during the year in between initial vaccination and booster vaccination. Neutralizing titers measured 12 weeks into the initial vaccination series and just prior to booster vaccination stayed in the 1,000-10,000 range for $PsVNA_{80}$ values (Fig 7). These results are important because during a typical response most animals and humans show a decrease in antibody titers a few weeks after vaccination and a drop would certainly be expected a year after vaccination. More importantly, when geese were booster vaccinated, IgY/IgY(ΔFc) antibodies from the sera of some geese reached $PsVNA_{80}$ neutralizing titers of 100,000 (Fig 7). Similar trends were observed for $PsVNA_{80}$ titers of goose anti-ANDV IgY/IgY(ΔFc) antibodies isolated from the eggs of vaccinated geese. After initial

vaccination $PsVNA_{80,}$ titers for individual eggs ranged between 1,000 and 10,000. Booster vaccination resulted in an increase in egg IgY/IgY (ΔFc) antibody titers to nearly 100,000 (Fig 8). In similarly designed experiments Brocato *et al.* reported that DNA vaccination of ducks with pWRG/AND-M resulted in the production of ANDV neutralizing antibodies[427]. But, ANDV specific duck IgY/IgY(ΔFc) antibodies purified from egg yolks of vaccinated ducks only reached neutralizing $PRNT_{80}$ titers of 10,240. These neutralizing titer values were lower than the neutralizing ANDV goose IgY antibody titers obtained in this study. Current results point out the advantage of administering a long-range booster vaccination in the use of pWRG/AND-M and that IgY/IgY(ΔFc) antibodies purified from egg yolks after booster vaccination represent a better option for post-exposure IgY antibody treatments due to the increased neutralizing capabilities of the antibodies.

Epitope mapping completed using IgY/IgY(ΔFc), IgY, and IgY(ΔFc) antibodies purified from egg yolks of vaccinated geese identified several regions of reactivity on the ANDV surface glycoproteins G_1 and G_2. Some of these regions are likely to represent neutralizing ANDV epitopes. In other hantavirus infections, neutralizing activity has been related to antibodies directed to the surface proteins. Monoclonal antibodies to G_1 and G_2, but not to N, have been shown to neutralize viral infection *in vitro* [437]. This matches what is seen in natural hantavirus infections in humans, where antibodies are made predominantly against the N protein, followed by the glycoproteins [438,439].

From the initial vaccination there were 11 epitopes recognized by IgY/IgY(ΔFc) antibodies and after the long-range booster vaccination there were an additional nine epitopes recognized by IgY/IgY(ΔFc) antibodies. There were two regions with the

highest reactivity; for all IgY antibody treatments, spanning peptides starting with aa 613-625 in G_1 and aa 727-742 in G_2. Looking at the predicted structure of the ANDV glycoproteins as part of a mature virion, aa 613-625 are part of the endomembrane domain prior to the WAASA cleavage site. This cleavage site is found at aa 647-651, where the glycoprotein precursor is cleaved into the two glycoproteins G_1 and G_2 [428]. The secondary structure of this region is predicted to be primarily α-helices. For the G_2 glycoprotein's highly reactive region, aa 727-742, is within the outer membrane domain of the protein and the secondary structure primarily made of β-sheets and random coils. The highly reactive regions in glycoproteins G_1 and G_2 do not overlap with regions previously identified as reactive by serum from human ANDV HPS patients, or serum from naturally infected rodents [428]. In addition, the highly reactive IgY antibody epitopes do not overlap with regions recognized by monoclonal antibodies (mAb) which neutralize other hantaviruses [440-442]. The only overlap with human and rodent epitopes was seen with IgY/IgY(ΔFc) anitbodies from egg yolks after long-range booster vaccination, specifically aa 691-710. After initial vaccination, this region was hardly recognized by IgY/IgY(ΔFc) antibodies, but after long–range booster vaccination IgY/IgY(ΔFc) antibody reactivity increased, especially the peptide starting with aa 694, which reached the highest reactivity potential (Fig 6). This is a potentially immunodominant domain since it is being recognized by antibodies from multiple species. The other two highly reactive regions recognized by the IgY/IgY(ΔFc) anitbodies from the egg yolks of booster vaccinated geese, represent novel, potentially neutralizing epitopes on the ANDV glycoproteins that can be used in the future to develop mAb therapies.

Future work will focus on using results from the epitope mapping experiments to develop cocktails of ANDV neutralizing mAbs. A treatment that has the potential to be more effective could be developed by specifically targeting epitopes that are neutralizing. This can lower the total antibody dose that needs to be administered and in turn lowers the chance of any adverse reactions to the treatment. This, coupled with the decreased reactogenicity due to the natural properties of goose IgY antibodies, leads to the opportunity to develop an all-around better treatment option for ANDV and other viral agents. This potential has been previously explored in our lab with Dengue virus and West Nile Virus (data not shown).

Previous research has already established the success of passive treatments for ANDV infection utilizing sera from animals vaccinated against ANDV. When passively transferred to Syrian hamsters one day prior to lethal challenged with ANDV (250 LD_{50} i.m.), serum from rehesus macaques vaccinated against ANDV was able to protect against infection [425]. The same serum was also able to reverse ANDV infections when administered either four or five days after challenge [425]. During this study the post-exposure treatment potential of plasma from a Chilean ANDV HPS patient that was collected during the convalescent-phase was also compared. When the convalescent plasma was administered at four or five days post-challenge only 50% of hamsters survived. Through previous survival studies in ANDV challenged hamsters using IgY/IgY(ΔFc) antibodies from the eggs of ducks vaccinated with pWRG/AND-M, it was concluded that high doses of ANDV- specific IgY/IgY(ΔFc) antibodies administered within eight days of exposure to virus is sufficient to contain and suppress the infection [427]. Notably, in this research, anti-ANDV goose IgY/IgY(ΔFc) antibodies were

administered at five and eight days post-challenge with ANDV in hamsters and there was 100% survival, while treatment with non-specific IgY antibodies, or no treatment led to death of nearly all the hamsters in those treatment groups (Fig 9).

These results demonstrate the *in vivo* efficacy of ANDV specific goose IgY/IgY(ΔFc) antibodies and the successful coupling of DNA vaccination with the extraction of therapeutic antibodies from the eggs of vaccinated geese. ANDV specific goose IgY/IgY(ΔFc) antibody treatments had a slightly better survival percentage than the anti-ANDV rabbit sera treated group. Therefore it is possible that antibodies from vaccinated animals have better virus neutralizing capabilities than those found in human convalescent serum, or that the antibody response is being directed towards highly neutralizing epitopes as a result of vaccination. To further support this, Brocato *et al.* also showed that IgY/IgY(ΔFc) antibodies isolated from the eggs of vaccinated ducks had higher plaque reduction values at lower concentrations than fresh frozen plasma (FFP) from a survivor of ANDV HPS[427].

This research demonstrates the potential to use goose IgY/IgY(ΔFc) antibodies as a post-exposure treatment for HPS caused by ANDV. Results both *in vitro* and *in vivo* clearly show the viral specificity and neutralizing capabilities of the anti-ADNV goose IgY antibodies. The decreased reactivity of IgY antibodies in the mammalian system makes it an ideal alternative to the rare and potentially dangerous human FFP.

CHAPTER IV

SAFETY OF GOOSE IGY FOR USE IN THE MAMMLIAN SYSTEM

Introduction

It has been demonstrated that avian IgY/IgY(ΔFc) antibodies do not interact with the human complement system, rheumatoid factor (RF), or bacterial pathogens that are Fc-binding, and generate less of a human anti-murine antibodies (HAMA)/anti-drug antibody (ADA) response than mammalian IgG antibodies. This lack of reactivity in the mammalian system is mostly due to the structure of the Fc portion of avian IgY/IgY(ΔFc) antibodies and how it differs from mammalian IgG.

HAMAs form often when antibodies are used for treatment or imaging; the murine antibody is seen as a foreign protein by the human body, which triggers an immune response resulting in the formation of HAMAs. In some cases HAMAs have also been found in the serum of patients who have not been treated with antibodies [66]. The HAMA response can persist in the blood for several months, and can be easily reactivated if memory B-cells are formed. This response can interfere with assays used in diagnosis and/or surveillance of disease as well as cause potentially painful symptoms for the patients. Even engineered antibodies have protein epitopes that can be seen as immunogenic by the immune system and ultimately cause an antibody response by the body. The HAMA/ADA responses within the body can result in a variety of symptoms from more mild allergic reactions, generalized pain, hyponatremia, fever, rigors, chills,

rash, paresthesia, weakness, chronic refractory postural hypotension [67,68], to hypersensitivity reactions that range from serum sickness with urticarial and bronchosapsms [68-72] to anaphylactic shock [73-75].

The structural differences between mammalian IgG antibodies and avian IgY antibodies are especially important in their interactions with complement proteins and RF. The heavy chain of the full length IgY antibody is made up of one variable domain and four constant domains, three of which make up the Fc region. The alternatively spliced (IgY(ΔFc)) antibody isotype has one variable domain and one constant domain [44]. In contrast, the heavy chain of mammalian IgG antibody has one variable domain and only three constant domains, two of which make up the Fc region [44]. The interaction of mammalian IgG antibody with complement proteins and RF can result in unwated inflammatory reponses in the body. When the complement pathway is inappropriately activated it can lead unnecessary inflammatory reactions. The anaphylatoxins, C4a, and C5a, products of the complement pathway, are often responsible for initiating parts of these inflammatory responses. Anaphylatoxins are able to stimulate mast cells to trigger IgE-independent histamine release as well as TNF-α release [89], wich can mimic allergic shock depending on the number of mast cells involved [90,91]. In humans, a Serum sickness-like illness can also develop depending on the number of complement immune complexes formed during the course of treatment.

Most studies on the differences in the reactivity of avian, compared to mammalian, immunoglobulin have been performed *in vitro.* In ELISAs comparing goat and chicken antibodies as capture antibodies to normal human serum (NHS), it has been shown that complement activation blocked up to 50% of antigen binding when using goat

antibodies, but an active complement system had no effect on antigen binding when chicken antibodies were used as a capture antibody [94]. In sandwich ELISAs used for the detection of antigens in human samples, the anti-mammalian IgG antibody RF can interfere and cause false positive results. These false positives can be avoided by the use of avian IgY antibodies [84].

The aim of this research is to determine the reactivity of goose IgY/IgY(ΔFc) and IgY(ΔFc) antibodies within the mammalian system utilizing *in vitro* peripheral blood monocyte assays, neutrophil assays, and *in vivo* injection studies to look at organ pathology for any damage due to inflammation. Previous research has proven the effectiveness of goose IgY/IgY(ΔFc) antibodies as potential treatments. The next step to using goose IgY/IgY(ΔFc) antibodies as human passive antibody treatments is to establish their safety. We show here for the first time *in vitro* and *in vivo* experiments testing the reactivity of IgY within the mammalian system.

Materials and Methods

Mice

Mice were injected intravenously (i.v.) with either 200μg of goose IgY in 200μL of 1X PBS pH 7.4 (Invitrogen, Carlsbad, CA, USA) or 200uL of 1X PBS alone on experimental day 0. Tissues were collected from IgY injected mice on experimental days 0, 6hr, 2, 4, 7, 14, and 28. Tissues were collected from control injected mice, PBS alone, on experimental days 0 and 14. On day 28 mice were euthanized and the following tissues were collected: brain, heart, lung, liver, spleen, and kidney. Blood was collected from mice prior to euthanization.

Histology

Mouse tissues were fixed in a 10% neutral buffered formalin solution (Fisher Scientific, Hampton, NH, USA). Fixed tissues were sent to AML laboratories (Baltimore, MD, USA) for paraffin embedding, sectioning, and hematoxylin and eosin staining. Stained tissues sections were then viewed and scored by a blinded pathologist.

Rabbits

Thirty adult female rabbits were divided into three experimental groups and one control group. Rabbits in experimental group one were injected with 10mg of commercially available human anti-rabies antibody HRIG, HyperRAB® (Grifols, Los Angeles, CA, USA). Rabbits in experimental group two were injected with 10mg of total goose anti-rabies IgY antibodies (IgY/IgY(ΔFc)). Rabbits in experimental group three were injected with goose anti-rabies IgY(ΔFc). Rabbits in the control group were inject with 1X PBS, p.H. 7.2. Treatments were administered via i.m. injections on experimental days 0, 7, 14, 28, and 42. Sera was collected from treated rabbits on experimental days 0, 14, 28, and 42. Four weeks after the last injection, experimental day 70, rabbits were terminally bled and euthanized. The spleen, thymus, lymph nodes, kidneys, hearts, liver, and brain were collected, fixed, H&E stained, and scored by a blinded pathologist.

Human PBMC Cell Culture

Peripheral blood monocytes (PBMCs) were isolated from whole blood of healthy human donors and resuspended at a concentration of $2.0x10^6$ cells/mL in RPMI 1640 culture media supplemented with 2mM L-glutamine, 10mM HEPES, 100ug/mL streptomycin, and 100U/mL penicillin. Then, 500µL of the cell suspension was added to

wells of a 24-well tissue culture plate. Next, 500µL of cell treatment at twice the final concentration in culture media or an additional 500µL of cells (negative control) was added to the wells. Cells were treated with lipopolysaccharide (LPS) (10µg/mL), mammalian IgG antibody (10µg/mL), and goose IgY/IgY(ΔFc) antibody (10µg/mL). Plates were incubated for 72 hours at 37ºC with 5% CO_2. Culture supernatants were collected at 12, 24, 48, and 72 hours and frozen for later cytokine analysis.

Human Neutrophil Cell Culture

Neutrophils were isolated from healthy human blood using Histopaque (Sigma Aldrich, MO, USA). Isolated neutrophils were resuspened in RPMI 1640 containing 1% BSA at a concentration of $5x10^5$ cells per mL. Then, 100uL of cell suspension was added to wells of a 96-well tissue culture plate. Cells were treated with PMA diluted 1:1000 (positive control), culture media alone (negative control), LPS (10µg/mL), mammalian IgG antibody (10µg/mL), and goose IgY/IgY(ΔFc) antibody (10µg/mL). Plates were incubated for 72 hours at 37ºC with 5% CO_2. Culture supernatants were collected at 12, 24, 48, and 72 hours and frozen for later analysis. Elastase levels in culture supernatants were quantified using a fluorometric assay kit (Cayman Chemical, Ann Arbor, MI, USA).

Cytokine Analysis

Cytokine levels in culture supernatants were quantified by ELISA using commercially available kits. The IL-1β kit was obtained from R&D systems, Minneapolis, MN. TNF-α and IL- 10 levels were quantified using BD Opt-EIA™ ELISA kits (BD Biosciences, San Diego, CA, USA). Nitric oxide (NO) levels in the culture supernatant were measured through a Griess assay utilizing a commercially available kit from Promega, Madison, WI. The colorimetric change of each well was

determined at various optical densities using a Thermomax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical Analysis

Statistical analysis was carried out using the two-way ANOVA and Bonferroni's multiple comparison tests. *P value* <0.05 was considered statistically significant unless otherwise noted. Data was plotted as calculated sample means with standard deviation.

Results

Injection of IgY/IgY(ΔFc) Antibodies Does Not Induce Abnormal Organ Pathology *in Vivo*

The effects of single and multiple injections of avian IgY/IgY(ΔFc) antibodies on organ pathology was examined *in vivo*. In the single injection study, mice were injected with either goose IgY/IgY(ΔFc) antibodies (n=35) or 1X PBS (n=10) via tail vein injection on experimental day 0. Organs collected from the experimental mice, included the brain, heart, lung, liver, spleen, and kidney; tissue was fixed, stained with H&E, and reviewed by a blinded pathologist. Of the six organs that were collected, there were no abnormal changes found in the brain, heart, liver, kidney, or spleen. The only tissue that showed any abnormalities was the lungs. Pulmonary hemorrhages were present in a large number of mice, across both treatment groups. The frequency and severity of hemorrhages in the lungs was increased on days 14 and 28 (Table 1).

Table 1. Pathology results from single injection study in mice.

PBS		**Brain**	**Heart**	**Liver**	**Spleen**	**Kidney**	**Lung**
	0 hours	-/-[1]	-/-	-/-	-/-	-/-	2FH/2FH[2]
	14 days	-/-	-/-	-/-	-/-	ARP/ARP[3]	2FH/2FH
IgY/IgY(ΔFc)		**Brain**	**Heart**	**Liver**	**Spleen**	**Kidney**	**Lung**
	0 hours	-/-	-/-	-/-	-/-	-/-	-/-
	6 hours	-/-	-/-	-/SC[4]	-/-	-/-	FH/FH
	2 days	-/-	-/-	-/-	-/-	-/-	3FH/3FH
	4 days	-/-	-/-	-/-	-/-	-/-	2FH/2FH
	7 days	-/-	-/-	-/-	-/-	-/-	3FH/3FH
	14 days	-/-	-/-	-/-	-/-	-/-	3FH/3FH, 1MH/1MH[5]
	28 days	-/-	-/-	-/-	-/-	-/-	4FH/4FH, 1MH/1MH

Results are shown for two pathology slides from a sample size of five mice for each experimental time point.
[1] no change
[2] focal hemorrhage (FH) in two of five mice
[3] acute inflammation of renal pelvis (ARP) in one of five mice
[4] small cyst (SC)
[5] moderate hemorrhage (MH) in one of five mice

Rabbits were injected in a multiple injection study, receiving five injections over a six-week period with either goose anti-rabies IgY/IgY(ΔFc), goose anti-rabies IgY(ΔFc), human anti-rabies antibodies (HRIG), or control 1X PBS. Four weeks after the last injection (day 70), all rabbits were euthanized and organs were collected including the spleen, thymus, lymph nodes, kidneys, heart, lungs, liver, and brain. Rabbit tissues were scored by a blinded pathologist for inflammation. Inflammation was almost

exclusively limited to the lungs of experimental rabbits, but there was no difference observed between treatment groups. Overall, the pathology results indicated that rabbits receiving multiple injections with either IgY/IgY(ΔFc) or IgY(ΔFc) antibodies showed no signs of abnormal pathology when compared to the rabbits that received multiple injections of HRIG or 1X PBS (Table 2). The results of the single and multiple injection studies further support the non-inflammatory nature of avian IgY in the mammalian system.

Table 2. Pathology results for organs collected from rabbits in the multiple injection study.

Rabbit	Brain[1]	Thymus[2]	Lung[3]	Heart[4]	Liver[5]	Kidney[6]	Spleen[7]	Treatment
1	0	0	2	0	0	0	0	HRIG
2	0	0	3	0	0	0	0	HRIG
3	0	0	1	0	2	0	0	HRIG
4	0	0	0	0	0	0	2	HRIG
5	0	0	0	0	0	0	0	HRIG
6	0	0	2	0	0	0	0	HRIG
7	0	0	0	0	0	0	0	HRIG
8	0	0	2	0	0	0	2	HRIG
9	0	0	0	0	0	0	0	IgY/IgY(ΔFc)
10	0	0	2	0	0	0	0	IgY/IgY(ΔFc)
11	0	0	2	0	0	0	1	IgY/IgY(ΔFc)
12	0	0	1	0	0	0	1	IgY/IgY(ΔFc)
13	0	0	0	0	0	0	1	IgY/IgY(ΔFc)
14	0	0	1	0	0	2	0	IgY/IgY(ΔFc)
15	0	0	1	0	0	0	2	IgY/IgY(ΔFc)
16	0	0	2	0	1	0	1	IgY/IgY(ΔFc)
17	0	0	0	0	0	0	0	IgY(ΔFc)
18	0	0	0	0	0	0	0	IgY(ΔFc)
19	0	0	2	0	0	0	0	IgY(ΔFc)
20	0	0	1	0	0	0	0	IgY(ΔFc)c
21	0	0	0	0	0	0	0	IgY(ΔFc)
22	0	0	3	0	0	0	0	IgY(ΔFc)
23	0	0	1	0	0	0	2	IgY(ΔFc)
24	0	0	1	0	2	0	1	IgY(ΔFc)
25	0	0	2	0	0	0	0	PBS
26	0	0	2	0	0	0	0	PBS
27	0	0	2	0	0	0	0	PBS

Table 2. Cont.

28	0	0	0	0	0	0	0	PBS
29	0	0	1	0	0	0	1	PBS
30	0	0	2	0	0	0	0	PBS

[1,4,6] Brain, Kidney, and Heart: 0= no change; 1= minimal change; 2= mild change; 3= moderate change; 4= marked changes but restricted extent; 5= marked and widespread changes; 6= very severe, diffuse changes

[3] Lung: 0= no change; 1= minimal change (or possible non-specific background or appearance compounded by lung collapse i.e. not inflated); 2= mild inflammation and/or pneumocyte hypertrophy; 3= moderate inflammation inflation and/or pneumocyte hypertrophy; 4= marked inflammation and/or pneumocyte hypertrophy; 5= severe inflammation and/or pneumocyte hypertrophy < 50% of lung lobe

[7] Spleen: 0= no change; 1= mildly increased extramedullary hematopoiesis (EMH); 2= moderately increased EMH and/or mild inflammation; 3= marked EMF and/or moderate inflammation; 4= marked inflammation; 5= severe inflammation; 6= severe necrotizing inflammation.

IgY/IgY(ΔFc) Antibodies Do Not Induce Notable Proinflammatory Mediator Release From Human PBMCs or Neutrophils

To better understand the reactivity of avian IgY/IgY(ΔFc) in the mammalian system and the capacity of avian IgY/IgY(ΔFc) antibodies to activate human immune cells and initiate an inflammatory reaction, human PBMCs and neutrophils were isolated from healthy human blood and then incubated *in vitro* with mammalian IgG antibodies or avian IgY/IgY(ΔFc) antibodies, and cells incubated with LPS served as a positive control and cells alone as a negative control. Culture supernatants were collected 0, 12, 24, 48, or 72 hours from start of incubation depending on the analysis being completed. Culture supernatants collected from treated PBMCs were analyzed for the release of cytokines, including proinflammatory cytokines IL-1β and TNF-α, anti-inflammatory cytokine IL-10, and nitric oxide (NO).

IL-1β was not detected as a result of IgY/IgY(ΔFc) antibody treatment at 12, 24, and 48 hours post-treatment, with a modest level of IL-1β observed at 72 hours (Fig 10). This is compared to a consistent amount of IL-1β being produced by mammalin IgG

treated PBMCs as indicated by the 5pg/mL concentration present in culture supernatants from 12 hours post-treatment to the final time point, and gnificant amounts of IL-1β generated by LPS treatment of PBMCs. TNF-α was not detected via ELISA in the culture supernatants collected from PBMCs incubated with IgY/IgY(ΔFc) antibodies (Fig 11). In contrast, TNF-α levels from cells incubated with LPS were at high throughout with the highest levels being measured at 72 hours post-treatment. The difference between the LPS treated PBMCs and all other groups was statistically significant. Only at one point, 24 hours post-treatment, did IgG treated PBMCs produce any TNF-α (Fig 11). The difference between IgY/IgY(ΔFc) antibody and IgG antibody treated cells was not significant. The IL-1β and TNF-α results correlate with the pathology results from the single and multiple injection studies, supporting the finding that IgY/IgY(ΔFc) antibody does not cause abnormal inflammatory responses. Levels of the anti-inflammatory cytokine IL-10 were also quantified in culture supernatants from treated PBMCs. No IL-10 was detected by ELISA with all treatments at all time points (data not shown).

Nitrite levels in culture supernatants collected from PBMCs were quantified as an indicator of NO production by the cells. Cells treated with IgY/IgY(ΔFc) antibodies showed a peak in nitrite levels at 24 hours with levels decreasing afterward (Fig 12). IgG antibody treated cells showed a stable increase in nitrite levels from 0 hours all the way up to 72 hours of incubation. There was no noticeable difference in the nitrite levels in culture supernatants collected from cells incubated with IgY/IgY(ΔFc) antibodies compared to those incubated with IgG antibodies. The nitrite levels generated by

IgY/IgY(ΔFc) antibody treatment of PBMCs were well below a level of concern during an inflammatory response.

The potential reactivity of IgY/IgY(ΔFc) with human neutrophils was also assessed. Neutrophils were isolated from the blood of healthy human donors and then co-cultured *in vitro* with mammalian IgG antibodies, avian IgY/IgY(ΔFc) antibodies, PMA (which is known to activate neutrophils leading to elastase release), and LPS. Untreated cells, served as a negative control. Activation of neutrophils by the various treatments was determined by the amount of elastase present in neutrophil culture supernatants at 12, 24, and 72 hours after treatment. Treatment of cells with IgY/IgY(ΔFc) antibodies resulted in the release of elastase by neutrophils, but levels were lower than all other treatments at 12 hours and slightly lower than all other groups at 72 hours as well (Fig 13). There was no difference in the release of elastase between neutrophils treated with mammalian IgG and those treated with IgY/IgY(ΔFc).

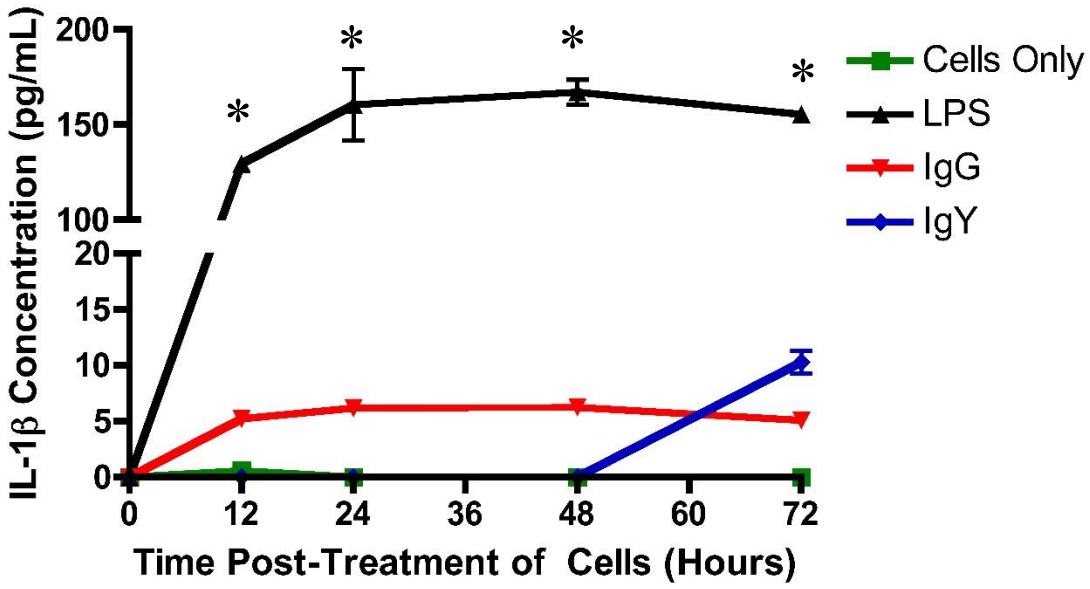

Figure 10. IL-1β concentration in culture supernatants from treated human PBMCs. IL-1β levels were analyzed in culture supernatants collected from PBMCs stimulated with mammalian IgG antibodies, goose IgY/IgY(ΔFc) antibodies, LPS (positive control), or cells only (negative control). IL-1β levels were quantified using an ELISA. Culture supernatants were collected at hours 0, 12, 24, 48, and 72 hours post-treatment of cells. Each data set is a mean of three samples with the bars representing the standard deviation. Two-way ANOVA and Bonferroni's Multiple Comparison tests were completed for statistical analysis. (*) indicatates significant distances between IgY/IgY(ΔFc) treatment of cells and LPS treatment of cells ($P < 0.001$).

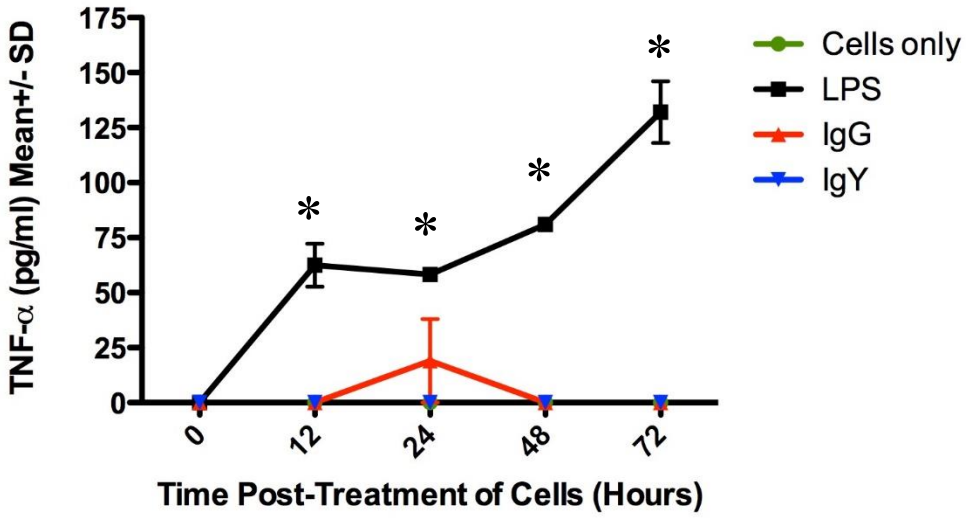

Figure 11. TNF-α levels in culture supernatants from treated human PBMCs. PBMCs were treated with either goose IgY/IgY(ΔFc) antibodies, mammalian IgG antibodies, LPS, or left untreated. Culture supernatants were collected 0, 12, 24, 48, and 72 hours after incubation. TNF-α levels in the culture supernatants were quantified using an ELISA. (*) indicates a significant difference between IgY/IgY(ΔFc) treated PBMCs and LPS treated PBMCs (P< .0001). Statiscal analysis completed was a Two-way ANOVA and Bonferroni's Multiple Comparison tests.

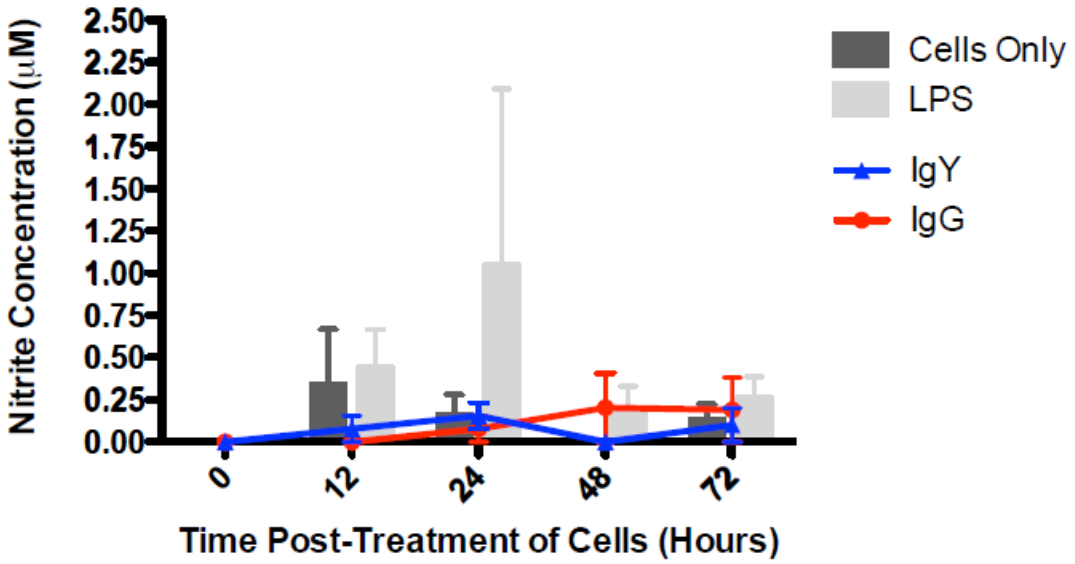

Figure 12. Nitrite concentration in culture supernatants from treated human PBMCs. A Griess assay measuring nitrite concentrations in culture supernatants was used as an indicator of the amount of nitric oxide released by treated PBMCs. Culture supernatants were collected from PBMCs treated with goose IgY/IgY(ΔFc) antibodies, mammalian IgG antibodies, LPS, or untreated cells. Nitrite levels in the culture supernatants collected at 0, 12, 24, 48, and 72 hours post-treatment were quantified using a Griess assay; absorbance was read at an OD between 520 to 540nm. The mean for each data point is shown, with the bars representing the standard deviation.

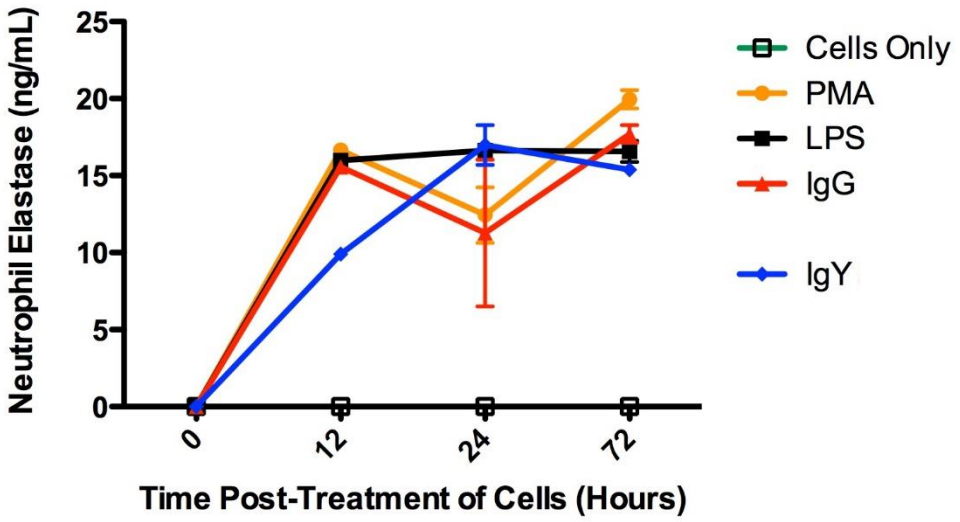

Figure 13. Neutrophil elastase levels in cultured supernatants from treated human neutrophils. Culture supernatants were collected from human neutrophils treated with IgY/IgY(ΔFc) antibodies, mammalian IgG antibodies, PMA (positive control), LPS (positive contro), or untreated cells (negative control) 12, 24, and 72 hours after treatment. Neutrophil elastase levels were quantified using a kit containing a non-flourescent elastase substrate, which is selectively cleaved by elastase to generate a highly fluorescent compound R110 that can be read at an excitation wavelength of 485nm and an emission wavelength of 525nm. Each data point represents a mean ± standard deviation.

Discussion

In this study we present data to support the structural evidence that the use of avian IgY and IgY(ΔFc) antibodies in the mammalian system does not result in abnormal inflammatory responses. The Fc portion of the mammalian antibody serves an important role in triggering many biological responses such as phagocytosis, endocytosis, antibody-dependent cellular cytotoxicity, and release of inflammatory mediators, but inappropriate interaction of the Fc portion of antibodies with FcR can result in unnecessary inflammatory reactions. These types of inflammatory reactions are sometimes seen when mammalian antibodies are used as passive antibody treatments. HAMA and ADA responses can also form, resulting in an array of painful symptoms. Structurally the Fc portion of avian IgY antibody is different than that of mammalian IgG antibody and is unable to interact with mammalian FcγR and activate immune pathways that can lead to unwanted inflammation [88,94]. Current and previous research has demonstrated that avian IgY/IgY(ΔFc) antibodies isolated from the eggs of vaccinated birds can be used effectively as a passive treatment against various antigens including bacteria and viruses. In our lab specifically, we have investigated and proven the effectiveness of goose IgY/IgY(ΔFc) antibodies *in vivo* as post-exposure treatment for hantavirus pulmonary syndrome caused by the Andes virus. The purpose of this study was to determine the reactivity of avian IgY/IgY(ΔFc) antibodies and IgY(ΔFc) antibodies in the mammalian system to ensure its safety moving forward towards an increased use in humans.

In both the single and multiple injection studies animals receiving IgY/IgY(ΔFc) antibodies or IgY(ΔFc) antibodies did not show any abnormal pathology or inflammation compared to control animals injected with PBS or HRIG. The only organ to show any

consistent abnormal pathology in both studies was the lungs. Abnormalities observed in the lungs were not dependent on treatment. Mice or rabbits in all treatment groups (IgY, PBS, or HRIG) developed hemorrhages or inflammation in the lung depending on how it was characterized in the study. In the single injection study, hemorrhaging did get worse at later time points, but again the severity and frequency of occurrence was the same between the control and experimental groups. The abnormal pathology in the lungs is therefore not dependent on treatment with IgY, PBS or HRIG specifically.

In this study we were able to show that IgY/IgY(ΔFc) antibody is safe for use in humans. Goose IgY/IgY(ΔFc) antibodies were incubated *in vitro* with humans PBMCs and the production of select pro- and anti-inflammatory mediators was measured. TNF-α and IL-1β are both proinflammatory cytokines produced by various immune cell types within the PBMC cell population. Both of these cytokines are produced early in the immune response. TNF-α is produced primarily by macrophages and promotes inflammation, activation of endothelial cells and allows extraversion of dendritic cells to the lymph node, further augmenting the immune response. IL-1β is produced by dendritic cells and macrophages; it can help to activate other immune cells including T-cells. Our results indicate the PBMCs treated with goose IgY/IgY(ΔFc) antibodies produce only negligible amounts of IL-1β and no TNF-α compared to the positive control (Fig 10 and 11). These results provide further evidence that IgY/IgY(ΔFc) antibody is safe for use within humans. IL-10 is an anti-inflammatory cytokine able to inhibit activation and effector function of T-cells, monocytes, and macrophages. Ultimately IL-10 is responsible for terminating inflammatory responses, but also plays a role in regulating growth and differentiation of some immune cell populations [443]. When

cells were treated with IgY/IgY(ΔFc) antibodies there was no IL-10 detected in culture supernatants collected. There was also no IL-10 produced by any other of the cell treatments. It is unlikely that IgY/IgY(ΔFc) antibody is immunomodulatory and able to induce an anti-inflammatory environment.

The activity of NO in inflammation is less well understood than that of the cytokine previously described. It is known that NO is released by activated macrophages and is known to regulate the activity, growth, and death of many immune and inflammatory cells including macrophages and T-lymphocytes [444]. Production of NO by PBMCs is indicative of immune cell activation and an ongoing inflammatory response. There was no difference between the NO levels of PBMCs treated with mammalian IgG and those treated with IgY/IgY(ΔFc) antibodies. Although there were low levels produced by cells treated with IgY/IgY(ΔFc) antibodies, the effects of NO are dependent on local concentration and levels produced by treated cells were low enough to not be of concern.

In order to look at the reactivity of IgY/IgY(ΔFc) antibodies with another population of innate immune cells, elastase release from activated neutrophils was measured. Neutrophils are relatively short lived white blood cells that can function in several ways at the site of inflammation including phagocytosis, cytokine release, and degranulation [445]. Neutrophil elastase is an active enzyme stored within the granules of neutrophils until the cells are activated. Upon activation neutrophil elastase can be excreted from the cell as a free protein or be associated with networks of extracellular traps (NETs) [446]. Neutrophil elastase is a serine proteinase that can attack a number of host proteins outside of the neutrophil and can have a damaging effect on host tissues if

release is triggered unintentionally [447]. Previous research seeking neutrophil elastase inhibitors alluded to the ability of mammalian IgG being able to actually induce NET formation [448]. It was therefore important that we test the ability of IgY/IgY(ΔFc) antibodies to activate neutrophils and lead to elastase release. Treatment of neutrophils with IgY/IgY(ΔFc) anitbodies led to elastase release, but it took until 24 hours post-treatment to reach levels similar to mammalian IgG (Fig 13). The reason for what appears to be more activation of human neutrophils over immune cells present in the human PBMC population is unknown. A better understanding of the direct interaction of IgY antibodies with mammalian cells is required.

This research demonstrates that IgY/IgY(ΔFc) antibodies are safe for use in the mammalian system. The single and multiple injection studies along with the *in vitro* cytokine data clearly support the structural evidence that IgY/IgY(ΔFc) and IgY(ΔFc) have limited immunoreactivity within the mammalian system. Based on these results, IgY continues to remain a viable option for use as passive antibody immunotherapeutics.

CHAPTER V

DISCUSSION

In recent years there has been an increased interest in avian-derived antibodies as a unique source of passive therapeutics. Avian antibodies have the potential to extend beyond current passive antibody therapeutic technology that primarily utilizes mammalian antibodies. The majority of mammalian antibodies are of murine origin and come with the risk of high immunogenicity in humans. IgY is the primary serum immunoglobulin of birds and is transferred to the egg yolk during embryo development; this results in the egg yolk having high IgY concentrations [21,44]. The egg yolk then becomes an easily accessible source of antigen- specific IgY antibodies. Most past and presently researched avian antibody treatments are polyclonal in origin; in an attempt to move IgY antibody therapeutics in a new direction part of this current research focused on the development of a goose monoclonal IgY antibody that could be used to treat DENV-2 infections. Currently, monoclonal antibody therapeutics are used to treat transplant rejection [449-451], cancer [452-458], and autoimmune disorders [459-464]. Surprisingly, the use of these treatments for infectious diseases is limited [465]. The advantages of using avian monoclonal antibodies are three fold: the antibodies produced by the hybridoma are homogenous, the response generated is consistent, and the need to collect raw materials from SPF birds is eliminated. In the present study, we were able to successfully complete the initial stages of hybridoma formation and mAb production, but

fusions did not last over time. To the best of our knowledge this is the first demonstration of successful fusion between avian B-cells and mammalian myeloma cells. Previous research in the area of avian monoclonal antibody development has utilized chicken myeloma cell lines fused with immune chicken spleen cells [339,341,342]. Despite being unable to develop stable long lasting hybridomas we were able to successfully show that the vaccinated geese did generate antigen- specific immune responses, including the production of antibodies, as indicated by the presence of antigen-specific antibodies in goose serum.

Polyclonal avian therapeutics have been developed utilizing chicken IgY antibodies against various antigenic targets, but the use of IgY antibodies from anseriformes birds has been limited to only a limited number of viral antigens [427,435]. In our research we focused on ANDV and the development of a polyclonal goose IgY antibody treatment for HPS caused by ANDV. It is well established that the humoral immune response is important for a favorable clinical outcome from HPS [350,422-424]. Consistent with findings that virus- specific antibodies play an important role in patient recovery from HPS, when ANDV- specific goose IgY/IgY(ΔFc) antibodies were given to hamsters after challenge with a lethal dose of ANDV all of the hamsters survived.

The neutralizing titers of ANDV specific IgY/IgY(ΔFc) antibodies in the serum of vaccinated geese provided insight into the humoral response of geese. Most interestingly, neutralizing titers were maintained during the year in between vaccinations. It is unknown if this type of response is typical of all birds and to all antigens. After booster vaccination neutralizing titers were nearly ten fold higher. Along with the increase in neutralizing titers there was also an increase in reactivity of IgY/IgY(ΔFc) antibodies

isolated from eggs of booster vaccinated geese with epitopes on the surface of both glycoproteins. The increase in titers and reactivity with epitopes suggests that collecting antibodies after a booster vaccination series may provide an improved passive IgY antibody treatment with better neutralizing capabilities.

For the epitope mapping experiments, IgY/IgY(ΔFc) antibodies from the egg yolks of geese, were separated into the full length (IgY) antibody and alternatively spliced (IgY(ΔFc)) antibody isotypes, after the initial vaccination series. In general, the two isotypes reacted with same epitopes on the virus glycoproteins, but there are potential advantages being able to use only the IgY(ΔFc) isotype. In the avian system IgY(ΔFc) is unable fix complement or sensitize the tissues to allergic reactions, functions that are thought to require the Fc portion of the IgY isotype antibody [49]. Even without the ability to carry out Fc-mediated functions IgY(ΔFc) antibody has still retained its ability to neutralize viruses within the avian system [44]. Because it is lacking the two constant domains compared to the full length isotype and one constant domain compared to mammalian IgG, reactivity within the mammalian system is likely to be limited. Results from our *in vivo* multiple injection study indicated there was no abnormal pathology in rabbits injected with IgY(ΔFc). Future studies looking directly at the reactivity of the IgY(ΔFc) isotype with human PBMCs may be useful to gain more information on the inflammatory potential of this specific isotype of IgY.

Regardless of the type of avian antibody treatment being used and the target, the safety of the treatment for use in the human body is important. In our study we were able to show through *in vivo* single and multiple injection studies that treatment of animals with IgY/IgY(ΔFc) or IgY(ΔFc) antibodies did not result in any abnormal pathology or

signs of inflammation compared to control animals. Also, IgY/IgY(ΔFc) antibodies did not induce inflammatory cytokine production by human PBMCs, another indication of the safety of IgY within the mammalian system. The positive safety results along with the success of the ANDV- specific IgY/IgY(ΔFc) antibodies as a post-exposure treatment for HPS caused by ANDV continues to advance the field of avian IgY antibody treatments towards more human trials.

An important next step in the area of avian antibody treatments is to identify the receptor on cells in the mammalian system, specifically humans, with which IgY and IgY(ΔFc) antibodies are able to interact with. Knowing this information would be helpful in determining what mechanisms IgY and IgY(ΔFc) antibodies use for clearance of antigens and to generate protection. Also, information about the receptor would be useful to better understand and test for the potential reactivity of the antibody within humans, and how potentially the antibody could be modified and still be functional.

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