IMMUNE ENHANCEMENT MECHANISMS BY THE COMPLEMENT PROTEIN C3d

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The final degradation product of the third (C3) component of complement, C3d, is a natural adjuvant. The adjuvant properties of this protein have been studied using several antigens fused to C3d. To determine the ability of this protein to enhance the humoral immune response in mice with different genetic backgrounds, inbred and outbred mice were immunized with C3d conjugated antigens. C3d induced enhanced immune responses that were comparable in all mouse strains. Analysis of the isotype class switch suggested that C3d favored the development of humoral immune responses. The classic mechanism of enhancement of the immune response involves interaction of C3d with its natural ligand, complement receptor 2 (CR2). This molecule is expressed mainly by follicular dendritic cells and B-cells. Simultaneous interaction of the antigen and C3d with the surface immunoglobulin M (sIgM) and CR2 on the surface of B-cells triggers two signaling pathways that cross-talk and synergize in cell activation. Most previous studies stressed the importance of the C3d-CR2 interaction for the adjuvant effect. However, the data supporting this hypothesis was derived from *in vitro* studies. To determine the importance of the C3d-CR2 interaction for enhancement of the immune response *in vivo*, mice deficient in CR2 ($CR2^{-/-}$) were immunized with antigens fused to C3d. Contrary to the predicted, $CR2^{-/-}$ mice immunized with antigens fused to C3d, developed almost similar humoral immune responses than wild-type mice. These results suggested that C3d enhances the immune responses by CR2-dependent and CR2-independent mechanisms. Finally, the ability of C3d to emulate the antigenic redundancy of T-cell independent antigens and thus induce class switch in the absence of CD4+ T-cells was explored in a MHC type II knock-out mouse model (MHC $II^{-/-}$). A soluble form of hemagglutinin (sHA) fused to C3d inefficiently induced IgG class switch in MHC $II^{-/-}$ mice. This demonstrated that C3d requires CD4+ T-cells to optimally enhance the immune responses. However, despite of the mild secondary immune responses, MHC $II^{-/-}$ sHA-$C3d_3$-vaccinated mice had reduced morbidity and enhanced survival following a lethal virus challenge, suggesting that besides B-cells, C3d activates other immune cells and that the final enhancement effect is the accumulation of various mechanisms.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

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I. Chapter 1: Introduction

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I.A. Vaccines

The term vaccine is derived from Edward Jenner's use of cowpox ("vacca" derived from Latin, which means "cow") in humans. In 1796, during the smallpox epidemic in Europe, Edward Jenner realized that milkmaids that occasionally catched cowpox, were "resistant" (immune) to smallpox. Jenner extracted infectious fluid from the pustules of one hand of a maid infected with cowpox. The fluid was injected into the arm of an 8 year-old boy ("vaccination"), James Phipps, who developed symptoms of cowpox. Forty-eight days latter, when Phipps was fully recovered, Jenner injected smallpox matter into the arm of the boy. Phipps, did not develop smallpox, demonstrating that cowpox derivatives induced protection against smallpox. Jenner's experiments were a variation of practices that started around the year 200 B.C. in China or India. In these countries a practice known as "variolation" involved the collection and grinding of drying pustules from people suffering from mild cases of smallpox. The powder produced was inserted into small autoinflicted skin abrasions or in the person's nose to help prevent smallpox (1, 2).

Vaccines are antigenic preparations that produce active immunity that intends to prevent or ameliorate the effects of infections by infectious agents. Vaccines are considered to be one of the most successful medical interventions against infectious diseases (3, 4). Vaccines have traditionally been used to prevent infectious diseases; however, the

"vaccinology" field has expanded and there is active research on vaccines intended to prevent different kinds of cancer (5, 6).

Vaccines have contributed to the improvement of public health worldwide. In 1979, after a successful vaccination campaign the World Health Organization (WHO) declared eradication of smallpox (1). Furthermore, in the 1980's all the countries suspended smallpox vaccinations. The morbidity and mortality of diseases such as rubella, polio, measles, mumps and chickenpox have been reduced due to vaccination campaigns. "Herd immunity" is part of the vaccine success. Herd immunity is a phenomenon in which a large portion of the population is vaccinated and this limits the spread of the disease to non-vaccinated people.

I.A.1. Types of Vaccines

There are four types of traditional vaccines: 1) live attenuated, 2) inactivated 3) toxoids, and 4) subunit vaccines. New technologies have been developed such as, 1) conjugated, 2) DNA, 3) viral delivery vectors and 4) virus-like particles vaccines.

I.A.1.a. Live Attenuated Vaccines

Live attenuated vaccines are composed of microorganisms that have been grown under conditions that reduce or disable their virulent properties. These microorganisms are still able to infect their target cells. However, infection is inefficient (mild) and there are limitations in the replication of the microorganisms. Because these vaccines cause a mild

infection, the immune responses induced are strong and long lasting. These vaccines stimulate both, humoral and cellular immune responses. The disadvantages of these vaccines involve: 1) development of mild symptoms that resemble the disease, caused by mild infection of the target cells, 2) potential reversion of the virulent properties of the microorganism that could cause a disease, 3) can not be used in immunocompromised patients, 4) difficulty of development, as not all microorganism can be attenuated. Examples of these vaccines include: yellow fever, tuberculosis (Bacillus Calmette-Guerin –BCG-) measles, mumps and rubella (MMR), smallpox (vaccinia virus), and the recently approved Live Attenuated Influenza Vaccine (LAIV) (1, 7-9).

I.A.1.b. Inactivated Vaccines

Inactivated vaccines are composed of microorganism that have been killed (inactivated) with chemicals (*i.e.* formalin) or heat. Since these microorganisms are not able to infect their target cells, they induce incomplete, short-lived immune responses that usually require booster shots. Furthermore, these vaccines only stimulate humoral immune responses. These vaccines induce small side effects. Examples of these vaccines include: trivalent inactivated influenza vaccine (TIV), cholera, bubonic plague and hepatitis A vaccines. (1, 10, 11)

I.A.1.c. Toxoids

Some microorganisms produce toxic compounds that are the responsible for causing the disease (*i.e.* tetanus toxin and diphtheria toxin). Toxoids are inactivated forms of these toxic compounds. Examples of toxoid-based vaccines include tetanus and diphtheria (12, 13).

I.A.1.d. Subunit Vaccines

Subunit vaccines involve inoculation of purified, soluble protein (antigen) that induces mainly a humoral immune response. These vaccines have benefited from the recombinant DNA technology that allows introducing foreign genes into yeast or bacteria expression systems. These microorganisms produce large amounts of the antigen, which is then purified. The recombinant protein produced is used as the vaccine. These vaccines induce short-lived immune responses that require several boosts to achieve protection. The vaccine against Hepatitis B Virus is the only subunit vaccine that has been approved by the Food and Drug Administration (FDA) for human use. There is active research for the development of subunit vaccines for other microorganisms such as HIV-1 (9, 14-16).

I.A.1.e. Conjugated Vaccines

The cell wall of certain bacteria, *e.i. Streptococcus pneumoniae*, is composed of polysaccharides that are poorly immunogenic, especially in children under 2 years of age. In order to improve the immunogenicity, these polysaccharides have been conjugated to

proteins (*e.g.* tetanus toxid). This conjugation step improves antigen presentation, as well as immunological memory formation, hence improving the immunogenic properties of the polysaccharides. The classic example of this kind of vaccine is the Pneumococcal conjugated vaccine (17, 18).

I.A.1.f. Viral-Vector Vaccines

Viral-vector or live-vectored vaccines have taken a proven safe and efficacious vaccine virus, such as vaccinia, and modifying its genome to include genes encoding for immunogenic proteins from other pathogens. An ideal viral vector should be safe and enable efficient presentation of required pathogen-specific antigen to the immune system. It should also exhibit low intrinsic immunogenicity to allow for its re-administration in order to boost relevant specific immune responses. Furthermore, the vector system must meet criteria that enable its production on a large scale basis. The success of the WHO's small pox eradication program through the use of vaccinia virus, opened the door for the use of this virus as a viral vector (19, 20). However, many other viruses are under investigation as potential vectors for vaccines such as adenoviruses (21, 22), alphaviruses (23), and polioviruses (24). Virus-derived vectors offer several advantages over traditional vaccine technologies. These include 1) high-level production of protein antigens directly within the cells of the immunized host; therefore, these proteins may elicit humoral and cellular immune responses, 2) potential adjuvant effects of the viral delivery system itself, and 3) the possibility of efficient delivery of the antigen directly to component of the immune system, such as antigen presenting cells (APC) (2).

I.A.1.g. DNA Vaccines

DNA (genetic) vaccination delivers the gene encoding for a protein rather the proteins or peptides themselves into a host. These genes are usually expressed from eukaryotic expression vectors, which use the transcriptional and translational machinery of the transfected eukaryotic cell to produce the associated protein. Plasmids (circular ds DNA) usually contain a eukaryotic promoter and poly adenylation signal for efficient transcription of the vaccine gene insert. In addition, most plasmids include a bacterial origin of replication and an antibiotic resistance gene for amplification and selection in media. The gene of interest is molecularly cloned into the expression vector at the multiple cloning site (MCS), which is located between the promoter and poly adenylation signal.

DNA vaccines can be inoculated into animals by various routes. Intramuscular (I.M.) (needle injection) and intradermal (I.D.)/gene gun (G.G.) inoculations are the two most common routes of DNA immunization. The primary cell type that expresses the DNA vaccine is different in these two systems. Muscle cells are the primary cell that express the DNA following intramuscular inoculation of DNA vaccines (25). Additionally, few immune cells such as dendritic cells (DCs) and macrophages are transfected by the inoculated DNA (26). However, the transfection of these cells is not efficient by I.M. DNA immunization. Muscle cells are not professional antigen-presenting cells (APCs), hence their main function is to produce protein in large quantities. The proteins are then

engulfed by APCs, which transport the immunogen to the regional-draining lymph nodes where the immune response is initiated (See Sections I.A.2.a. and I.A.2.b.) (27, 28). Macrophages, can also be transfected

In contrast, gene gun inoculation results in direct and efficient transfection of DCs. Proteins are expressed in these transfected cells and presented on MHC class I molecules. In addition, proteins may also be engulfed by other untransfected APCs and presented on MHC class II molecules. Both types of inoculation lead to cellular and humoral immune responses.

Several methods have been implemented to increase the immunogenicity of DNA vaccines: 1) the addition of strong transcription and translation enhancers into the vaccine vector (29), 2) codon optimization of gene sequences (30-32), and 3) the use of adjuvants (interleukin (IL) 12 (IL-12), RANTES, IL-2) (33-36).

I.A.1.h. Virus-like Particles Vaccines

Virus-like particles (VLPs) or "pseudovirions" are defined as self-assembling, non-replicating, non-pathogenic, genomeless particles that are similar in size and conformation to intact virions (37). The development of VLP has been possible thanks to a deep understanding of the microorganism, their structural proteins and the immunogenicity of each antigen. A major advantage of a VLP approach compared to live-attenuated virus is that VLP expresses multiple viral epitopes in their native

conformational form. These epitopes stimulate a diverse set of immune responses without many of the deleterious effects of a live-attenuated virus. VLPs have the potential for activating both the endogenous and exogenous antigen pathways leading to the presentation of viral peptides by MHC class I and class II molecules. Furthermore, these multi-epitope vaccines are more likely than their single component (subunit vaccines) counterparts to generate a broad-based immune response. This is mainly because the antigens are in their native form and thus are able to induce neutralizing antibodies more efficiently. Currently, there is active research in the development of VLPs for Human Papillomavirus (HPV) and HIV-1. HPV VLPs, which consist of the major capsid protein L-1 (HPV16 L1 VLP), are highly immunogenic, well tolerated and have shown 100% effective in preventing HPV infection un humans (38, 39). HIV-1 VLPs are in active development and several animal trial are currently being performed.

I.A.2. Immune Responses to Vaccines

The design of effective vaccines requires several elements to be taken into account. First, the antigen against which a memory immune response needs to be targeted. It is important to choose the most immunogenic molecule from the pathogen. Maintenance of the tertiary structure of the antigen is important for eliciting neutralizing antibodies. Second, vaccines need to stimulate the innate, as well as the adaptive immune responses. The innate immune responses react quickly (within minutes) to molecular patterns found in microbes, while the acquired immune responses develop slowly (over days to weeks) (40). Innate immune responses, among their many effects, lead to a rapid burst of inflammatory cytokines and activation of antigen-presenting cells (APCs) such as

macrophages and DCs. These non-clonal responses also lead to a conditioning of the immune system for subsequent development of specific adaptive immune responses (41). Adaptive immunity uses selection and clonal expansion of immune cells harboring made-to-order somatically rearranged receptor genes (T- and B-cell receptors) recognizing antigens from the pathogen, thereby providing specificity and long-lasting immunological memory (42).

I.A.2.a. Innate Immune Responses and Vaccines

Innate immune responses have been classically described as non specific, present at birth and limited in diversity. The innate immune system involves: physical barriers (e.g. skin and mucosal surfaces), the complement system and a vast array of phagocytic, granulocytic and immune cells (e.g. macrophages, eosinophiles, basophiles and natural killer cell). It has been considered that the system does not develop immunological memory; however, there is new evidence suggesting the contrary.

To distinguish pathogens from self-components, the innate immune system uses a wide variety of relatively invariable receptors that detect evolutionarily conserved signatures from pathogens (pathogen-associated molecular patterns (PAMPs) (43). PAMPs are recognized by pattern-recognition receptors (PRRs), which are differentially expressed on a wide variety of immune cells, including neutrophils, macrophages, dendritic cells, natural killer cells, B cells and some nonimmune cells such as epithelial and endothelial cells. Engagement of PRRs leads to the activation of some of these cells and their

secretion of cytokines and chemokines, as well as maturation and migration of other cells. PRRs consist of 1) non-phagocytic receptors, such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) proteins, and 2) receptors that induce phagocytosis like scavenger receptors, mannose receptors and β-glucan receptors (44).

Non-phagocytic receptors (TLRs and NOD) lead to an elaborate signal transduction cascade that activates transcription factors (e.g. nuclear factor – κB (NF-κB) and interferon regulatory factor 3 (IRF-3)) resulting in expression of inflammatory cytokines and other cellular activation events. The NF-κB pathway controls expression of proinflammatory cytokines like interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α). IRF-3 controls the pathway that leads to the production of antiviral type I interferons (IFN-α and IFN-β). Activation of PRR signaling by PAMPs results mainly in the activation of transcription factors such as NF-κB and IRF-3, which ultimately provide the inflammatory context for the rapid activation of host defenses. The NF-κB pathway controls the expression of proinflammatory cytokines such as IL-1 and TNF-α. Meanwhile, the IRF3 pathway leads to the production of IFN-α and INF-β (45).

Receptors that induce phagocytosis recognize ligands on the surface of pathogenic microbes and lead to: 1) engulfment of the microorganism into phagocytic cells like macrophages, and 2) activation of the complement system (lectin pathway). In the lectin pathway (**Figure 1**), a protein known as mannan-binding lectin (MBL) binds to mannose residues present on the surface of microorganisms. Binding of MBL to the surface of

pathogens exposes residues that collect MBL-associated proteins (MASP) -1 and -2, which subsequently activate complement proteins 4 and 2 (C4 and C2), leading to the formation of C3 convertases. C3 convertases cleave C3, which products are involved in opsonization of microorganisms, the formation of anaphylotoxins and activation of the pathway that leads to assembly of the membrane attach complex (MAC). Some of the C3 products involved in opsonization of pathogens, like C3b, iC3b and C3d, are recognized by complement receptors 1 and 2 (CR1 and CR2). CR1 and CR2 are present on the surface of various immune cells like phagocytes (CR1), B-cells (CR2) and FDC (CR2). Complement proteins facilitate the recognition of microorganism by the immune cells and thus bridges the innate with the acquired immune responses. Furthermore, some products like C3d, when interact with CR2 on the surface of B-cells, activate a pathway that enhance B-cell activation and reduce the amount of antigen needed to trigger an immune response (Sections I.C.3 - I.C.6) (46-48)

The inflammatory cytokine environment produced by activation of TLRs, NODs, and the activation of phagocytic cells generated an appropriate setting for the development of adaptive immune responses (Section I.A.2.b). For this reason, various experimental vaccines have introduced in their formulations elements (*e.g.* CpG motifs and LPS) that stimulate the innate immune responses.

I.A.2.b. Adaptive Immune Responses and Vaccines

After parenteral inoculation, the antigenic portions of the vaccine are recruited to the draining lymph node (LN). The recruitment is performed via the afferent lymphatic channel. The antigen can reach the lymph node in free form or after being uptaken by antigen-presenting cells (APC). Macrophages, B-cells and dendritic cells (DC) are APCs; however, DCs are considered the most efficient ones. DCs are located in the peripheral tissues as sentinels in a quiescent state sampling for antigens. When an antigen is encountered, it is internalized and processed to peptide fragments. These peptides are then bound to MHC class I (recognized by CD8+ T-cells) or class II (recognized by CD4+ T-cells) molecules that are presented on the cell surface. DCs are known to be one of the most important cell types for initiating the priming of naive CD4+ T-helper-cells (CD4+ Th) and for inducing CD8+ T cell differentiation into killer cells (49). At least three different subsets of DCs have been described based on their origins (derived from myeloid, lymphoid and Langerhans cells) and phenotypic characteristics, which include expression of different TLRs repertoires (50). In this way, activation of different DCs has the potential to induce qualitatively distinct immune responses. For example, lymphoid DCs have the preponderance to secrete IL-12. DC activated CD4+ T-cells in the presence of IL-12 can become CD4+ Th1 cells, which produce IL-2, TNF-β and IFN-γ. INF-γ is especially important for macrophage activation and thus in the development of "cellular" immune responses. On the other hand, activation of myeloid DCs may lead to the development of Th2-like immune responses. CD4+ Th2 cells produce mainly IL-4, IL-5 and IL-10, which activate B-cells, thus helping in the development of "humoral" immune responses. (51). TLR signaling also has an important role in determining the quality of

these helper T cell responses. For example, TLR2 engagement by its ligand Pam3Cys leads to a preferential production of Th2 cytokines, whereas lipopolysaccharide, which is a TLR4 ligand, leads to Th1 responses (51, 52). DCs not only initiate the priming of naïve $CD4^+$ helper T-cells, but also induce $CD8^+$ T-cell differentiation into killer cells (53).

For a wide rage of infectious diseases, neutralizing antibodies are the key immunological parameter relevant for vaccination-induced immune responses (54). The part of the immune response that leads to the production of antibodies is known as "humoral" immune response. Most antibody responses that intend to induce high affinity IgG molecules are dependent on help from CD4+ helper T-cells (Th2), which receive their activation signals from APCs. Activation and maturation of B cells and differentiation into memory and plasma cells initially requires direct recognition of antigen by membrane bound surface IgM (sIgM) receptors. This recognition also occurs in secondary lymphoid tissues. B-cells that recognize antigens through the IgM receptor, internalize and process the antigen. The processed antigen is then presented in MHC molecules to activated CD4+ T-helper-cells, which stimulate B-cells through surface proteins (*e.g.* CD40 ligand) and soluble factors (*e.g.* IL-4 and IL-5) that induce B-cell proliferation, antibody production and differentiation into plasma cells. Some antigens, especially highly ordered, repetitive structures are able to cross-link these B cell receptors and with help of co-ligating complement-derived CD21 ligand are able to activate and mature B cells in the absence of T cell help (55).

Once activated by DCs, $CD8^+$ T cells become cytotoxic (killer) T-cells, which scan and kill target cells that display peptide fragments of cytosolic pathogens on MHC class I molecules. MHC class I molecules are expressed ubiquitously and present molecules that come from the destruction of self-proteins and intracellular microorganisms like viruses and bacterias (*e.g. Mycobacterium tuberculosis*). Cytotoxic T-cells activity is initiated through effector molecules such as perforin, granzymes and Fas ligand. Perforin is a molecule that creates holes in the target cell membrane. Granzymes are proteases that act intracellularly to trigger apoptosis. Fas-ligand is a membrane bound molecule whose interaction with Fas-bearing cells activates apoptosis. Cytotoxic T-cells are also able to produce cytokines such as IFN-γ, TNF-α and TNF-β.

T and B cells critically depend on the amount of antigen being able to reach the cognate T and B cells in lymph nodes and the duration of its presence before being denatured (56). For this reason, for a vaccine to be effective, its antigen requires to be present in sufficient quantity and for a certain period of time in the draining lymph node.

I.B. Adjuvants

The goal of vaccination is the generation of a strong immune response able to provide long-term protection against infection. Live-attenuated vaccines, which produce an active, though minor infection, are considered the gold standard in vaccine immune

response induction. However, these vaccines have many inconveniencies such as 1) important side effects, 2) attenuated microorganisms can potentially revert to a pathogenic form, 3) can not be used in people with immune impairments (elderly, young-children), 4) require a cold chain, and 5) difficult to develop, as not all microorganisms can be attenuated. Advances in the understanding of the microorganisms, as well as the immune system, have lead to the development of "intelligently designed" vaccines, like subunit, DNA, viral-delivery-vectors and VLP vaccines (See Section I.A.1). These vaccines are able to induce immune responses; however, these are far less immunogenic than live-attenuated vaccines. In order to improve the immunogenicity of these vaccines several kind of adjuvats have been developed.

Adjuvants are compounds that enhance the specific immune response against co-inoculated antigens. The word adjuvant comes from the Latin word adjuvare, which means "to help" or "to enhance". The idea and concept of adjuvants arose in the 1920s from observations such as those of Ramon *et al.* who noted that horses that developed an abscess at the inoculation site of diphtheria toxoid generated higher specific antibody titers (57, 58). The adjuvant properties of aluminium compounds were initially demonstrated by Glenny et al. in 1926 with diphtheria toxoid absorbed to alum. Currently, aluminium-based compounds (principally aluminium phosphate or hydroxide) remain as the predominant adjuvants for human use. In 1936, Freund developed an emulsion of water and mineral oil containing killed mycobacteria, thereby creating one of the most potent known adjuvants, Freund's complete adjuvant (FCA) (59). Despite being the gold standard adjuvant, FCA causes severe local reactions and is considered too toxic

for human use. The oil in water emulsion without added mycobacteria is known as Freund's incomplete adjuvant (FIA). FIA is less toxic and thus, has been used in human vaccine formulations (60). In the 1950s, it was found that lipopolysaccharides (LPS) from Gram-negative bacteria exhibited adjuvant activity and detoxified LPS or related compounds such as lipid A have since been used as adjuvants in human studies (61, 62). In 1974, muramyldipeptide (MDP) was identified as a mycobacterial component with adjuvant activity contained in FCA. Bacterial components are often potent immune activators; however, these are commonly associated with toxicity, for example, bacterial DNA with immunostimulatory CpG motifs is one of the most potent cellular adjuvants. Immunostimulatory CpG are unmethylated cytosine-guanine dinucleotides found in bacterial DNA, but absent in mammalian DNA (63, 64). In general, several hundred natural and synthetic compounds have been shown to have adjuvant activity. The mechanism of enhancement of the immune response is different for each kind of adjuvant. Several of these compounds are more potent than aluminum; however, more toxic at the same time, which has limited their use in humans.

Adjuvants are mainly used to 1) enhance the immunogenicity of highly purified or recombinant antigens, 2) reduce the amount of antigen or the number of immunizations needed for protective immunity, and 3) improve the efficacy of vaccines in newborns or immunocompromised persons.

I.B.1. Adjuvant Classification

Adjuvants can be classified according to their source, mechanism of action or physicochemical properties. Edelman classified adjuvants into three groups: 1) active immunostimulants, which are substances that increase the immune response to the antigen; 2) carriers, being immunogenic proteins that provide T-cell help; and 3) vehicle adjuvants, which are oil emulsions or liposomes that serve as a matrix for antigens as well as stimulating the immune response (65). A more recently proposed system of classification divides adjuvants into the following major groups: mineral-salt, tensoactive agents, bacteria derived, oil emulsions, liposomes, human protein-based and carbohydrate adjuvants (Table 1).

I.B.1.a. Mineral Salt Adjuvants

I.B.1.a.1. Alum-Based Adjuvants

Alum salts, principally aluminium phosphate or hydroxide, remain as the most widely used adjuvants in human. Unfortunately, alum salts are relatively weak adjuvants and rarely induce cellular immune responses (66). Several studies suggest alum salts work by causing the formation of an antigen depot at the inoculation site from where antigen is released slowly. The trapping of soluble antigen in the alum gel may also increase the duration of antigen interaction with the immune system. Other mechanisms of action involve complement, eosinophil and macrophage, activation and increased efficiency of

antigen uptake by APCs cells (67). Alum-based vaccines are usually well tolerated. However, granulomas are common when the subcutaneous or intradermal route is used rather than intramuscular injection. Other specific limitations of alum adjuvants are increased IgE production, allergenicity and neurotoxicity. Low doses of aluminium are normally secreted by the kidney. In conditions such as reduced renal function, however, aluminium can accumulate till toxic levels that affect the brain, bone tissue (63).

I.B.1.a.2. Other Mineral Salt Adjuvants

Calcium phosphate has similar properties than alum salts; however, has the advantage that it is a natural compound to the human body and is therefore exceptionally well tolerated. Calcium phosphate has been used, in particular, for diphtheria-tetanus-pertussis vaccines (68). The salts of iron and zirconium have also been used to adsorb antigens; however, to a lower extent than alum salts.

I.B.1.b. Tensoactive Adjuvants

Quil A is a saponin derived from an aqueous extract from the bark of *Quillaja saponaria*. Fractions purified from this extract by reverse phase chromatography, mainly QS-21, have been studied as alternatives to alum when strong cellular responses are required for a particular vaccine (69, 70). Saponins are tensoactive glycosides which induce a strong adjuvant effect to T-dependent as well as T-independent antigens. Saponins also induce strong cytotoxic CD8+ lymphocyte responses and potentiate the response to mucosal

antigens (69). Quil A has been used successfully for veterinary applications; however is generally considered too toxic for human use.

I.B.1.c. Bacteria-derived Adjuvants

Bacteria-derived substances constitute a major potential source of adjuvants because of their potent immunostimulatory capacity. Cell wall peptidoglycan or lipopolysaccharide (LPS) of Gram-negative bacteria, enhance the immune response against co-administered antigens despite themselves not being very immunogenic. This adjuvant activity is mediated by activation of Toll-like receptors that mediate the danger signals activating the host immune defence system (71). Different species of bacteria have been used as a source of adjuvants, for example *Mycobacterium spp.*, *Corynebacterium parvum*, *C. granulosum*, *Bordetella pertussis* and *Neisseria meningitidis*. The adjunvanticity of these bacteria is mediated by N-acetyl muramyl-L-alanyl-D-isoglutamine (MDP). Furthermore, the adjuvanticity of MDP depends on the administration conditions. For example, in saline, it mainly enhances humoral immunity, while, when incorporated into liposomes or mixed with glycerol, it induces strong cellular immunity (63). MDP activates many different cell types including macrophages, leucocytes, mastocytes, endothelial cells and fibroblasts inducing the secretion of cytokines such as IL-1, B-cell grow factor and fibroblast activating factor.

LPS is a potent B-cell mitogen, but it also activates T-cells to produce IFN-γ and TNF and therefore enhances cellular immune responses. The major structural element

responsible for the toxicity and adjuvant effect is lipid A. In low acid conditions, lipid A can be hydrolysed to obtain monophosphoryl lipid A, a compound which retains the adjuvant activity of Lipid A with reduced toxicity (72).

The demonstration that mycobacterial DNA had adjuvant activity, led to the discovery that the adjuvant activity correlated with a higher content of CpG motifs present in bacterial nucleic acids. DNA containing CpG motifs is one of the most potent cellular adjuvants (73, 74).

I.B.1.d. Adjuvant Emulsions

This class includes oil in water or water in oil emulsions such as FIA, Montanide, Adjuvant 65, and Lipovant. The mechanism of action of adjuvant emulsions includes the formation of a depot at the injection site, enabling the slow release of antigen and the stimulation of antibody producing plasma cells (63, 71). In general, these adjuvants are too toxic for routine human prophylactic vaccine use, although they may be suitable for use in terminal conditions such as cancer where there is a greater tolerance of side-effects. Frequent side-effects of emulsions include inflammatory reactions, granulomas and ulcers at the injection site.

I.B.1.e. Liposome Adjuvants

Liposomes are synthetic spheres consisting of lipid layers that can encapsulate antigens and act as both a vaccine delivery vehicle and adjuvant. Liposomes have been used widely in experimental vaccines. The potency of liposomes depends on the number of lipid layers, electric charge, composition and method of preparation. Liposomes enhance both humoral and cellular immunity to protein and polysaccharide antigens. Liposomes help extend the half-life of antigens in blood ensuring a higher antigen exposure to antigen presenting cells after vaccination (75-77)

I.B.1.f. Human Protein-Based Immunomodulators

Cytokines are included in the modern classification of adjuvants. IFN-γ is a pleiotropic cytokine able to enhance cellular immune responses through a variety of mechanisms (78). Granulocyte-macrophage colony stimulating factor (GM-CSF) enhances the primary immune response by activating and recruiting APCs (79). The practical application of GM-CSF as an adjuvant has been limited by the requirement for multiple doses, toxicity and the immunogenicity of heterologous cytokines. Cytokines as adjuvants have potential in DNA vaccines, where the cytokine can be expressed by the same vector as the antigen or co-inoculated in separate DNA plasmids (80). There is also active research on ILs as adjuvants. Currently, IL-1, IL-2, IL4, IL-12, IL-15 and IL-18 are being tested in DNA and subunit vaccines (64). Co-stimulatory molecules such as CD80, CD86 and MHC molecules have been incorporated into live vectors and cells in order to enhance the immune responses (81).

Products derived from the complement system have also been used as adjuvants. C3b and C3d are degradation products of the third component of complement (Section I.C.1.). C3b as well as C3d have been fused to different antigens and the chimera proteins generated, enhance mainly humoral immune responses. Of these molecules, C3d has been extensively studied using several antigens derived from infectious pathogens (82-85) (Section I.C.6.).

I.B.1.g. Carbohydrate Adjuvants

I.B.1.g.1. Inulin-derived Adjuvants

Various carbohydrates of natural origin stimulate cells from the immune system. For example, Gamma inulin, a carbohydrate derived from plant roots of the Compositae family, is a potent humoral and cellular immune adjuvant. Gamma inulin is a potent alternate complement pathway activator increasing production of activated C3 and thereby activating macrophages. Gamma inulin is effective at boosting cellular immune responses without the toxicity exhibited by other adjuvants such as FCA. Inulin-based adjuvants have successfully been tested in multiple animal models in combination with such antigens as diphtheria and tetanus toxoid, respiratory syncytial virus, the E7 protein from the human papilloma virus, herpes simplex virus-2 glycoprotein D, hepatitis B surface antigen, influenza haemagglutinin, *Haemophilus influenzae* antigens and antigens from *Plasmodium falciparum*. Major advantages of inulin-derived adjuvants are that they

induce both Th1 and Th2 immune responses unlike alum do not induce IgE, and are not associated with any significant local or systemic toxicity (86). Inulin is metabolizable into simple sugars fructose and glucose.

I.B.1.g.2. Other carbohydrate Adjuvants

Some polysaccharides based on glucose and mannose have adjuvant action. These polysaccharides include glucans, dextrans, lentinans, glucomannans and galactomannans. Levans and xylans also have immuno-enhancing activity. Macrophages have glucan and mannan receptors, activation of which stimulates phagocytosis and cytokine secretion plus release of leukotrienes and prostaglandins. Polysaccharides have been used for immune stimulation in patients with cancer. *In vitro*, mannan activates monocytes and macrophages to secrete IFN, TNF, GM-CSF, IL-1 and IL-6 (87).

I.B.2. Adjuvant Formulations

New adjuvant formulations have resulted from the mixture of different adjuvants in the same formulation. As a general rule, two or more adjuvants with different mechanisms of action are combined to enhance the potency and type of the immune response to the vaccine antigen. For example, alum salts can be formulated in combination with other adjuvants such as Lipid A to increase immunogenicity. Saponins such as Quil A have also been used as a part of immunostimulatory complexes (ISCOMS). ISCOMS are spheric particles of 30–40 nm and dodecahedric structure, composed by Quil A, lipids

and cholesterol. Antigens can be inserted in the membrane or encapsulated. A wide variety of proteins have been inserted in these cage-like structures (88, 89).

In conclusion, the goal of a vaccine is to generate a strong immune response against the administered antigen. This immune response needs to be strong enough to provide long-term protection against the infection. The majority of the newly designed vaccines do not induce strong enough immune responses and this has opened the door for the development and use of adjuvants. Adjuvants have become a crucial portion of the formulation of new vaccines. With the use of appropriate adjuvants, the immune responses can be modulated either for the humoral or cellular side. Despite the hundreds of molecules with adjuvant properties that have been isolated, only few are used in humans; aluminium salts are the most common. The limited use of adjuvants in human is due to the extensive side effects caused by most adjuvants.

The derivatives of complement system, like C3d, have become part of the adjunvants studied with potential use for different kinds of vaccines. In DNA, as well as protein immunizations, C3d enhanced vaccines are efficient and safe. No side effects have been reported in small animal studies and every day a larger number of antigens are tested. C3d is considered a "natural" adjuvant (See Section I.C.6.) because one of its roles in a normal immune response. C3d opsonizes pathogens and through interaction with its receptor CR2, enhances antigen uptake, processing and presentation (90). Furthermore, C3d reduces the amount of antigen needed to activate the B-cell (47). Hence, the use of

this molecule exploits normally occurring events that lead to a natural enhancement of the immune response and therefore lowers the chance to develop undesired side effects.

Table 1. Adjuvant Classification

Adjuvant Class	Examples
Mineral-Salt (Gel-type)	Aluminium hydroxide (alum) Aluminium/Calcium phosphate Iron and zirconium salts
Tensoactive	Quil A QS-21
Bacteria Derived	Bacterial DNA (CpG) BCG Inactivated *Mycobacterium vaccae* *Bordetella pertussis* Mycobacterial cell wall skeleton and Monophosphoryl lipid A (MPL) Genetically-attenuated cholera toxin (CT) and *E. coli* heat-labile toxin (LT) Muramyl di- and tripeptide (MDP/MTP) and derivatives Streptococcal cell wall Tetanus toxoid (TT), Diphtheria toxoid (DT) and other bacterial protein carriers of T-cell help
Emulsions	Freund's incomplete adjuvant MF-59 Syntex Adjuvant Formulation (SAF)
Liposomes and Particulates	Immune-stimulating complexes (ISCOMs) Liposomes Virosomes Poly(lactic-co-glycolic) (PLGA) microspheres
Human Protein-Based Immunomodulators	Interferon-gamma (IFN-γ) Interleukin (IL)-1, IL-2, IL-4, IL-12, IL-15 IL-18 Granulocyte-macrophage colony-stimulating factor (GM-CSF) C3d Costimulatory (CD80; CD86) and MHC molecules for incorporation into live vectors and cells
Carbohydrate	Gamma inulin Glucans, dextrans, lentinans, glucomannans and galactomannans Levans and xylans

I.C. The Complement System

The complement system, which was first identified as a heat-labile principle in serum that "complemented" antibodies in bacteria killing, is composed of more than 30 soluble serum proteins that are present as zymogens and activated in cascade. These proteins are classically cataloged as part of the innate immune system. However, there is a large body of information indicating that this system bridges the innate and acquired immune responses. The main proteins in the bridging function are the products derived from the cleavage of the third component of complement (C3): C3b, iC3b and C3d. Additionally, there is accumulating information that demonstrates that C5a may play a similar role (91).

I.C.1. Complement System Pathways

Activation of complement system can occur via three distinct pathways: the classical, alternative, and lectin pathways (**Figure 1**). The classical pathways is activated by 1) immune complexes containing antigen and IgM or IgG (IgG complement fixing isotypes), 2) C reactive protein (CRP) - bound microbial pathogens, 3) serum amyloid P (SAP) – bound microorganisms, and 4) apoptotic bodies (92). The lectin pathway is initiated by binding of the mannan binding lectin (MBL) to 1) mannose residues present on the surface of invading microorganism, 2) ficolins (93) or 3) immune complexes containing IgA (94). Binding of MBL to the surface of pathogens exposes residues that collect MBL-associated proteins (MASP) -1 and -2, which subsequently activate complement proteins 4 and 2 (C4 and C2), leading to the formation of C3 convertases.

Finally, the alternative pathway is activated by 1) foreign microorganisms, 2) immune-complexes containing IgA, 3) nephritic factors, which are autoantibodies that stabilize C3 activating enzymes, and 4) the "amplification loop", where C3b that is deposited on targets either by the classical or lectin pathways binds Factor B and results in activation of the alternative pathway.

Each pathway that activates the complement system leads to the formation of C3 convertases (C4b2a, classical; C3bBb, alternative), which cleave C3 (**Figure 1**). This step is critical because leads to the downstream effector functions of complement: 1) formation of anaphylotoxins (C3a, and C5a), 2) lysis of pathogens through the membrane attack complex (MAC) (C5b-9) and 3) opsonization of pathogens (C3b, iC3b and C3d) (95, 96).

C3 cleavage by C3-convertases generates C3a and C3b. C3b binds to C4b (classic pathway) or C3bBb (alternative pathway) and generates C5-convetases, which cleaves C5 thereby releasing C5a and C5b. C3a and C5a are known as anaphylotoxins because of their role in the development of anaphylactic allergic reactions. Even though C5a maybe more potent than C3a, both proteins display powerful biological activities that stimulate inflammatory sequelae. Anaphylotoxins are strong chemoattractants and are involved in the recruitment of inflammatory cells, such as, neutrophils, eosinophils, monocytes, and T lymphocytes. They also activate phagocytic cells and release granule-based enzymes and generate oxidants, all of which contributes to innate immune functions or tissue damage. On the other hand, C5b generates C5b6 complexes, which interact with C7, C8

and multiple C9 molecules. This large molecular complex C5b-9 (MAC) acts as a transmembrane pore and can cause cell death (95-97). These functions, dedicated to the eradication of infections, have been known for decades and initially cataloged the complement as part of the innate immune system.

I.C.2. Complement Bridges the Innate and Acquired Immune Responses

In the recent years, it has become clear that the complement system not only plays a role in the innate immune system, but importantly bridges the innate with the acquired immune responses. The main proteins in this bridging function are the products derived from the cleavage of C3. As previously indicated, cleavage of C3 (by C3-convertases) generates C3a and C3b. Activated C3b, in addition to the formation of C5-convertases, can covalently attach to available protein amine groups or to hydroxyl groups present on carbohydrate-containing glycoproteins of invading microorganisms. This covalent interaction is mediated by the presence of a highly reactive internal thiol ester group in C3b. C3b attaches to the surface of microorganisms, interacts with complement receptor 1 (CR1 / CD35) and is converted to iC3b by fluid phase factor I and H. This fragment is subsequently cleaved to C3d(g) by factor I, and CR1 as a cofactor (**Figure 1**). This results in C3d-tagged microorganisms that have the capacity to bind to complement receptor 2 (CR2 / CD21) (98). The interaction of C3 derived products with receptors (CR1 and CR2) present on the surface immune cells that are part of the adaptive immune response, bridges the innate with the acquired immune responses.

I.C.3. Complement Receptors 1 (CR1/CD35) and 2 (CR2/CD21)

In mice CR1 (CD35) and CR2 (CD21) are encoded by the same gene. Each protein is expressed by alternative splicing of the mRNA (99, 100). In contrast, in humans, CR1 and CR2 are encoded by two separate genes (101, 102). CR1 (molecular weigth 190 kDa) binds activated products from C3 (i.e.C3b, iC3b, C3d,g and C3d) and C4 (i.e. C4b and iC4b). CR1 is assembled from 21 repeat units (also known as short consensus repeats – SCR-), each one consisting of 60-70 amino acids, a transmembrane region and a cytoplasmic of 35 amino acids (103, 104). Besides binding to C3 and C4 activated products, CR1 works as a cofactor in the inactivation and cleavage of C3b by factor I. CR2 (molecular weight 150 kDa) is composed of 15 SCRs and is almost identical to CR1, except for the absence of six SCRs at the N-terminal region (105). CR2 binds the same ligands that CR1, but C3b and C4b. CR2 is present on the surface of follicular dendritic cells (FDC), B-cells and some T-cells (101, 106-110).

I.C.4. CR2 and Follicular Dendritic Cells

FDC are located in the germinal center of lymphatic nodes and play an important role in antigen presentation, which is not dependent on MHC class I or II molecules, but on Fc receptors γII (FcRγII) (111). These receptors collect and maintain antigens coated (opsonized) with immunoglobulins (Igs) (**Figure 2**) (112, 113). The antigen is retained by FDC in these tissues for long periods of time. Therefore, FDCs also play a role in the maintenance of immunological memory. The role of CR2 on the surface of FDC appears to be similar to FcRγII. Microorganisms coated with C3d can interact with CR2 and thus

help in antigen presentation, as well as in maintenance of memory (**Figure 2**). Additionally, CR2 bound immune complexes facilitate important signaling events that are required for the survival of maturing B-cells, as well as for the selection of high affinity B-cells (90, 114-117)

I.C.5. CR2, CD19 and the B-cell Signaling Complex

On B-cells, CR2 enhances cell activation. B-cells are usually activated when antigens bind to the surface IgM (sIgM), which triggers a cascade of events that activates the cell. Binding of C3d to CR2 aggregates CD19, CD81 (TAPA-1) and Leu-13. CD19 has a long intracellular domain that also participates in signaling for cell activation (46). Thus, co-binding of sIgM and CR2 activates pathways that crosstalk, enhances cell activation, reduces the amount of antigen needed to activate the cell, reduces inhibitory signals and prevent apoptosis (**Figure 3**) (101, 118-123). Additionally, sIgM-CR2 co-ligation enhances antigen uptake (124) and improves its presentation by selective trafficking to MHC II molecules (125).

I.C.6. C3d as Natural Adjuvant – Enhancement of Secondary Humoral Immune Responses to Different Antigens

Since co-ligation of sIgM-CR2 can enhance B-cell activation, antigen presentation and consequently antibody production, it was hypothesized that C3d could be used as a molecular adjuvant (

Figure 4). Dempsey *et al*. was the first report the use of C3d as adjuvant. This study demonstrated that the conjugation of hen egg lysozime (HEL) to two or three tandem copies of murine C3d enhanced the secondary immune responses by as much as 1,000 and 10,000 fold, respectively (126). Similar results were achieved conjugating C3d to viral, bacterial, parasitic and cellular (self) antigens. Different mechanisms to conjugate C3d to antigens have been used, such as gene fusions that produce antigen-C3d chimera proteins (83-85, 127), covalent conjugation (128), and biotinylation of C3d (129-131).

Several antigens derived from infectious microorganisms have been used to further explore the adjuvant properties of C3d. DNA plasmids expressing the HIV-1 envelope (Env) gp120 gene fused to two or three copies of murine C3d were constructed and used to immunize mice (132). Intradermal immunizations of these DNAs via gene gun in various mouse strains demonstrated the ability of C3d to enhance not only secondary humoral immune responses (IgG), but also the maturation of antibody avidity, the ability of the antibody to neutralize infection, as well as stimulate B-cell proliferation (85, 132, 133). Three repeats of C3d conjugated to a soluble trimeric form of the HIV-1 Env was more effective at inducing neutralizing antibodies to primary isolates than plasmids encoding for non-C3d conjugated Env glycoproteins (134). Codon-optimizing the gene insert to use the most prevalent codons found in mammalian cells also enhances anti-Env antibodies. Fusing C3d to these codon-optimized genes allowed for even a greater reduction of the dose of DNA by 100-fold and demonstrated that C3d and codon-optimization enhance the immune response by two distinct and non-synergistic mechanisms (83, 135). Immunizations of modified HIV-1 Env gp120 proteins

(gp120ΔC1/C5-C3d2) in physiological solutions (PBS) also enhanced anti-Env immune responses. However, the C3d enhancement was not synergized following immunizations performed in the emulsified adjuvant, Ribi, as both gp120ΔC1/C5 and gp120ΔC1/C5-C3d2 induced similar antibody titers (136).

A soluble form of hemagglutinin (sHA) from the influenza virus [A/PuertoRico/8/34 (H1N1)] has also been conjugated to three tandem copies of C3d. Mice immunized with sHA-C3d3 induced anti-HA antibody titers similar to those induced by the highly immunogenic transmembrane HA (tmHA). Even though, both DNAs induced similar total antibody titers, only sHA-C3d3 enhanced antibody avidity maturation and hemagglutinin inhibition activity, which correlated with a more rapid appearance of protective immunity following lethal virus challenge (84). C3d conjugated to sHA also induced antibodies able to protect mice from heterologous virus challenge (A/Aichi/2/68-x31 – H3N2) and a stronger production of IL-4 than sHA or tmHA DNA vaccines alone (127). In a different approach, Watanabe *et al*. performed protein intranasal immunizations with tmHA, sHA or sHA-C3d3. These immunizations were performed either in the presence or absence of cholera toxin B subunit with a trace of holotoxin (CTB-H) as an adjuvant. All these vaccines induced nasal IgA and serum IgG antibodies against viral HA, which protected from lethal virus challenge, when inoculated with CTB-H. However, in the absence of CTB-H, only sHA-C3d3 induced locally secreted IgA and serum IgG that protected from lethal virus challenge (137). Finally, a soluble form of hemagglutinin from measles virus (sHMV) fused to C3d (sHMV-C3d3)

generated a more rapid appearance and higher levels of neutralizing antibody activity against measles virus than DNA expressing sHMV alone (138).

E2 glycoprotein from type 2 bovine viral diarrhea virus (BVDV) is an important target of neutralizing antibody in BVDV-infected cattle. Purified E2 proteins fused to murine C3d3 were 10,000 more immunogenic than E2 alone and anti-E2 antibodies neutralized virus infection (139). Similar results were observed when the bovine homolog of C3d was fused to E2 (140).

T-cell independent antigens, such as the capsular polysaccharide of serotype 14 *Streptococcus pneumoniae* (PPS14) conjugated to C3d, induced a significant increase in serum anti-PPS14 concentrations compared to native PPS14 or control PPS14-glycine conjugates (128). Furthermore, class switch from IgM to IgG was detected and IgG1 was the main isotype induced. Even though, the primary antibody responses to PPS14-ovalbumion (OVA) were higher than those induced by PPS14-C3d, a second immunization with PPS14-C3d induced almost similar serum anti-PPS14 responses than a boost of PPS14-OVA (128). This study also suggested that T-cells are not required for enhancement of primary immune responses, but necessary for enhancement of the memory responses after a second immunization with PPS14-C3d conjugates (128). In a follow up study, the "quality" of the IgG antibodies induced after the second immunization of either PPS14-C3d or PPs14-OVA were evaluated and the results indicated that OVA induced antibodies with higher avidity and enhanced opsonization

functions than C3d (141). This suggests that at least with T-cell independent antigens, C3d is not as effective as T-dependent carrier proteins.

In a recent study, the self-antigen collagen type II (CII) was conjugated to C3d and inoculated into a mouse model for collagen-induced arthritis. Animals that received heterologous CII conjugated to C3d developed rheumatoid arthritis (RA) in the absence of Complete Freund's Adjuvant (CFA), while mice that received CII alone did not develop RA, unless inoculated with CFA (131). CFA is necessary for the development of autoimmune disease in several animal models because it causes local inflammation, enhances antigen uptake and promotes TLR-dependent cytokine release. Thus, the adjuvant effect of C3d bypassed the need for the broad immunostimulatory effect induced by CFA. This report was the first to demonstrate that C3d can induce pathogenic autoantibodies and thus suggests that C3d may be involved in the development of other B-cell dependent antoimmune diseases. However, it also suggests that C3d can break the natural tolerance to self proteins and thus could be used to induce immune responses to some kinds of tumor antigens, which may broaden the uses of C3d as a vaccine adjuvant.

I.C.7. The Complexity of the C3d-CR2 Interaction and Its Relevance for the In vivo Adjuvant Effect.

The proposed mechanism for the enhancement of the immune response by C3d involves binding and signal transduction via CR2 on the surface of B-cells. However, some aspects of this interaction remain unclear. Initial studies based on synthetic mimetic peptides reported that the minimal CR2-binding region of C3d was located between

residues 1199-1210 (mature C3 numbering) (142). A polymeric synthetic peptide (P28) that included this region (1187-KFLTTAKDKNRWEDPFKQLYNVEATSKYA-1214), not only bound to CR2 on Raji cells, but also stimulated cell proliferation (143, 144). However, a study by Diefenbach *et al*. demonstrated that extensive mutagenesis of the segment 1199-1210 of human C3 reduced binding of iC3b or C3d(g) to CR2 by less than 20% (145). X-ray crystallography indicated that the structure of C3d has a negatively charged (acidic) pocket on the concave side of the molecule, which was proposed to be the site of interaction with CR2 (146). Replacement of two clusters of negatively charged residues on the opposite site of the pocket led to severe inhibition of the interaction with CR2 and thus supported this hypothesis (147). Surface plasmon resonance technology was used to analyze the kinetics of the interaction between iC3b, C3d and P28 to CR2 and the results suggested the possibility of more than one interacting region (148). However, when the X-ray crystal structure of CR2 [short consensus repeats (SCR) 1 and 2] complexed to C3d was revealed, many unexpected features were exposed such as 1) only SCR2 contacts C3d and not SCR1, 2) extensive SCR1-SCR2 side-by-side packing in a folded back structure, 3) extensive main-chain, rather than side-chain interactions between C3d and CR2, 4) receptor contact sites on C3d that were not previously predicted by mutagenesis, which also disproved the hypothesis that the acidic pocket was the binding region of C3d and 5) formation of CR2-CR2 dimers by interaction of the SCR1 domain of each molecule (149, 150). A model based on theoretical electrostatic potential and pK_a calculations tried to reconcile the controversial results and suggested that the association of C3d with CR2 is predominantly electrostatic in nature and involves the whole molecule and not only the limited association sites that were previously

studied. This model suggests that recognition and binding is due to electrostatic attraction (controlling K_{on} rates) and that van del Waals and hydrogen bonding interactions are responsible for making the two molecules stick together (controlling K_{off} rates). In particular, charged residues in regions of C3d that are remote to the association site appear to affect CR2 binding and this may help to explain the previous mutagenesis results (151).

Despite all the information gained from X-ray crystal structures, mutational analysis and theoretical models, we still do not have a complete understanding of the C3d and CR2 interaction and its consequences for *in vivo* models. This lack of understanding is most likely because the crystal structures involve just the first two SCRs of CR2 and not the full length molecule, which is composed of 14-16 SCRs. Interactions between C3d and the remaining SCRs may influence binding as suggested by surface plasmon resonance studies that demonstrate that although the binding affinity of SCR1-SCR2 and SCR1-SCR15 (full length CR2) for C3d were the same, association and dissociation rates of SCR1-15 were approximately 10-fold slower that SCR1-2 (149, 152). Dimerization of CR2 also remains to be clarified as monomeric or dimeric complexes could have different outcomes in the cells.

I.C.8. A C3d Minimal Binding Domain and the Enhancement of the Immune Response

Based on 1) the importance the C3d-CR2 interaction for the enhancement of the immune response and 2) surface plasmon studies that demonstrates P28 binding to CR2 (148), our

laboratory designed DNA vaccines that use four copies of this molecule as adjuvant. P28 was almost as immunogenic as C3d since mice immunized with DNA plasmids expressing HIV-1 Env gp120-(P28)4 elicited similar antibody titers than mice vaccinated with Env gp120-C3d3. Furthermore, similar numbers of IFN-γ and IL-4 secreting splenocytes were elicited by both vaccines (153). Also, fusion of the hepatitis B virus (HBV)-preS2/S antigen to four copies of P28 enhanced anti-preS2/B titers, as well as the maturation of high avidity antibodies (154).

I.C.9. C3d and Impairment of the Immune Response

Although several studies have reported enhancement of the immune response by C3d, other reports indicated that C3d fusion proteins inhibited immune responses. Antibodies specific to the non-toxic diphtheria toxin fragment B (DT), human chorionic gonadotropin, bovine rotavirus VP7, bovine herpes virus type 1 plycoprotein D and malaria circumsporozite protein were inhibited by conjugating to C3d (118, 155-157). The dose and individual immune properties of each antigen appear important for either inhibiting or enhancing immune responses by C3d. Low doses of an antigen fused to three copies of C3d enhance the immune responses, but high doses inhibit (158). Bergmann-Leitner *et al.* reported that the individual characteristic of an antigen is important for C3d adjuvanticity. Fusing C3d to the malaria circumsporozite protein (CSP) inhibited anti-CSP immune responses most likely because the immunogenic epitopes in the carboxy-terminus of CSP were covered by C3d (155).

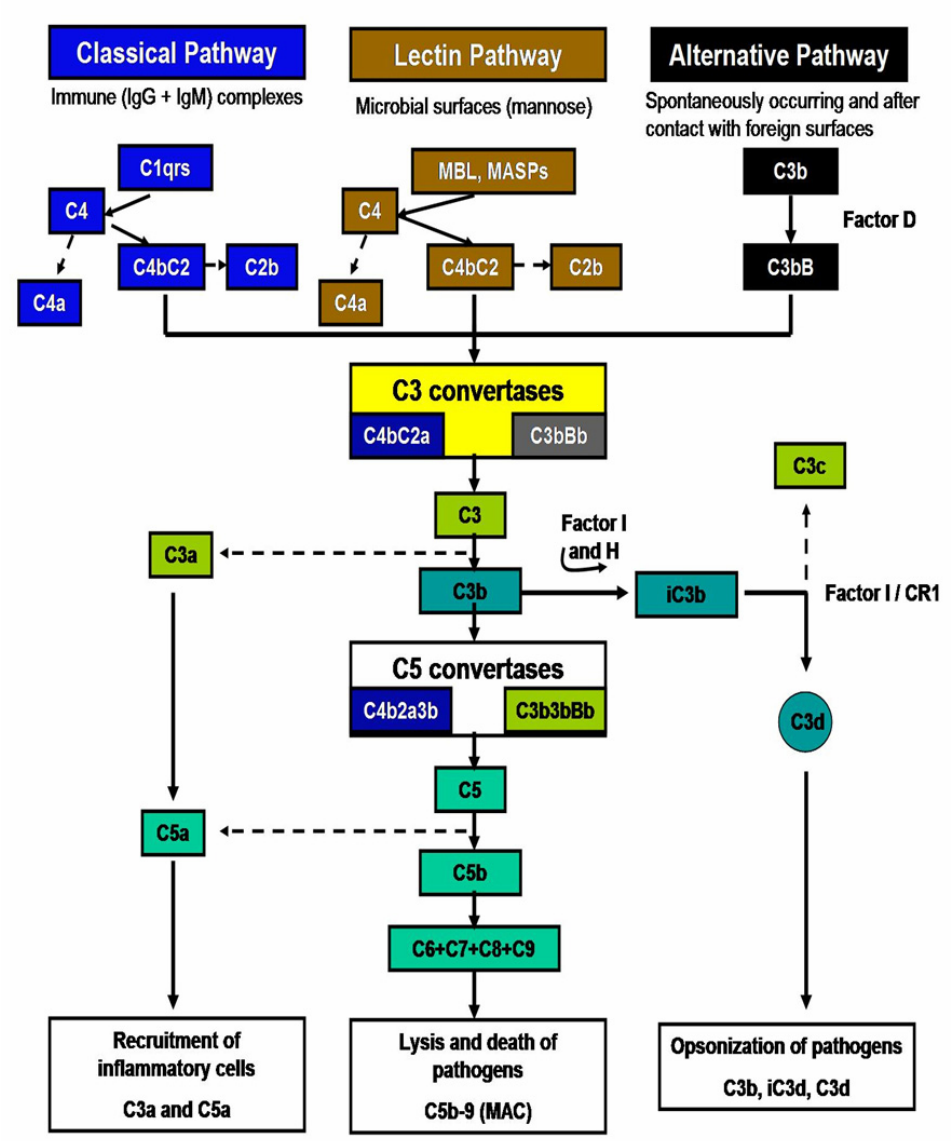

Figure 1. The Complement System

The complement system can be activated by the classical, lectin and alternative pathways, which lead to the formation of C3 convertases (C4bC2a, classical and C3bBb, alternative). Cleavage of C3 is a critical step that leads to the effector branches of the complement system: 1) recruitment of inflammatory cells by anaphylotoxins, 2) lysis and death of microorhanisms by formation of the membrane attach complex, and 3) opsonization of pathogens. C3d is formed from the subsequent cleavage of C3 into C3b and iC3b by factors I, H and CR1 as cofactor.
MBL: Mannan Binding Lectin. MASPs: MBL-associated proteins

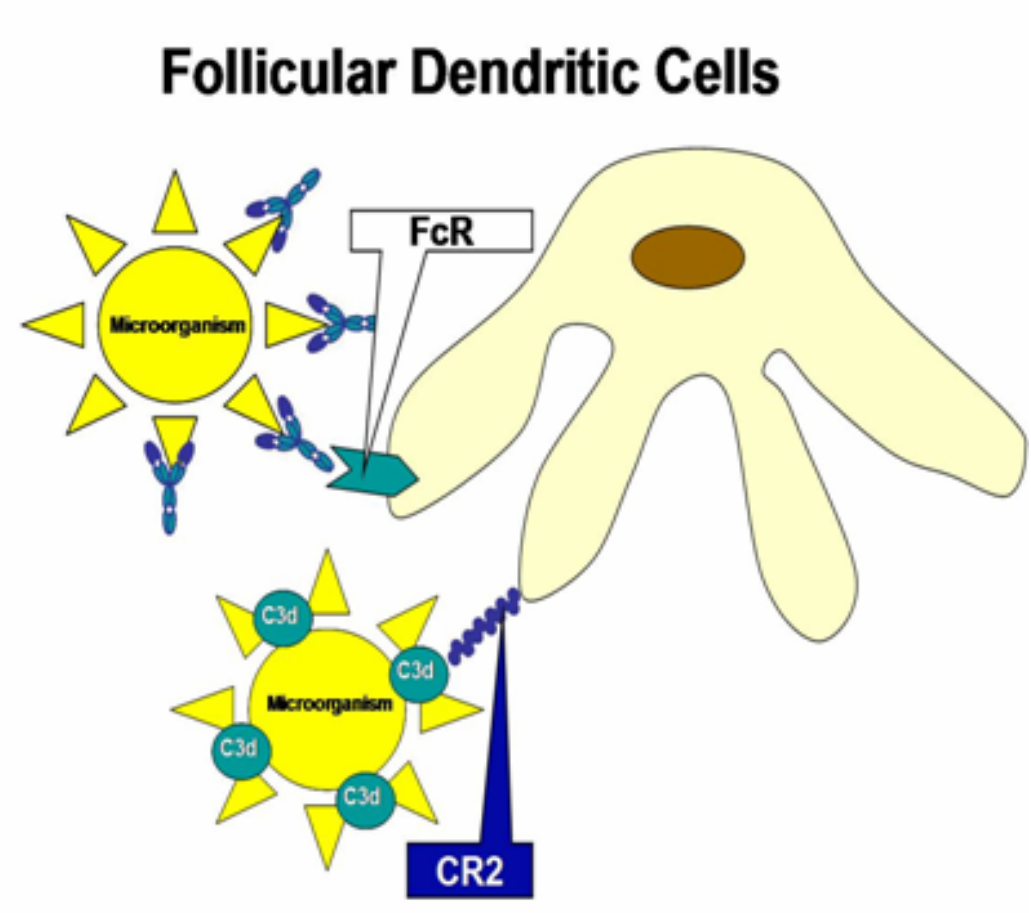

Figure 2. CR2 and Follicular Dendritic Cells

Follicular dendritic cells (FDCs) bind microorganisms through FcRγII and CR2. These antigens remain attached for long periods of time, hence helping in antigen presentation and maintenance of memory.

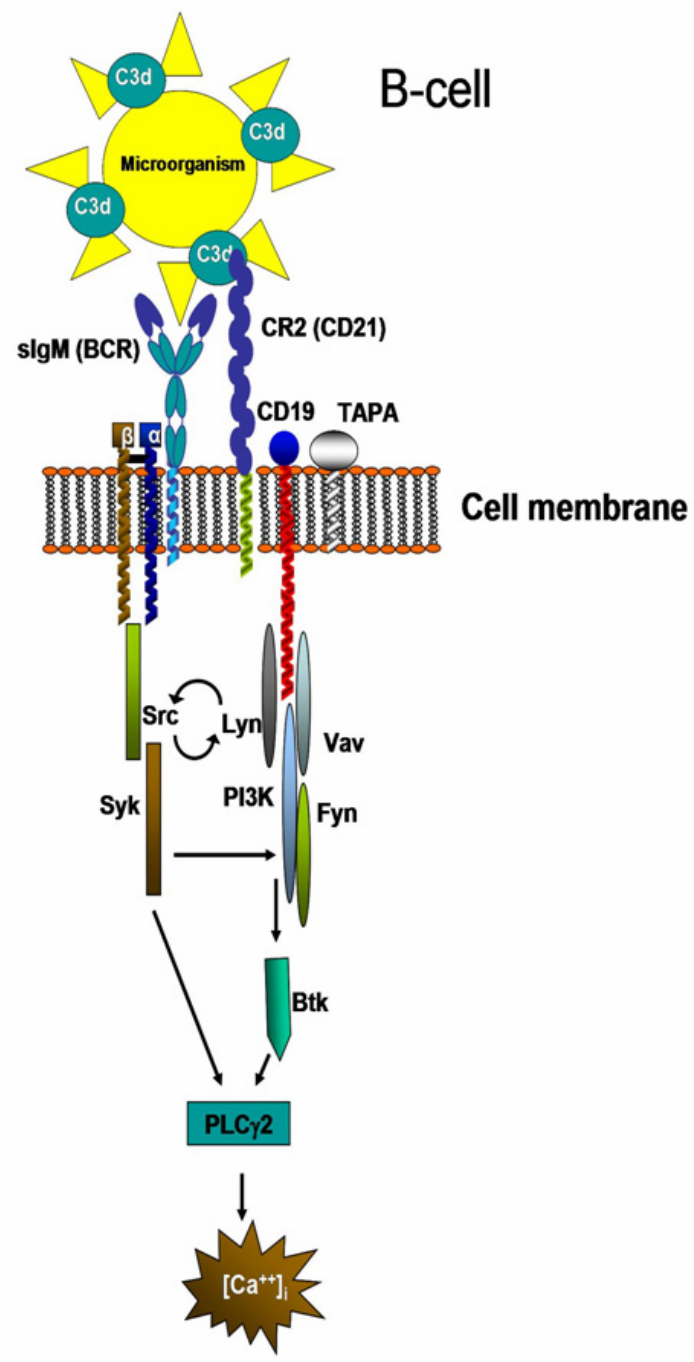

Figure 3. C3d: A Natural Adjuvant

Invading microorganisms coated with C3d interact with B-cells through the surface IgM (sIgM) and CR2. Co-ligation of these two receptors activate pathways that cross-talk and lead to activation of the cell.

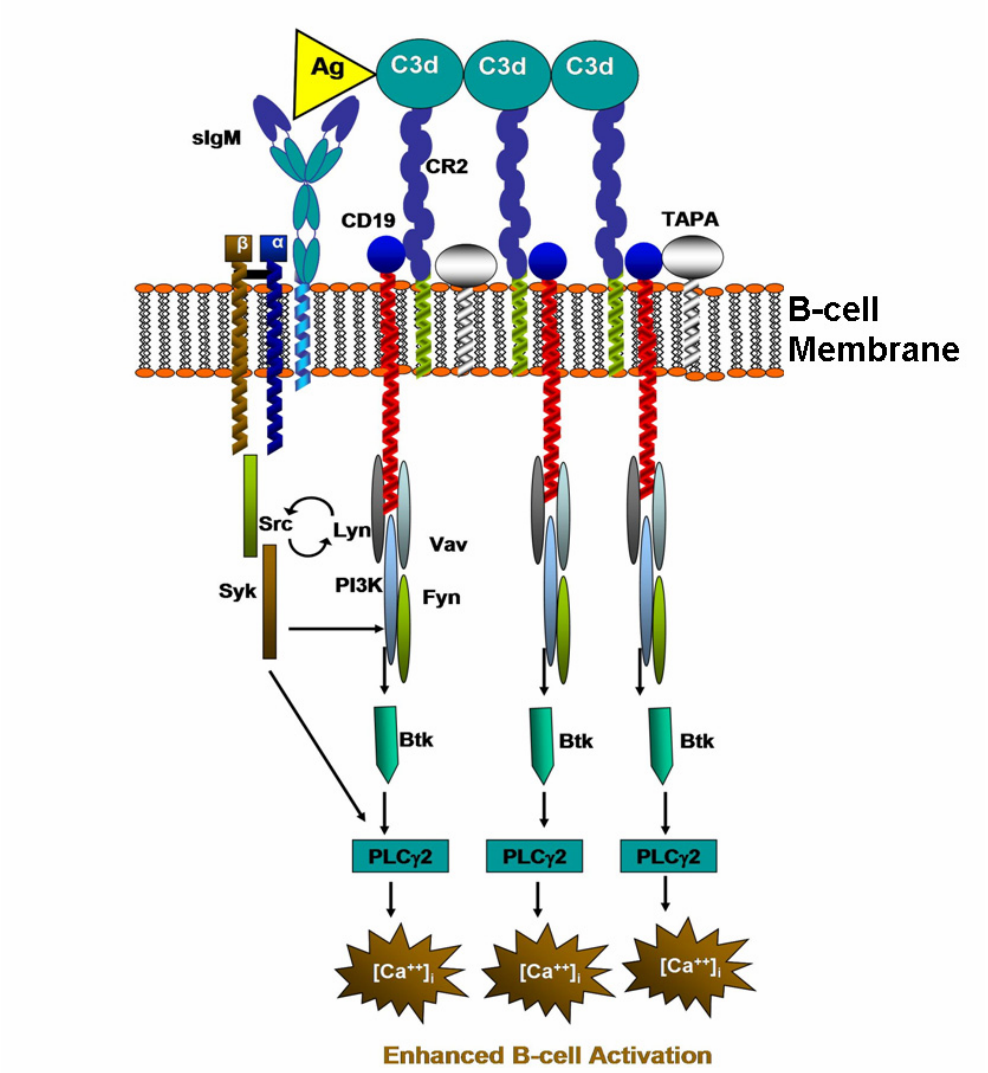

Figure 4. C3d: A molecular Adjuvant

Ag-C3d$_3$ fusion proteins bind the sIgM, as well as several copies of CR2. This redundant interaction activates a signaling cascade that results in B-cell activation. In addition, this mechanism reduces the amounts of antigen needed to activate the cell.

I.D. Antigens

Antigens are the portion of a vaccine against which an immune response is desired. There are several kinds of model and experimental antigens. These are used as tools for the study of the development of immune responses and mechanism of action of adjuvants. In order to study the mechanism of enhancement of the immune responses by C3d, three different antigens were selected: 1) the gp120 subunit of the envelope glycoprotein (Env_{gp120}) from the human immunodeficiency virus (HIV-1), 2) a soluble form of hemagglutinin (sHA) from influenza virus A/PR/8/34 (H1N1) and 3) streptavidin (SA). The first two proteins are medically relevant antigens derived from microorganisms that induce chronic and acute infections in large portions of the human population. The last one represents a model antigen used in the study of the development of immune responses. These three antigens will be described, including a brief review of the microorganism from where they are derived.

I.D.1. Human Immunodeficiency Virus (HIV-1)

I.D.1.a. HIV-1 General Aspects

The human immunodeficiency virus (HIV-1) is the causative agent of the Acquired Immune Deficiency Syndrome (AIDS). HIV-1 was isolated in the early 80's (159-161). The virus was simultaneously identified by several laboratories and as result, each laboratory gave each isolate a different name: i.e. 1) lymphadenopathy-associated virus

(LAV), 2) human T-cell lymphotropic virus type III (HTLV-III), and 3) AIDS-associated retrovirus (ARV). It was not until 1986, that the International Committee on Taxonomy of Viruses officially named this virus as HIV-1.

HIV-1 is part of the *Retroviridae* family, which is characterized by an RNA genome that is converted into a DNA form by reverse transcription and integrates into the chromosomal DNA of the host (proviral genome). Simple and complex retroviruses have been described. Simple retroviruses (i.e. alpharetroviruses, betaretroviruses, gammaretroviruses) contain only the genes that encode for Gag, reverse transcriptase (RT), protease (PR) and envelope (Env) proteins. On the other hand, complex retroviruses (i.e. deltaretroviruses, epsilonretroviruses, lentiviruses, spumaviruses) contain the genes for these four proteins and an assortment of regulatory and accessory genes. HIV-1 is a complex virus member of the *Lentivirus* genus.

The envelope of HIV-1 virus is composed of a lipid bilayer that is derived from the host cell membrane and acquired during the budding process. The envelope is embedded with multimeric envelope (Env) glycoproteins that are composed of two domains: 1) a globular surface domain (gp120) and 2) a transmembrane domain (gp41). Env glycoproteins associate to form trimeric structures that spike on the surface of the virion. The matrix (MA, p17) protein lines the inner surface of the viral envelope and surrounds the capsid. The capsid (CA, p24) layer contains approximately 2,000 molecules and encases the nucleocapsid (NC, p9), which surrounds the viral genome and associated viral proteins (**Figure 5**A).

I.D.1.b. HIV-1 Groups, Strains and Clades

Based on the genetic sequence, HIV-1 has been divided in three distinct major groups: major (M), outlier (O), and non-M/non-O (N). The strains of HIV-1 that infect most humans worldwide are part of the M group. The group M has evolved into several different clades (~10) (A, B, C, D, F, G, H and J) and 13 different circulating recombinant forms (CRF) (394, 470).

The genetic diversity of HIV-1 has been analyzed in various group M viruses (393, 394, 622). The genetic diversity of the HIV-1 population can vary from 6-10% within an infected individual. Moreover, intraclade nucleotide diversity can vary 15% (Gag) or up to 30% (Env_{gp120}), whereas interclade variability may range between 30-40% depending on the gene examined.

I.D.1.c. Epidemiology

As of December of 2005, between 36 million and 45 million people were estimated to be living with HIV infection, of which 17.6 million were women and 2.2 million were children. Approximately half of the infected population was between 15 and 24 years of age (162). An estimated 4.9 million new HIV infections occurred in 2005, including 4.2 million new infections in adults and 700,000 new infections in children less than 15 years of age (163). The highest prevalence of HIV infection is observed in sub-Saharan Africa,

especially in Botswana, South Africa, and Lesotho, Namibia, and Swaziland, where as much as 30-40% of pregnant women are infected. Three fifths of individuals with HIV infection reside in sub-Saharan Africa.

It is estimated that one-fourth of the one million United States (U.S.) residents living with HIV are unaware that they are infected (164). In addition, half of the 400,000 newly infected individuals are younger than 25 years of age each year in the U.S. (70% men, 30% women) (165). HIV/AIDS is the leading cause of death in Africa and the fourth leading cause of death worldwide.

I.D.1.d. Disease Course and Immune Response to HIV-1 in Humans

There are four major modes of HIV-1 transmission: 1) sexual intercourse, 2) blood products, 3) contaminated needles from intravenous drug use and 4) mother-to-child during the perinatal period (166). Many immunological and viral hallmarks are observed during the course of infection of HIV-1 (167, 168). The timeline for disease progression from infection with HIV-1 to the development of AIDS varies between individuals. In general, the first symptoms of clinical AIDS become evident 8-15 years after infection. HIV infection typically follows an established course: 1) primary acute infection often with a mononucleosis-like disease, 2) a prolonged period without obvious, visible symptoms and 3) a severe immunodeficiency that results in the development of opportunistic infections and tumors that lead to the major causes of death in AIDS

patients (169). The rate of progression through these phases varies among infected individuals.

In the first days after infection, the acute phase is characterized by high levels of viral replication in activated lymphocytes located in the lymph nodes (lymphadenopathy). Individuals generally experience flu-like symptoms during this phase of HIV-1 disease (6-12 weeks). During this time, the viral population is relatively macrophage-tropic (M-tropic). M-tropic HIV-1 isolates, seen during the early stage of infection, infect cells expressing the chemokine receptor, CCR5. The activation of cytotoxic T cells (CTL) and induction of anti-HIV antibodies result in containment of the initial viremia. An increase in CD8+ cytotoxic T lymphocytes is seen in the acute phase, but autologous neutralizing antibodies are not detected until 6 months after infection. CD4+ T lymphocytes decrease during this phase but return to near normal levels after six months. The levels of CD4+ T-cells do not always return to normal levels. In some cases the levels remain low and the patients rapidly progress to the symptomatic or AIDS phase.

The asymptomatic phase occurs approximately 3-4 months after infection. Minimal viral replication occurs during this stage, and the level of HIV detected in the blood remains relatively stable for many years. The amount of virus in the blood (viral load) decreases to a setpoint (steady state level of virus) and is prognostic for the course of infection and disease; higher setpoints correlate with a more rapid disease progression. Viral load set points of $< 10^3$ copies of viral RNA/mm^3 of plasma generally are associated with a slower progression to AIDS (170, 171). During the asymptomatic phase, patients experience

mild symptoms that may include fatigue, weight loss and shingles. Despite the immune response to HIV-1, virus replication continues at a low rate. At the beginning of the asymptomatic phase, the viral population consists mainly of M-tropic strains. However, the viral population becomes more heterogeneous (M-tropic, dual tropic and T cell tropic (T-tropic) HIV-1 strains) towards the end of the asymptomatic stage.

The symptomatic or AIDS phase is the end stage of HIV-1 disease and is characterized by a dramatic drop in CD4+ T lymphocyte population (<200 cells/mm^3 of blood) and is associated with a rise in viremia. Normal healthy adults usually have greater than 1 x 10^3 CD4+ T cells per mm^3 of blood (166, 170, 171). In the lymph nodes, HIV-1 replication increases and lymphoid cells and tissue are destroyed. In the last phase, the virus population becomes more heterogeneous with the emergence of the virulent, T-tropic viruses. T-tropic HIV-1 isolates appear later in the course of HIV infection and infect cells expressing the chemokine receptor, CXCR4. The mechanism by which CD4+ T cells are depleted remains to be identified. Different mechanisms for the destruction of CD4+ and CD8+ T cells have been proposed: 1) direct infection of the cell, 2) the induction of apoptosis 3) syncytium formation of healthy T cells with infected T cells, and 4) bystander killing by some viral proteins (*e.g.* nef) (172). AIDS is characterized by a state of immunodeficiency that allows for the development of secondary, opportunistic infections. During this stage, opportunistic infections develop and eventually the patient succumbs to an AIDS-related illness. Some of the most prevalent opportunistic infections of AIDS are *Pneumocystis carinii, Cryptosporidium, Toxoplasma, Mycobacterium avium/tuberculosis* and *Salmonella* (173-175). Tumors usually associated with HIV

infection are Kaposi's sarcoma (skin), non-Hodgkin's lymphoma (lymphatic tissues) and primary lymphoma of the brain (176).

I.D.1.e. Replication Cycle of HIV-1

The HIV replication cycle occurs in an orderly fashion. HIV-1 entry into target cells is mediated through a complex interaction between the viral envelope glycoprotein and specific cell surface receptors. HIV-1 infects susceptible cells by binding to CD4 on the CCR5 and CXCR4. After coreceptor binding, a subsequent conformational change exposes the fusion domain in Env_{gp41} and results in fusion of the viral and plasma membranes. This process culminates in viral entry and release of the viral core into the cytoplasm of the cell (177, 178). The HIV single stranded (ss) RNA genome is transcribed into double stranded (ds) DNA by the virally encoded reverse transcriptase upon successful entry into the target cell (179). After translocation of the preinfectious complex (PIC) to the nucleus, the viral DNA is integrated randomly into the host chromosomal DNA via the viral integrase and long terminal repeats (LTRs) (180). At this stage, the viral genome is called the provirus. The integrated provirus is flanked by repeat sequences known as LTRs, which are important for integration. In addition, the 5' LTR contains the promoter/enhancer elements necessary for viral gene expression (180). Upon cellular activation by environmental and cellular transcription factors as well as the HIV transactivator protein, Tat, transcription of the proviral genome is initiated. Using host cell proteins and machinery three different viral mRNAs are produced: 1) multiply-spliced, 2) singly-spliced and 3) unspliced mRNAs (181). Initially, the multiply-spliced

mRNAs are transcribed during the early phase of HIV-1 transcription (Tat, Rev and Nef). The singly-spliced mRNAs encode for Env, Vpu, Vif and Vpr. Unspliced mRNAs are transcribed in the late phase of HIV-1 transcription (Gag, Gag-Pol, and genomic RNA). Nuclear export of singly and unspliced viral mRNAs is provided by the viral protein, Rev along with cellular proteins and machinery. Once in the cytoplasm, these mRNAs are then translated into viral proteins. The envelope proteins are synthesized, glycosylated and processed in the endoplasmic reticulum and Golgi apparatus. Following cleavage by furin, the envelope proteins form multimers (trimers) that migrate to the cell surface (182). The structural gene products accumulate at the cell surface and assemble into an immature viral particle, which encapsidates two copies of the viral genome along with the associated proteins. The virus undergoes budding and is released from the infected cell (183). In addition to HIV-1 Env, the viral membrane contains host-derived proteins, such as MHC class I. The particle undergoes a maturation process that involves the proteolytic processing of the Gag and Gag-Pol polyproteins by the viral protease. Gag_{p55} is processed to yield the MA, CA, NC and p6, while Gag-Pol_{p160} is cleaved to produce the Gag gene products plus PR, RT and IN (184).

I.D.1.f. Proviral HIV-1 Genome and Its products

The HIV-1 proviral genome [double-stranded DNA (dsDNA)] contains nine open reading frames that encode for 15 viral proteins (185) (**Figure 5**B and **Table 2**). Like all retroviruses, HIV-1 contains three major genes (*gag*, *pol* and *env*), which encode precursor polyproteins that are cleaved to produce the core structural (CA, MA, NC), enzymatic (PR, RT, IN) and envelope (gp120, gp41) proteins, respectively. The HIV-1

genome also contains two regulatory genes (*tat* and *rev*) and 4 accessory genes (nef, *vif*, *vpu*, and *vpr)* that are required for efficient virion replication and maturation (**Table 2**). The proviral DNA genome also contains two long terminal repeat (LTRs) which flank both the 5' and 3' ends. The 5' LTR contains the HIV-1 promoter and enhancer sequences that regulate gene expression.

Only the products of the major structural and enzymatic genes are described in the text. Regulatory and accessory proteins are described in (**Table 2**)

I.D.1.f.1. Products of the *gag* Gene: Gag, Matrix, Capsid and Nucleocapsid

The group associated antigen (*gag)* gene encodes a 55 kDa precursor protein (Gag_{p55}), which is expressed from the unspliced viral messenger RNA (mRNA). The precursor protein is proteolytically cleaved into three main structural gene products that are incorporated into mature virions: matrix (MA), capsid (CA) and nucleocapsid (NC). Assembly and maturation of HIV particles is dependent on the Gag gene products.

The MA (p17) protein undergoes post-translational myristylation at the N-terminus, promotes attachment of Gag_{p55} to the cell membrane and forms the submembrane layer of the virion. Two distinct features of MA are involved in membrane targeting: 1) N-terminal myristate group and 2) basic residues found within the first 50 amino acids (together known as the "membrane-binding" or "M" domain). Trimeric MA associates with the cell membrane by insertion of the 3 myristate groups into the lipid bilayer

located directly above the trimer, and the interaction occurs between the basic residues on MA and bilayer phospholipid head groups. In addition to Gag/Gag-Pol membrane targeting, MA assists in Env incorporation into the virions by association with the cytoplasmic tail of Env (186, 187).

The CA (p24) protein is the most abundant viral protein found in the virus and is required for Gag-Gag multimerization. CA is composed of two domains: 1) N-terminal region or core domain (virion maturation and incorporation of cyclophilin A (CypA)), and 2) C-terminal "dimerization" domain (Gag-Gag interactions). CA is also required for the incorporation of Gag-Pol polyprotein into virions, which is essential for the recruitment of PR, RT and IN into the virus particle (188).

NC is located within the capsid layer and is responsible for packaging of the viral RNA genome (189). NC contain 2 zinc-finger motifs found in many cellular DNA binding proteins (190). NC is tightly associated with the viral RNA in virions by binding to the packaging signal, *psi* (ψ), located near the major splice donor site (immediately 5' of *gag*) (191, 192). The interaction between NC and ψ requires intact zinc fingers and the flanking basic residues (193, 194). In addition to RNA binding and encapsidation, NC plays a role in: 1) RNA dimerization (195), 2) Gag-Gag interactions, 3) virus assembly (196), 4) tRNA incorporation and annealing to the primer binding site (pbs)/strand transfer during RT (188, 197), and 3) stability of the PIC (198).

I.D.1.f.2. Products of the *pol* Gene: Protease, Reverse Transcriptase and Integrase

The *pol* gene encodes for three viral enzymes: protease (PR), reverse transcriptase (RT), and integrase (IN) (**Table 2**). The *pol* gene products are derived from the Gag-Pol_{p160} precursor, which is generated by a ribosomal frameshifting during translation of Gag_{p55}. The frameshift only occurs 5-10% of the time, which ensures that *pol* gene products are expressed at low levels (compared to Gag gene products). The three enzymes are associated with the viral genome.

PR is responsible for the proteolytic processing of the Gag-Pol_{p160} and Gag_{p55} precursor polyproteins and thus plays a critical role in the maturation of the virion. HIV-1 PR uses two apposed Asp residues at the active site to direct a water molecule that catalyzes the hydrolysis of a peptide bond in the target protein. HIV-1 PR functions as a dimer (199, 200) and the substrate binding site is located within a cleft formed between the two monomers. The first cleavage event in all retroviral Gag-Pol polyproteins is the autolytic processing of PR. Following its release, PR forms a dimer and cleaves a number of sites in the Gag and Gag-Pol precursors. Gag processing by PR occurs at junctions between MA/CA, CA/p2, p2/NC, NC/p1 and p1/p6, but each site is cleaved at different rates. Proteolytic cleavage of Gag/Gag-Pol precursors results in a dramatic change in the morphology of the particle, which is known as maturation.

RT converts the viral ssRNA genome into the double-stranded (ds) DNA form known as the provirus (201). Although each virus contains two strands of RNA, only one provirus is made (202). The mature RT holoenzyme is a heterodimer (p51/p66; 250 molecules per virion) and has three enzymatic functions: 1) RNA-directed DNA polymerization (minus-strand DNA synthesis), 2) RNaseH activity (degradation of the tRNA primer and genomic RNA in the DNA/RNA hybrid intermediates) and 3) DNA-directed DNA polymerization (plus-strand DNA synthesis). The final product of reverse transcription is a ds DNA molecule that can integrate into the host chromosomal DNA. The high mutation rate of HIV-1 is largely due to the error-prone nature of RT, which lacks proofreading activity and frequently switches templates (203).

IN (p32) mediates the integration of the viral DNA into host cell chromosomes during the replication cycle. Retroviral INs are comprised of 3 structural/functional domains: 1) N-terminal zinc-finger-containing domain, 2) core domain and 3) the relatively conserved C-terminal domain. Integration of all retroviruses follow the same series of events (204, 205). IN removes 2-3 nucleotides from the blunt 3' terminus of both strands of full-length, ds DNA forming the pre-integration substrate. Randomly, IN catalyzes a staggered cleavage of the cellular target sequence once inside the nuclease. The 3' recessive ends of viral DNA are joined to the 5' ends of the cleaved cellular DNA (strand transfer). Host cell repair machinery fills in the gaps thus completing the integration process (204, 206). The integrated viral DNA (provirus) is flanked by a 5 bp direct repeat (5'-TG, CA-3').

I.D.1.f.3. Products of the *env* gene: Env_{gp120} and Env_{gp41}

The *env* gene encodes for glycoproteins that are important in receptor binding and entry (**Figure 5** and **Table 2**) (182, 207). The *env* gene is expressed as a polyprotein precursor, Env_{gp160}. Env_{gp160} is cleaved by the cellular protease furin in the two Env glycoproteins: gp120 and gp41. Env_{gp120} and Env_{gp41} associate through noncovalent interaction to form a multimeric structure (trimer) on the surface of the virion (208-210). Env_{gp41} forms the transmembrane domain of the Env complex, while Env_{gp120} is presented on the surface of infected cells or virions. Env_{gp120} has five hypervariable (V) (V1-V5) and five constant (C) (C1-C5) regions. The amino acid sequence in the variable loops can vary greatly among HIV-1 isolates (211-215). One of these regions, the V3 loop, is an important determinant in cell tropism for HIV-1 and contains the chemokine receptor binding domain (214, 215). Env_{gp120} as well as Env_{gp41} mediate entry of the virus into host cells. Initially, Env_{gp120} binds to human CD4 (hCD4) on the surface of target cells (177, 216). This interaction results in a conformational change in Env_{gp120}, which exposes the chemokine receptor-binding domain. Binding of the virus to the chemokine receptor leads to another conformational change in Env_{gp120} that exposes the fusogenic domain in Env_{gp41}. This domain instigates entry of the viral core by fusing the viral and host cell membranes (217, 218).

I.D.1.g. Env as Antigen for Vaccine Design

Env is the only realistic target for neutralizing antibodies against HIV-1, because it is the only viral protein found on the outer surface of the virion and mediates entry of the virus into susceptible cells. For this reason, several experimental vaccines have targeted this molecule. In early Env subunit vaccine studies in mice and non-human primates, neutralizing antibodies were enhanced (219-221) and some (not all) chimpanzees were protected from infection following challenge with HIV (14, 222). Based on these results, recombinant Env_{gp120} was evaluated for safety and immunogenicity in humans. Antibodies to Env were detected in almost all vaccinees, and neutralizing antibodies were detected in the majority of recipients (223, 224). It later became evident that these neutralizing antibodies were transient and limited to homologous, laboratory-adapted HIV strains (225, 226). HIV Env subunit vaccines do not generally induce significant CTL responses against Env (227, 228), nor do they neutralize primary isolates (225, 229). In order to improve Env based vaccines researcher developed strategies that may produce more immunologically relevant antibodies to the native structure of Env such as oligomeric gp140 and particle based-vaccines. Various approaches to construct soluble, trimeric forms of Env that more closely mimic the native Env_{gp160} on the surface of virions have been employed. Some of these include stabilized $Env_{gp120/gp41}$ subunits (230), Env_{gp160} and Env_{gp140} oligomers (210, 231) and trimeric Env (232). Soluble, trimerized Env_{gp140}, unstablized or stabilized with domains (GCN4, T4 bacteriophage fibritin motifs or by additional disulfide bonding (SOS)), elicited modest levels of enhancement of neutralizing antibody compared to antibody elicited by monomeric forms of Env (233) (234). Furthermore, several DNA vaccine approaches have been developed for Env. One

of the advantages of DNA vaccines over subunit vaccines is the potential to induce humoral as well as cellular immune responses (See Section I.A.1.g). Initial studies using wild-type Env_{gp120} sequences concluded that these genes were not immunogenic enough due to the poor expression in mammalian cells. In order to enhance the immunogenicity of the *env* gene, several approaches have been taken, such as the use of molecular adjuvants (cytokines and complement derived C3d) (81, 85, 132, 235) and codon optimization of the gene sequence for optimal expression in mammalian cells (135, 172).

The extensive use of Env in the development of HIV-1 vaccines has provided a large body of knowledge about this antigen. Several immune assays to measure the anti-Env immune response in small as well large animals, including humans, have been created. Furthermore, the array of reagents available for the study of Env is extensive and can be easily accessed. All this, has made HIV-1 Env a good model antigen for the study of adjuvants.

I.D.1.h. Antiretroviral Therapy

Currently, highly active anti-retroviral therapy (HAART) is a treatment regimen widely used by physicians. HAART involves using an assortment of antiretroviral drugs to reduce or prevent viral replication (usually inhibitors of HIV-1 PR and RT). A combination of two or more antiretroviral medications is generally more effective than using just one of these medications (monotherapy) for treating HIV infection. The regimen usually consists of one protease inhibitor (e.g. lamivudine) and one or more reverse transcriptase inhibitors (e.g. zidovudine or stavudine) and results in reduced

levels of virus (<50 copies of viral RNA/mm^3 blood) after one year of treatment in approximately 60-80% of patients (236). The use of HAART has enhanced both the longevity and quality of life for infected individuals by controlling viral replication (236, 237). Some of the advantages of combination antiretroviral drug therapy for the treatment of HIV are: 1) minimal incidence of HIV-related complications, 2) decrease in viral loads/induction of lower viral setpoints, 3) lessened severity and delayed onset of symptoms and 4) prolonged survival of infected individuals (269, 498). Despite the effectiveness of HAART, several drawbacks are accompanied with this treatment that limit its worldwide use (particularly in developing nations). First, HAART does not protect patients against initial infection nor does HAART clear viral infection. Other disadvantages include: toxicity, non-adherence, lack of efficacy, interactions with other drugs and food, unfavorable pharmacokinetics, transportation and storage, high production cost and drug resistance (238).

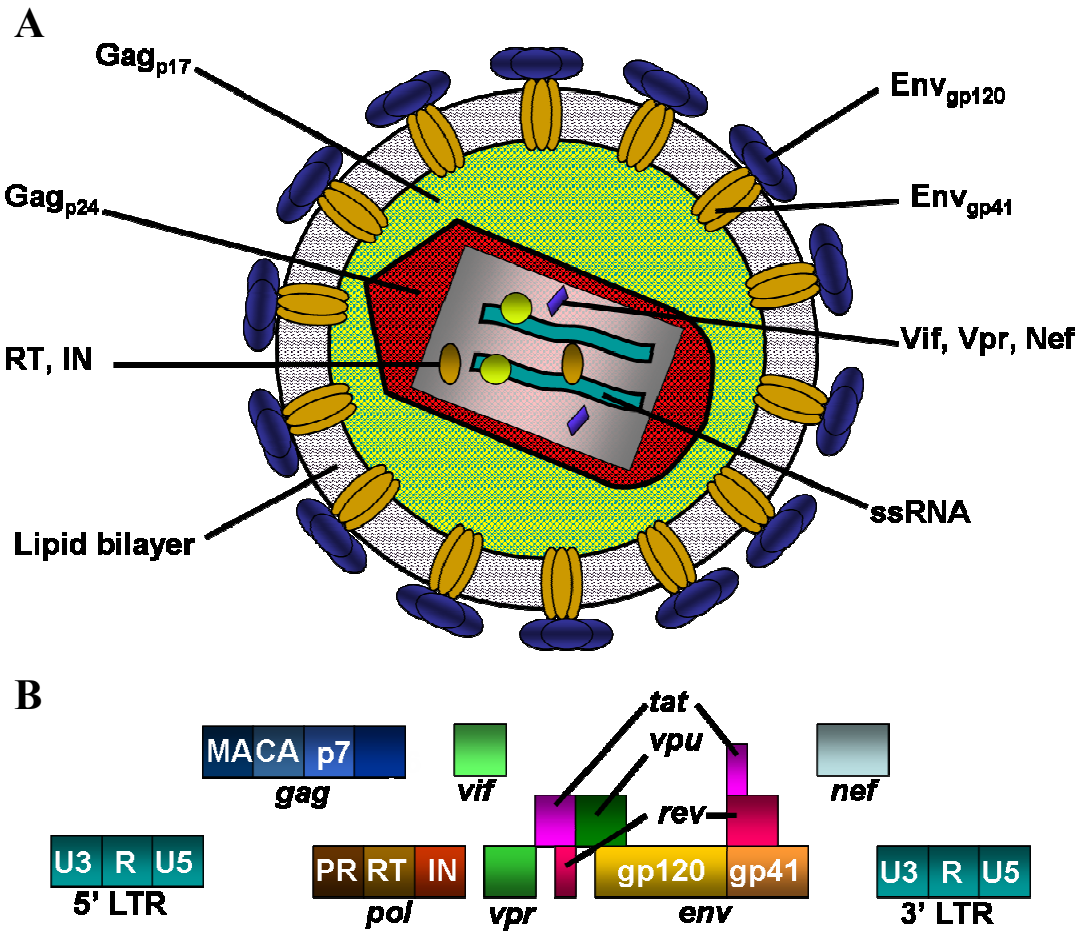

Figure 5. Representation of HIV-1 Virion and Proviral Genome.

A) The mature HIV-1 virion contains multiple viral gene products. Gag and Env are the main structural proteins that form the spherical shape with spiked glycoproteins protruding from the outer surface. The gag gene products include the capsid (Gagp24), matrix (Gagp17), and nucleocapsid (Gagp6) proteins. The env gene products include surface (Envgp120) and transmembrane (Envgp41) glycoproteins. The Env glycoproteins are inserted in the lipid bilayer, which is derived from the host cell membrane during the budding process. In addition, there are 2 enzymatic (RT and IN) and 3 accessory (Vif, Vpr, Nef) proteins found in the mature virion. HIV-1 contains 2 copies of its single stranded RNA genome (ssRNA).
B) The proviral genome is flanked by 2 long terminal repeats (LTRs). Transcription of viral genes is initiated from the promoter found in the 5'LTR. In the HIV-1 genome, there are 9 open reading frames, which generate 15 different gene products. The Gag-Pol precursor polypeptide is cleaved into gag and pol gene products. The Env/Vpu mRNA is singly spliced to produce Vpu or the env gene precursor (Envgp160) that is processed to Envgp120 and Envgp41. Regulatory (Tat and Rev) and accessory (Vif, Vpr, Nef) proteins are generated by multiply splicing of the Gag-Pol precursor mRNA

Table 2. HIV-1 Proteins and Their Main Function

Type of Protein	Protein	Main Function
Structural	Gag (CA, MA, NC)	Forms sphere of virion. Encapsidation of vRNA
Structural	Env (Env_{gp120} and Env_{gp41})	Virus binding and entry into susceptible cells
Enzymatic	Pol-IN	Directs proviral integration into host chromosome
Enzymatic	Pol-RT	Converts genomic viral RNA to proviral DNA
Enzymatic	Pol-PR	Cleaves Gag-Pol pr55 into 7 gene products
Regulatory	Tat	Promoting and enhancing viral transcription
Regulatory	Rev	Nuclear export of unspliced and singly spliced vRNAs
Accessory	Nef	Downregulation of CD4 and MHC I, increases infectivity
Accessory	Vpu	Downregulation of CD4 and enhances virus release
Accessory	Vpr	Nuclear localization of PIC, cell cycle arrest (G2)
Accessory	Vif	Required for replication in vivo, enhances infectivity

I.D.2. Influenza Virus

I.D.2.a. Influenza Virus – General Aspects

Influenza viruses are the causative agents of an acute febrile respiratory disease called influenza (commonly known as "flu"). Influenza is one of the most prevalent and significant viral infections worldwide. There are even descriptions of influenza epidemics (local dissemination of influenza) that occurred in ancient times. Probably the most famous influenza pandemic (wordwide dissemination of influenza) is the one that swept the world in 1918 and 1919 (Spanish flu), killing around 20 million people. Several other pandemics have occurred since then in 1947, 1957, 1968 and the last one in 1977. New virus strains have been detected since the last pandemic, including a limited outbreak in Honk Kong in 1997 (chicken flu).

Influenza is caused by Influenza virus, which is a member of the Orthomixoviridae family. There are three types of Influenza viruses; however, only influenza A and B can cause significant human disease. Orthomixoviruses possess segmented, negative stranded RNA genomes (vRNA) and are enveloped, usually spherical and bud from the plasma membrane (more specifically, the apical plasma membrane of polarized epithelial cells). Complete virus particles, therefore, are not found inside infected cells. Virus particles consist of three major subviral components, namely the viral envelope, matrix protein (M1), and core (viral ribonucleocapsid [vRNP]) (**Figure 6**). The viral envelope surrounding the vRNP consists of a lipid bilayer containing spikes composed of viral

glycoproteins (HA, NA, and M2) on the outer side and M1 on the inner side. Viral lipids, derived from the host plasma membrane, are selectively enriched in cholesterol and glycosphingolipids (239). M1 forms the bridge between the viral envelope and the core. The viral core consists of helical vRNP containing vRNA (minus strand) and NP along with minor amounts of NEP and polymerase complex (PA, PB1, and PB2). For viral morphogenesis to occur, all three viral components, namely the viral envelope (containing lipids and transmembrane proteins), M1, and the vRNP must be brought to the assembly site, i.e. the apical plasma membrane in polarized epithelial cells. Finally, buds must be formed at the assembly site and virus particles released with the closure of buds (240) (**Figure 6**).

The segmented genome of these viruses facilitates the development of new strains through the mutation and reassortment of the gene segments among different human and animal strains of virus. This genetic instability is responsible for the annual epidemics and periodic pandemics in influenza infection worldwide (241).

I.D.2.b. Influenza Types, Subtypes and Strains

There are three types of influenza viruses: A, B, and C. The members of each type are serologically cross-reactive with each other but not with members of the other types. This serological cross-reactivity is primarily attributable to antibodies to the major internal structural proteins, the matrix (M1) and nucleocapsid (NP). These antibodies are detected by complement fixation or ELISA assays and are useful for diagnosis, but do not confer

protection against infection. Only influenza A viruses are further classified by subtype on the basis of the two main surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) (e.g. H1N1). Influenza A subtypes and B viruses are further classified by strains(242).

I.D.2.b.1. Influenza Type A and Its Subtypes

Influenza type A viruses can infect people, birds, pigs, horses, and other animals, but wild birds are the natural hosts for these viruses. Influenza type A viruses are divided into subtypes and named on the basis of two proteins on the surface of the virus: hemagglutinin (HA) and neuraminidase (NA). For example, an "H7N2 virus" designates an influenza A subtype that has an HA 7 protein and an NA 2 protein. There are 16 known HA subtypes and 9 known NA subtypes. Many different combinations of HA and NA proteins are possible. Only some influenza A subtypes (i.e., H1N1 and H3N2) are currently in general circulation among people. Other subtypes are found most commonly in other animal species. For example, H7N7 and H3N8 viruses cause illness in horses, and H3N8 also has recently been shown to cause illness in dogs.

Only influenza A viruses infect birds, and all known subtypes of influenza A viruses can infect birds. However, there are substantial genetic differences between the influenza A subtypes that typically infect birds and those that infect both people and birds. Three prominent subtypes of the avian influenza A viruses that are known to infect both birds and people are:

Influenza A H5

Nine potential subtypes of H5 are known. H5 infections, such as the high pathogenic avian influenza (HPAI) H5N1 viruses currently circulating in Asia and Europe, have been documented among humans and sometimes cause severe illness or death.

Influenza A H7

Nine potential subtypes of H7 are known. H7 infection in humans is rare but can occur among persons who have direct contact with infected birds. Symptoms may include conjunctivitis and/or upper respiratory symptoms. H7 viruses have been associated with both LPAI (e.g., H7N2, H7N7) and HPAI (e.g., H7N3, H7N7), and have caused mild to severe and fatal illness in humans.

Influenza A H9

Nine potential subtypes of H9 are known; influenza A H9 has rarely been reported to infect humans. However, this subtype has been documented only in a low pathogenic form.

Influenza A Strains

Different strains of influenza A virus are classified by the next 4 characteristics: 1) Type (A, B and C), 2) place of original isolation, 3) date of original isolation, and 4) antigen HA and NA. For example, a strain of virus can be designated as A/Bangkok/1/79 (H3N2), meaning that was first isolated in Bangkok in January 1979 and contains HA (H3) and NA (N2) antigens. New strains of influenza viruses appear and replace older strains. This process occurs through antigenic drift. When a new strain of human influenza virus emerges, antibody protection that may have developed after infection or vaccination with an older strain may not provide protection against the new strain. Therefore, the influenza vaccine is updated on a yearly basis to keep up with the changes in influenza viruses(242).

I.D.2.b.2. Influenza Type B

Influenza B viruses are usually found only in humans. Unlike influenza A viruses, these viruses are not classified according to subtype. Influenza B viruses can cause morbidity and mortality among humans, but in general are associated with less severe epidemics than influenza A viruses. Although influenza type B viruses can cause human epidemics, they have not caused pandemics.

I.D.2.b.3. Influenza Type C

Influenza type C viruses cause mild illness in humans and do not cause epidemics or pandemics. These viruses are not classified according to subtype.

I.D.2.c. Human Influenza Viruses and Avian Influenza A Viruses

Humans can be infected with influenza types A, B, and C viruses. Subtypes of influenza A that are currently circulating among people worldwide include H1N1 and H3N2 viruses.

Wild birds are the natural host for all known subtypes of influenza A viruses. Typically, wild birds do not become sick when they are infected with avian influenza A viruses. However, domestic poultry, such as turkeys and chickens, can become very sick and die from avian influenza, and some avian influenza A viruses also can cause serious disease and death in wild birds.

I.D.2.c.1. Low Pathogenic versus Highly Pathogenic Avian Influenza A Viruses

Avian influenza A virus strains are further classified as low pathogenic (LPAI) or highly pathogenic (HPAI) on the basis of specific molecular genetic and pathogenesis criteria that require specific testing. Most avian influenza A viruses are LPAI viruses that are usually associated with mild disease in poultry. In contrast, HPAI viruses can cause severe illness and high mortality in poultry. More recently, some HPAI viruses (e.g., H5N1) have been found to cause no illness in some poultry, such as ducks. LPAI viruses

have the potential to evolve into HPAI viruses and this has been documented in some poultry outbreaks. Avian influenza A viruses of the subtypes H5 and H7, including H5N1, H7N7, and H7N3 viruses, have been associated with HPAI, and human infection with these viruses have ranged from mild (H7N3, H7N7) to severe and fatal disease (H7N7, H5N1). Human illness due to infection with LPAI viruses has been documented, including very mild symptoms (e.g., conjunctivitis) to influenza-like illness. Examples of LPAI viruses that have infected humans include H7N7, H9N2, and H7N2.

In general, direct human infection with avian influenza viruses occurs very infrequently, and has been associated with direct contact (e.g., touching) infected sick or dead infected birds (domestic poultry).

I.D.2.d. Antigenic Drift and Shift

New strains of influenza A are generated through mutation and reassortment. The genetic diversity of influenza A is fostered by its segmented genomic structure and ability to infect and replicate in humans and many animal species (zoonose), including birds and pigs. Hybrid viruses are created by co-infection of a cell with different strains of influenza A virus, allowing the genomic segments to randomly associate into new virions. An exchange of the HA glycoproteins may generate new virus that can infect an immunologically naïve population. For example, an H1N1 bird virus and an H3N2 human virus infected pigs, reassortants were isolated from the pig, and the resulting virus was able to infect humans. This type of reassortment is proposed to be the source of

pathogenic human strains. Minor antigenic changes resulting from mutation of the HA and NA genes are called "antigenic drift". This process occurs every 2-3 years, and is responsible for local outbreaks (epidemics) of influenza A infection. Antigenic drift apperars to involve the selection under antibody pressure of antigenic mutants with the ability to reinfect at least a proportion of the population. The precise mechanism of this selective process, however, remains unknown (243). Major antigenic changes, known as "antigenic shift", result from the reassortment of genomes among different strains, including animal strains. Antigenic shift is associated with the occurrence of major pandemics (wordwide dissemination of influenza). The mechanism of antigenic shift involves simultaneous infection of an individual with two different influenza viruses (*e.g.* H2N2 and H3N1). Reassortment of the RNA segments of these different viruses results in the production of a completely new virus (*e.g.* H3N2) (243). Antigenic drift occurs both in influenza A and B viruses, while antigenic shift occurs only in influenza A virus. Antigenic shifts occur infrequently, taking place on average every 10-15 years. In 1947, the prevalent influenza A virus was the H1N1 subtype. By 1957, there was a shift in both antigens, which resulted in the H2N2 subtype. The H3N2 subtype appeared in 1968 and the in 1977 the H1N1 reappeared (241, 242).

I.D.2.e. Epidemiology

Influenza causes both endemic and epidemic disease. In both the Northern and Southern hemispheres, disease is largely seen during the winter months. In the Northern hemisphere, the season runs from November to March; in the Southern hemisphere the

season runs from April to October. In equatorial regions, the incidence is relatively even. The mechanisms for seasonal variation are unknown. Each year, influenza occurs in distinct outbreaks of varying severity. Some years, disease can be relatively insignificant, but then have major consequences in other years. This epidemiologic pattern reflects the antigenic changes undergone by the envelope glycoproteins, hemagglutinin and neuraminidase, and the subsequent susceptibility of the population (Section I.D.2.d.). Factors that determine the extent and severity of outbreaks are not fully understood, but are determined in part by the prevalence of antibodies to the circulating virus, and the intrinsic virulence of the virus (e.g., efficiency of transmission, ability to cause symptomatic infection etc.).

An outbreak of influenza A virus typically begins abruptly, peaks over a 2-3 week period, and last for 2-3 months. In most outbreaks, an increase in febrile respiratory illness in children, followed by an increase in influenza-like illnesses in adults is the earliest indicatory of influenza activity. During most outbreaks, the attack rate is between 10-20% of the susceptible population, but can exceed 50% during pandemics. Outbreaks caused by influenza B virus are generally less extensive and associated with less severe disease, but influenza B can still be lethal.

Due to high attack rates, influenza has great social impact. It is the major cause of work and school absenteeism. During an interpandemic year, 75 million work days and greater than 50 million school days are lost. During an interpandemic year, there are more than 110,000 excess hospitalizations. During a pandemic, that number can rise to 200,000 to

greater than 300,000. Mortality associated with influenza and associated pneumonia occurs largely in adults, especially those with underlying disease that puts them at high risk for complications. However, during pandemics, approximately 50% of the deaths have occurred in those less than 65 years old.

I.D.2.f. Influenza Virus Genome and Its Proteins

Influenza, which possesses segmented, negative stranded RNA genomes (vRNA), is an enveloped virus that buds from apical plasma membrane of polarized cells. Virus particles (usually spherical ~100 nm in diameter) consist of three major subviral components, namely the viral envelope, matrix protein (M1), and core (viral ribonucleocapsid [vRNP]) (**Figure 6**). The viral envelope surrounding the vRNP consists of a lipid bilayer containing spikes composed of viral glycoproteins (HA, NA, and M2) on the outer side and M1 on the inner side. Viral lipids, derived from the host plasma membrane, are selectively enriched in cholesterol and glycosphingolipids (239). M1 forms the bridge between the viral envelope and the core. The viral core consists of helical vRNP containing vRNA (minus strand) and NP along with minor amounts of NEP and polymerase complex (PA, PB1, and PB2). The products of the influenza gene segments are summarized in **Table 3**.

The genomic segments of the influenza A virus range from 890 to 2340 bases. All the proteins are encoded on separate segments, with the exception of the nonstructural

proteins (NS1 and NS2 [now known as nuclear export protein –NEP-]) and the M1 and M2 proteins, which are transcribed from one segment each (**Table 3**). The most relevant gene products are briefly described.

I.D.2.f.1. Hemagglutinin (HA)

Hemagglutinin (HA), a type I transmembrane, is the major envelope protein (~ 80%). HA forms a spike-shaped homotrimer (**Figure 6**) (244). Each unit is activated by a protease and cleaved into two subunits that are held together by a disulfide bond. Influenza HA has several functions: 1) HA is the viral attachment protein (binding to sialic acid [N-acetyl-neuraminic acid] on epithelial cell surface receptors); 2) promotes fusion of the envelope to the cell membrane; 3) hemmaglutinates (binds and aggregates) human, chicken and guinea pig red blood cells; and 4) elicits the protective neutralizing responses (245).

The actual sialic acid binding site is a very small part of the large HA trimer protein. This allows HA to change antigenically under selective pressure from the immune system while preserving a functional binding site. Mutation derived changes in HA are responsible for the minor (drift) and major (shift) changes in antigenicity. Shifts occur only in influenza A virus and are designated H1, H2 and so on (246).

In order to be infectious, the HA polypeptide must be cleaved by a host cell serine protease. If the cleavage is not performed, HA can still attach to a host cell, but the virus cannot enter it. Antibodies against HA are the most important for anti-flu immunity, since they are able to prevent infection. However, antibodies to neuraminidase have also been shown to confer protection (247).

HA is one of the major influenza viral antigens. An efficient immune response to this protein confers protection against infection (247). HA is part of the current anti-flu vaccines used in humans and has been used as a model antigen for the development of new vaccines technologies (84, 127, 248, 249). One of the advantages of using HA in vaccine studies is the availability of well characterized small animal models that can be infected by adapted influenza virus strains. These adapted influenza virus strains cause morbidity (illness) and/or mortality of the animals. Hence, the immune response induced by an experimental vaccine can be readily evaluated by a lethal virus challenge. For these reasons, HA from influenza virus is an antigen than can also be used in the study of adjuvants.

I.D.2.f.2. Neuraminidase (NA)

Neuraminidase (NA), a type II transmembrane glycoprotein, is present as a homotetramer on the viral envelope (**Figure 6**). NA has enzyme activity. NA cleaves the sialic acid on glycoproteins, including the cell receptor. Cleavage of sialic acid on glycoproteins prevents clumping and facilitates the release of virus from infected cells. NA also

hydrolyzes mucoproteins in nasal secretions, which may help to spread the virus. Antibodies against NA help to prevent cell to cell spread, thus decreasing the severity of the illness. NA is the target of two antiviral drugs: zanamivir and oseltamivir (250, 251). NA of influenza virus also undergoes antigenic changes and major differences acquire designations N1, N2 and so on.

I.D.2.f.3. . Matrix (M1) and Membrane (M2) proteins

Matrix (M1), membrane (M2) and nucleoprotein (NP) proteins are type-specific and are therefore used to differentiate among influenza A, B and C viruses.

M1 and M2 proteins encoded in the same gene segment and are products of alternative spliced mRNA. M1 proteins line the inside of the virion, promote assembly and have a role in the budding process. M2, a type III transmembrane protein, is a minor protein component of the viral envelope (only 16-20 molecules/virion) (**Figure 6**). M2 is a homotetramer, functions as an ion channel (252, 253), and is crucial during uncoating for dissociating the vRNP from M1 in the early phase of the infectious cycle. M2 protein of influenza A virus is the target for influenza drugs amantadine and rimantadine (250, 251).

I.D.2.f.4. . Nucleoprotein (NP)

Influenza A virus RNA segment 5 encodes for the nucleoprotein NP (a polypeptide of 498 amino acids in length), which is rich in arginine, glycine and serine residues.

Suggestions that NP contains kinase activity have not been confirmed and it is not certain that the protein possesses an enzymatic function. NP binds ssRNA and is able to self-associate to form large oligomeric complexes. It also binds the PB1 and PB2 subunits of the polymerase complex and the matrix protein M1. NP has also been shown to interact with at least four cellular polypeptide families: nuclear import receptors of the importin α class, filamentous (F) actin, the nuclear export receptor CRM1 and a DEAD-box helicase BAT1/UAP56. (254).

NP is part of the viral core, which consist of 1) helical ribonucleocapsids containing vRNA, 2) minor amounts of the nuclear export protein (NEP), and 3) three polymerase proteins (PB1, PB2 and PA) which form the viral RNA polymerase complex (3P complex) (254).

I.D.2.g. Replication Cycle of Influenza Virus

Influenza replication cycle begins with the binding of HA to sialic acid structures on cell surface glycoproteins. The virus is then internalized into a coated vesicle and transferred to an endosome. Acidification of the endosome causes HA to bend over and expose hydrophobic fusion–promoting regions of the protein. The viral envelope then fuses with the endosome membrane (245). Cleavage of HA is an absolute requirement for infectivity and the nature of the HA cleavage site is an important virulence determinant for influenza viruses. Cleavage efficiency of HA varies depending on the presence of single or multiple basic residues at the cleavage site of HA1 and HA2 and the plasminogen binding ability

of NA. Viruses containing HA with a single positive charge at the cleavage site can be cleaved by specific enzymes such as *tryptase Clara* present in the lungs, whereas HA containing multiple basic residues at the cleavage site are cleaved ubiquitously by proteases (255). In the acid pH of the endosome, the cleaved HA undergoes conformational changes releasing the NH_2 terminal fusion peptide of HA2 and causing fusion of viral and endosomal membranes (256). Virus particles containing uncleaved HA can bind and be endocytosed but cannot undergo fusion and are therefore non-infectious. The M2 protein promotes acidification of the envelope contents to break the interaction between M1 protein and the vRNP to allow uncoating and delivery of the nucleocapsid into the cytoplasm. The nucleocapsid travels to the nucleous where it is transcribed into messenger RNA (mRNA) (257).

The influenza transcriptase (PA, PB1, PB2) (polymerase complex) (**Table 3**) uses host cell mRNA as a primer for viral mRNA synthesis. Therefore, it steals the methylated cap region of the RNA, which is the sequence required for efficient binding to ribosomes. All the genomic segments are then transcribed into 5' capped, 3' polyadenilated (poly A) mRNA for individual proteins, except the segment for the M and NS proteins. These segments are differentially spliced, using cellular enzymes, to produce two different mRNAs. The mRNAs are translated into proteins in the cytoplasm. The HA and NA glycoproteins are processed by the endoplasmic reticulum and Golgi apparatus. The M2 protein inserts into cellular membranes. Its proton channel prevents acidification of Golgi and other vesicles, thus preventing acid-induced folding and inactivation of the HA within the cell. HA and NA are then transported to the cell surface (240).

Positive-sense RNA templates for each segment are produced and the negative-sense RNA genome is replicated in the nucleus. The genomic segments are then transported to the cytoplasm and associated with polymerase and NP proteins to form nucleocapsids, which interact with the M1 protein lining plasma membrane sections containing M2, HA and NA. The genomic segments are enveloped in a random manner, with 11 segments per virion. This process produces a small number of virions with a complete genome and numerous defective particles. The particles are antigenic and can cause interference, which may limit the progression of the infection. The virus buds selectively from the apical surface of the cell as a result of the preferential insertion of the HA in this membrane. Virus is released about 8 hours after infection (240).

I.D.2.h. Pathology of the Disease and Clinical Course

Influenza is an acute febrile illness that is usually self-limited. However, significant mortality results from complications following an influenza infection. The virus is transmitted by airborne droplets and enters the nasopharynx. Influenza virus infects ciliated epithelial cells of the respiratory tract. Nasal and bronchial biopsies of patients with acute uncomplicated influenza show desquamation of the ciliated columnar epithelial cells of the bronchi. At the beginning of the second week of infection, patients begin to develop neutralizing antibodies against HA and NA. Protection from new infections is mediated by antibodies to HA and NA. However, cell mediated immunity is important for clearance of infection and recovery (258).

Depending on the degree of immunity to the infecting strain of virus and other factors, infection may range from asymptomatic to severe. Patients with underlying cardiorespiratory disease, people with immune deficiency (even that associated with pregnancy), and smokers are susceptible for a severe case of the flu. Risk of serious complications include: 1) people over the age of 65 years (people over the age of 50 years require a flu shot); 2) people of any age with chronic medical conditions, such as chronic obstructive pulmonary disease (COPD), diabetes and cardiopulmonary disease; 3) very young children, and 4) pregnant women. These individuals should receive a yearly vaccination, as should all healthcare providers (258-261).

After an incubation period of 1 to 4 days, the "flu syndrome" begins with a brief prodrome of malaise and headache lasting a few hours. The prodrome is followed by the additional abrupt onset of fever, severe myalgia and usually a non-productive cough. The illness persist for approximately 3 days and unless a complication occurs, recovery is complete within 7-10 days. Complications of influenza include bacterial pneumonia, myositis and Reye's syndrome (262). Influenza can directly cause pneumonia, but it more commonly promotes a secondary bacterial superinfection that leads to bronchitis or pneumonia. The tissue damage caused by progressive influenza virus infection of alveoli can be extensive, leading to hypoxia and bilateral pneumonia. Secondary bacterial infections usually involve *Streptococcus pneumoniae*, *Haemophilus influenzae* or *Staphylococcus aureus* (258, 263)

I.D.2.i. Immunology

Influenza immunity is usually measured as serum IgG antibodies to the HA and NA antigens of the circulating influenza A and B viruses and high titers typically correlate with a lower attack rate for infection and less severe influenza disease (247). While convenient, this approach does not evaluate other immune mechanisms, such as mucosal antibodies and cell-mediated immunity. The higher susceptibility of the host to serious influenza at the extremes of age is likely to reflect diminished capacities of the innate and adaptive immune system in the very young and the elderly (258). Young children are often immunologically naïve hosts and may also have intrinsic limitations in immune cell function. On the other hand, immunosenescence in the elderly is characterized by poor responses to infection or vaccination, despite repeated priming of memory immunity. While HA antibodies in serum provide a correlate of protection (247, 248, 264, 265), influenza protection is likely to depend on a repertoire of effector mechanisms provided by innate responses, as well as effector and memory B- and T-cells. Such responses at both mucosal and systemic sites are presumed to be necessary for natural and vaccine-induced protection, but the immune mechanisms and relative importance of each component in the exposed or infected host is not known. Memory CD4+ and CD8+ T-cells that recognize influenza virus particles and antigens are detectable by cytokine flow cytometry methods (266). In addition to HA, cytotoxic T cells can recognize the nucleoprotein (NP), matrix protein (M1), nonstructural protein 1 (NS1), and polymerases (PB1 and PB2). Although these T cell responses are detected, their role in the control of acute influenza infection or in protecting the human host against re-infection is not well defined. Little is known about influenza-specific B cells at the single cell level or the

homing of effector T or B cells to the respiratory tract and the contribution of NK cells is poorly understood. Recent experiments demonstrated IFN-γ production by peripheral blood NK cell subsets, as well as by influenza A-specific memory CD8 T cells after exposure to influenza A virus; IL-2 production by T cells was required for the IFN-γ response of NK cells, indicating that memory T cells enhance innate NK-mediated antiviral immunity (267).

I.D.2.j. Antiviral Agents Used in the Treatment of Influenza

There are several antiviral drugs that can be used to treat influenza. Briefly, amantadine and rimantadine are approved for the treatment and prophylaxis of influenza type A (268). These drugs target M2, which limits the formation of the proton channel and thus inhibiting infection of the cell. Zanamivir and oseltamivir are neuraminidase inhibitors that are active against both influenza type A and type B viruses (250). Zanamivir is approved for treatment of influenza in patients over 7 years old. Oseltamivir is approved for both treatment (18 years and older) and prophylaxis (13 years and older) of influenza.

I.D.2.k. Influenza Vaccines

Inactivated influenza vaccines have been used for 50 years and provide substantial, although not complete protection in pediatric and adult populations; levels of protection appear to be lower in the elderly. The standard influenza vaccine is a trivalent inactivated (TIV) preparation, given by intramuscular injection, which has generally been very

effective except in the elderly and during annual epidemics when components of TIV do not match the circulating influenza strain. Experience with TIV efficacy in young children is also more limited than in adults. The major immunogenic components of standard TIV vaccines consist of HA and NA proteins from circulating flu strains that are partially purified from detergent extracted, inactivated virions and administered by intramuscular injection (11)

A new live attenuated influenza (LAIV) vaccine that is administered intranasally was licensed in 2002 (7) with the intent of providing an alternative approach to influenza vaccination. LAIV is made using cold-adapted A/Ann Arbor/6/60 and B/Ann Arbor/1/66 strains as the genetic 'backbone' into which HA and NA genes from circulating strains are inserted by gene reassortment; the genetic stability of the attenuation phenotype of the vaccine viruses results from changes in several of the 'backbone' genes that reduce virulence (269, 270). LAIV is given as a large particle intranasal spray. Although LAIV was licensed recently, vaccines derived from this 'backbone' have been evaluated for more than 25 years (R.B. Belshe, Current status of live attenuated influenza virus vaccine in the US, *Virus Res.* **103** (2004) (1–2), pp. 177–185.). Several clinical studies carried out over the past 10 years have documented LAIV safety and capacity to induce protective immunity in children and healthy adults. In addition, several studies have provided data to suggest that LAIV induces considerable heterosubtypic immunity in recipients. Studies are in progress to compare the efficacy and safety of LAIV and TIV in young children less than 5 years of age and the relative capacity of the two vaccine preparations to

protect when one or more of the vaccine components is mismatched with circulating influenza strains.

HA antibodies provide a good correlate of protection after TIV immunization, but this correlation is not as definitive after LAIV immunization, suggesting differences in the mechanisms of protective immunity. Both vaccines induce neutralizing antibodies, but the extent to which TIV and LAIV induce T cell and mucosal immunity and whether the profiles of cell-mediated and mucosal responses depend upon the vaccine characteristics, the age of the vaccine recipient, or both is not known. Protection following TIV correlates with circulating antibody levels to HA 3–4 weeks after immunization while immunity following natural infection or LAIV seems to correlate better with mucosal IgA and anti-HA antibodies. However, cellular immune correlates have not been thoroughly examined. Some subjects given LAIV appear to have protection without detectable serum or even nasal antibody responses (271). Whether protection is mediated by T cells or by B cell responses that are not measured by current serologic assays is not known. Recipients of LAIV also have evidence of protection even when the circulating virus differs from vaccine strain (8, 271, 272).

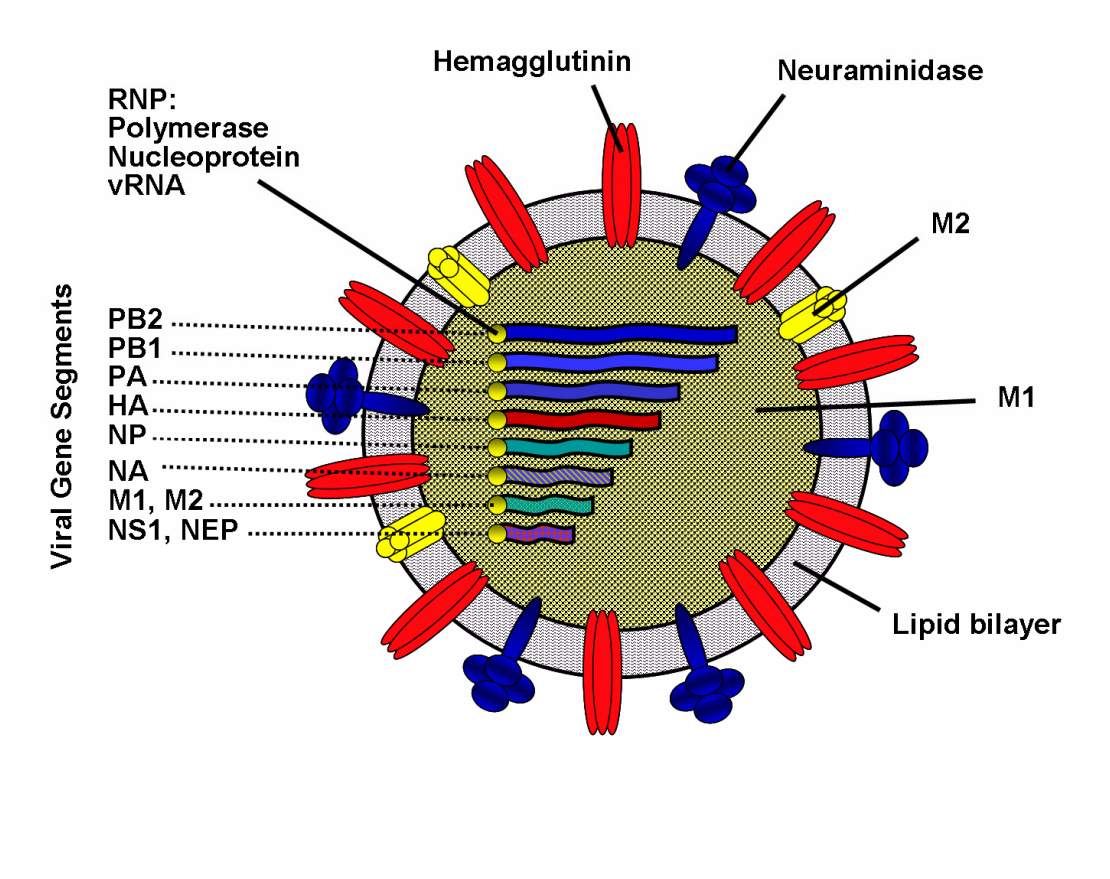

Figure 6. Influenza Virus and Gene Segments

Influenza virions have segmented, negative stranded RNA genomes (vRNA). Virus particles consist of three major subviral components, namely the viral envelope, matrix protein (M1), and core (viral ribonucleocapsid [RNP]). The viral envelope is derived from the host cell and consists of a lipid bilayer containing spikes composed of viral glycoproteins: hemagglutinin (HA), neuraminidase (NA) and M2. M1 is a structural protein that forms the matrix. M1 forms the bridge between the viral envelope and the core. The viral core consists of helical RNP containing vRNA (minus strand) and nucleoprotein (NP) along with minor amounts of the nuclear export protein (NEP) and the polymerase complex (PA, PB1, and PB2).

Table 3. Products of Influenza Gene Segments

Segment†	Protein	Function
1	PB2	Polymerase component
2	PB1	Polymerase component
3	PA	Polymerase component
4	HA	Hemagglutinin Viral attachment protein: binds to Sialic Acid Fusion protein Target of neutralizing antibodies
5	NP	Nucleocapsid
6	NA	Neuraminidase Cleaves Sialic acid and promotes viral release
7*	M1	Matrix Protein Viral structural protein Interacts with nucleocapsid and envelope Promotes assembly
	M2	Membrane protein Forms membrane ionic channel Facilitates uncoating and HA production
8*	NS1	Non-structural protein Inhibits cellular messenger RNA translation
	NEP (NS2)	Nuclear export protein (formerly known as non-structural protein 2) Help in the export of the vRNPs from the nucleous

† Listed in decreasing order size
*** Encodes two messenger RNAs**

I.D.3. Streptavidin

Streptavidin is a tetrameric protein from *Streptomyces avidinii* that binds very tightly to the vitamin biotin. The binding constant for this interaction is very high and has made the streptavidin/biotin system the focus of a number of studies aimed at determining what particular intermolecular interactions give rise to the tight binding (273, 274). On the other hand, streptavidin has been used as an antigen in some vaccine studies (275). This molecule is a relatively poor immunogen, which makes it perfect for the study of adjuvants.

II. Chapter 2: Materials and Methods

II.A. DNA Plasmids

The plasmids used are outlined in Table 1. Digestion (Section II.A.1), ligation (Section II.A.2) bacterial transformation and amplification (Section II.A.3), extraction and purification from agarose gels (Section II.A.4), and PCR (Section II.A.5) were all performed as described. The plasmids were stored at 4°C (short term) or –20°C (long term).

II.A.1. DNA Digestion with Restriction Enzymes.

Plasmid DNA was digested in a 1.5 ml centrifuge tube in the following reaction: 500 ng (vector plasmids) or 1 µg (insert plasmid) of plasmid DNA, 2 µl of enzyme buffer (final concentration, 1X) and 1 µl of each restriction enzyme in a final volume of 20 µl. The samples were placed in a water bath at 37°C for 1-4 h (1 h for diagnostics and 2-4 h for molecular cloning). The digested DNA was analyzed by agarose gel electrophoresis. Digested DNA was mixed with 1X blue sucrose loading dye (Invitrogen Life Technologies, Carlsbad, CA, USA) and loaded onto a 1% agarose gel (1% agarose in TAE buffer (40mM Tris acetate, 2mM Na_2 EDTA (pH 8.0) in distilled water) along with a 1kb ladder (Invitrogen Life Technologies, Carlsbad, CA, USA). The samples were electrophoresed (~80V) for 45-60 min. Digested DNA was analyzed for fragment size using a Transiluminator ultraviolet light (Fisher Scientific, Pittsburgh, PA, USA) and a Photo-Documentation Camera (Fisher Scientific, Pittsburgh, PA, USA).

Table 4. DNA Plasmids

Plasmids †	Description
$pEnv_{gp120(IIIB)}$	Wild type HIV-1 envelope gp120, isolate IIIB
$pEnv_{gp120\ (IIIB)}$-$C3d_3$	Wild type HIV-1 envelope gp120, isolate IIIB, fused to three tandem copies of murine C3d
$pcoEnv_{gp120(YU2)}$	Codon optimized HIV-1 envelope gp120, isolate YU2
pco-6(X)His-$Env_{gp120(YU2)}$	Codon optimized HIV-1 envelope gp120, isolate YU2, with a 6(X)-Histidine Tag at the 5' end
$pcoEnv_{gp120(YU2)}$ -$C3d_3$	Codon optimized HIV-1 envelope gp120, isolate YU2, fused to three tandem copies of murine C3d
psHA	Soluble wild type influenza virus hemagglutinin, isolate A/PR/8/34 (H1N1)
psHA-6(X)-His	Soluble wild type influenza virus hemagglutinin, isolate A/PR/8/34 (H1N1), with a 6(X)-Histidine Tag at the 3' end
psHA-10(X)-His-$C3d_3$	Soluble wild type influenza virus hemagglutinin, isolate A/PR/8/34 (H1N1), with a 10(X)-Histidine Tag at the 3' end, fused to three tandem copies of murine C3d

† TR600 is the base vector in all these plasmids

Table 5. Polymerase Chain Reaction Cycling Parameters.

1. Start	1 cycle	Hot Start	95 ºC	1 min
2. Extension	30 cycles	Melting	95 ºC	1 min
		Annealing	55 ºC	1 min
		Polymerizing	72 ºC	1 min per 1kb
3. End	1 cycle		72 ºC	15 min

Each PRC reaction contained 200 ng of plasmid DNA, 5 µl of 10X reaction buffer (100mM Tris-HCl, pH 8.3 (at 42°C) 500mM KCl, 25mM $MgCl_2$, 0.01% gelatin), 50mM of dNTP (12.5mM dATP, 12.5mM dCTP, 12.5mM dGTP, 12.5 mM dTTP, neutralized at pH 8.0 in water), 1 µg of each oligonucleotide primer, 1 U of Taq DNA polymerase (1 U/µl) (Stratagene Cloning System, La Jolla, CA, USA) and distilled water added to final volume of 50µl.

II.A.2. Ligation of DNA Fragments.

Plasmid DNA was digested and purified as described in Sections II.A.1 and II.A.4. DNA fragments were ligated using the following reaction: vector and insert fragments (vector to insert ratios: 1:1, 1:3 and 1:6) were added to 1 µl of 10X T4 DNA ligation buffer, 1 µl (1 U/µl) of T4 DNA ligase (Invitrogen Life Technologies, Carlsbad, CA, USA) in a total volume of 10 µl of distilled water. The samples were incubated at 16° C for 4 h and followed by transformation into *E. coli* XL-gold cells (see Section II.A.3).

II.A.3. DNA Amplification and Purification from Bacteria

II.A.3.a. . Competent cells

Bacterial transformation was used to introduce plasmid DNA into competent bacterial cells. The bacterial cells used for transformation, chemically competent XL10-Gold cells, were derived from XL10-Gold ultracompetent cells (Invitrogen Life Technologies, Carlsbad, CA, USA). XL10-Gold ultracompetent cells (50 µl) were gently thawed on ice for approximately 10 min. The cells were added to 200 ml of sterile Luria Broth (LB) (EZmix: enzymatic casein digest 10 g/L, yeast extract 5 g/L, NaCl 5 g/L, and inert binder 0.6 g/L) (Sigma, St. Louis, MO, USA) and incubated for 16 h at 37°C (shaking at 225 rpm). 2.5% of the overnight culture was added to 200 ml of fresh LB and incubated at 37°C with shaking until the O.D. reading at 550 nm was 0.3 using a BioMate 3

spectrophotometer (Thermo Spectronics, Rochester, NY, USA). LB alone was used as the blank. The bacterial culture was then placed into four 50 ml conical tubes (U.S.A. Scientific, Ocala, FL, USA) and incubated on ice for 15 min. The cells were pelleted by centrifugation (3,000 rpm for 5 min) at 4°C using a RC5 centrifuge (Sorvall Instruments, Newtown, CT, USA). The supernatants were decanted, and each pellet was resuspended in 16 ml of Transformation Buffer #1 (99.3M RbCl, 48.5M $MnCl_2$, 1M KOAc, 10.2M $CaCl_2$, 15% glycerol, pH 5.8). The samples were mixed thoroughly, and the cells were pelleted by centrifugation (3,000 rpm for 5 min) at 4°C. The supernatants were decanted, and each pellet was resuspended in 4 ml of Transformation Buffer #2 (0.5M MOPS (pH, 6.8), 19.9M RbCl, 150M $CaCl_2$, 30% glycerol) and pooled together. The sample was mixed thoroughly and placed into 1.5 ml centrifuge tubes (200µl each). Finally, the cells were flash-frozen in an ethanol-dry ice bath for 1 min and stored at -80°C.

II.A.3.b. Bacterial Transformation and DNA Amplification

Plasmid DNA (50-250 ng) was mixed with 20 µl (~1.0 x 10^6 cells) of chemically competent XL10-Gold cells in a 17x100 mm culture tube (USA Scientific, Woodland, CA, USA). The mixture was incubated on ice for 20 min, and then incubated in a 42 º C water bath for 45 sec. The mixture was returned to ice for an additional 5 min. 80 µl of sterile LB was added to the mixture. The mixture was incubated at 37° C with shaking at 225 rpm for 1 h. The entire mixture was then transferred onto a pre-warmed (37 ºC), antibiotic agar plate (ampicillin or kanamycin) (Sigma, St. Louis, MO, USA) (50 µg/µl). Sterile glass beads (10-12 beads per plate) (Fischer Scientific, Middletown, VA, USA)

were added onto the plate and used to spread the mixture evenly by shaking the plate back and forth several times. The plate was inverted and incubated in at 37°C in a bacterial incubator (Precision, Winchester, VA, USA) for 18 h. A bacterial colony was picked from the plate and used to inoculate 5 ml of sterile LB containing 0.01µg/µl antibiotic (kanamycin or ampicillin). The culture was incubated at 37°C with shaking at 225 rpm for 8 h. 1 ml of the culture was then added to 200 ml of LB containing 0.01 µg/µl antibiotic. The culture was then incubated at 37°C with shaking at 225 rpm for 15-18 h.

II.A.3.c. Plasmid DNA Purification from Bacterial Cultures

Plasmid DNA was purified from bacterial cultures using a Qiagen Plasmid Midi Kit (Qiangen Inc., Valencia, CA, USA), following the recommendations of the manufacturer. Briefly, bacterial cells were harvested by centrifugation at 6,000 rpm for 20 min at 4°C in a 250 ml polyclear centrifuge tube (Nalgene, Rochester, NY, USA) using a RC5C centrifuge (Sorvall Instruments, Newtown, CT, USA). The supernatant was removed and the bacterial pellet resuspended in 4 ml of Qiagen Buffer P1 (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, and 100 µl/ml RNase A) (Quiagen Inc, Valencia, CA, USA). The resuspended bacteria were transferred to a 50 ml round centrifuge tube (Nalgene, Rochested, NY, USA) and 4 ml of Qiagen Buffer P2 (200 mM NaOH and 1% SDS) were added to lyse the cells. The solution was mixed by gently inverting the tube 4-6 times and incubated at room temperature for 5 min. Lysis was neutralized by adding 6 ml of pre-chilled Qiagen Buffer P3 (3.0 M potassium acetate, pH 5.5). The solution was gently

mixed by inverting the tube 4-6 times and incubated on ice for 20 min. The sample was centrifuged at 20,000 rpm for 30 min at 4°C. The supernatant containing plasmid DNA was decanted into a new round centrifuge tube. The sample was then centrifuged at 20,000 rpm for an additional 15 min at 4°C. A Qiagen-tip 100 column was equilibrated by applying 4 ml of Qiagen Buffer QBT (750mM NaCl, 50mM MOPS, pH 7.0, 15% isopropanol, 0.15% Triton X-100). The column was allowed to drain by gravity flow. The supernatant containing the plasmid DNA was then applied to the column and allowed to drain by gravity flow. The column was washed two times with 10 ml of Qiagen Wash Buffer QC (1.0M NaCl, 50mM MOPS, pH 7.0, 15% isopropanol). The plasmid DNA was eluted with 5 ml of Qiagen Buffer QF (1.25 M NaCl, 50mM Tris-Cl, pH 8.5, 15% isopropanol) into a new round centrifuge tube. The plasmid DNA was precipitated by adding 3.5 ml of room temperature isopropanol to the 5 ml of eluted DNA. The sample was immediately mixed and centrifuged at 15,000 rpm for 30 min at 4°C. The supernatant was carefully discarded, not disturbing the DNA pellet. The plasmid DNA pellet was washed with 2 ml of 70% ethanol and centrifuged at 15,000 rpm for 10 min at 4°C. The supernatant was carefully discarded and the pellet was air-dried for 1 hour at 37°C. The plasmid DNA pellet was resuspended in distilled water. A sample of each DNA was diluted 1:200 (5 µl of DNA in 995 µl of distilled water) and the DNA concentration was determined using a BioMate 3 spectrophotometer (Thermo Spectronics, Rochester, NY, USA). The optical density (wavelength, 260/280 nm) of the diluted DNA sample was measured by the spectrophotometer. The optical density of non-DNA containing distilled water was subtracted from the recorded optical density of the diluted DNA to calculate the final optical density. The concentration of DNA was

determined by the following: DNA concentration (C, µg/µl) = [A_{260}/ 0.020 $(\mu g/ml)^{-1}$] x (1 ml/1000µl).

II.A.4. Extraction and Purification of DNA from Standard Agarose Gels

Restriction enzyme-digested DNA was extracted and purified from 1% agarose gels. This DNA was used for the construction of the various molecular clones described in Table 1. The restriction enzyme-digested DNA was electrophoresed until the DNA bands were clearly separated. The DNA band of interest was removed from the agarose gel (1% agarose in TAE, 40 mM Tris Acetate, 2 mM Na_2EDTA (pH 8.0) in distilled water) (Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) by cutting the segment of agarose that contained the DNA with a razor blade and placed in a 1.5 ml microcentrifuge tube and weighed. DNA fragments were separated from the agarose using a QIAquick gel extraction kit (QIAGEN Inc, Valencia, CA, USA) according to the manufacturer's recommended protocol. Briefly, three volumes of Buffer QG (proprietary compound mixture, 50-100% guanidinium thiocyanate, pH indicator) were added to one volume of agarose gel. For example, 300 µl of Buffer QG was added to 100 mg of gel. The microcentrifuge tube was incubated at 50°C for 10 min or until the agarose was completely dissolved. The tube was vortexed every three minutes to help dissolve the gel. One volume of isopropanol (in example 100 µl) (Sigma, St. Louis, MO, USA) was then added to one volume of gel. The dissolved mixture was overlaid into a QIAquick ion-exchange spin column was placed in a collection tube and centrifuged for 1 min at 10,000 rpm. The flow-through was discarded, and the QIAquick spin column was placed back in the same collection tube. The sample was washed with 750 µl of Buffer PE (proprietary

compound mixture, 44.4% ethanol) and centrifuged for 1 min at 10,000 rpm. The flow-through was discarded and the QIAquick spin column was placed back in the same collection tube. The QIAquick spin column was centrifuged for an additional 1 min at 10,000 rpm and the flow-through was discarded. The QIAquick spin column was placed in a new 1.5 µl microcentrifuge tube. The DNA was eluted by adding 40 µl of distilled water to the center of the QIAquick membrane and incubated at room temperature for 2 minutes. The sample was then centrifuged for 2 min at 10,000 rpm, the spin column was then discarded, and the eluted DNA was stored at 4°C if used within 24 hours. Alternatively, the samples were stored at -20°C for long term use.

II.A.5. Polymerase Chain Reaction (PCR) and cloning into TOPO 2.1

In vitro polymerase chain reaction was used to amplify gene products. Plasmid DNA (50 ng) with the sequence of interest was added to a microcentrifuge tube with 2 synthetic oligonucleotide primers (1 µg/µl), 25-80 nucleotides in length. Each reaction mixture (**Table 5**) was placed in a Robocycler® Gradient 96 thermal cycler (Stratagene, La Jolla, CA, USA). A DNA fragment was amplified using a three step reaction cycle (**Table 5**). The amplified product was cloned into pCR2.1-TOPO plasmid vector using the TOPO TA Cloning Kit® (Invitrogen Life Sciences, Carlsbad, CA, USA), following the manufacturer's recommendations. Briefly, each amplified fragment (3 µl) was incubated in a 1.5 ml centrifuge tube in the following conditions: 1 µl of salt solution (1.2 M NaCl, 0.06 M $MgCl_2$), 1 µl of pCR2.1-TOPO vector (10 ng/µl plasmid are in: 50% glycerol, 50 mM Tris-HCl, pH 7.4 (25°C), 1 mM EDTA, 1 mM DTT, 0.1% Triton-X 100, 100 µg/ml BSA, phenol red), and 1 µl of distilled water for 5-20 minutes at 25°C. 2 µl of the

ligation reaction was then incubated in 50 µl One Shot® TOP10 Competent Cells (*E. coli*, F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G) (Invitrogen Life Sciences, Carlsbad, CA, USA) on ice for 20 min. The samples were heat-shocked for 45 sec at 42°C and then returned to ice for 3 min. S.O.C. medium (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) (250 µl) was added to the competent cell mixture. The mixture was incubated at 37°C for 1 h with shaking (200 rpm). The samples were spread using glass beads onto pre-warmed (37°C) antibiotic selective agar plates (ampicillin or kanamycin), and incubated for 16-18 h at 37°C.

II.B. DNA Microcarriers

II.B.1. DNA Gold Bullets Preparation

II.B.1.a. DNA/Gold Mixture Preparation

Plasmid DNA (Table 1) was purified, resuspended in distilled water, and the concentration determined by spectrophotometry as described in Section II.A.3. Approximately, 60 bullets of 1µg DNA per 0.5 mg of gold were prepared for each plasmid. DNA was resuspended at a concentration of 1µg/µl (if the DNA concentration was not the required, the samples were either diluted of concentrated using an Eppendorf Vacufuge™ concentrator (Eppendorf AG, Hamburgh, Germany)) and 90 µl this DNA were used. 45 mg of 1µm gold beads (Bio-Rad, Hercules, CA, USA) were weighted out in 1.5 ml microcentrifuge tube. The gold was weighted directly in the centrifuge tube to

reduce gold loss. 100 µl of freshly prepared 0.05 M spermidine (Sigma, St. Louis, MO, USA) were added to the gold beads and this mixture was sonicated using a Sonic Cleaner water bath (Fisher Scientific, Fair Lawn, NJ, USA) for ~5 sec. Subsequently, the plasmid DNA (90 µg) was added to the gold/spermidine mixture and the sample was vortexed briefly. Following the addition of plasmid DNA, 100 µl of freshly prepared 1M $CaCl_2$ were added dropwise. After each $CaCl_2$ drop, the sample was carefully vortexed. The plasmid DNA was then allowed to precipitate by incubating the gold beads for 5 minutes at room temperature. The tube was briefly (~ 20 sec) centrifuged at 12,000 rpm and the supernatant discarded.

II.B.1.b. Slurry preparation

500 µl of fresh, never opened before, stored at -20°C, 100% ethanol (Pharmco, Brookfield, CT, USA) were added to the gold/plasmid DNA pellet. The microcentrifuge tube was then vortexed, centrifuged (~20 sec, 12,000 rpm) and the supernatant discarded. This procedure was repeated twice. 5.3 ml of 100% ethanol were added to a 15 ml conical tube (USA Scientific, Woodland, CA, USA). The gold/plasmid DNA pellet was resuspended in 300 µl of 100% ethanol taken from the 15 ml conical tube. The gold/plasmid DNA pellet was then briefly vortexed, sonicated (5-10 sec) and carefully transferred, using a 1 ml pipette, to the 15 ml conical tube. This process was repeated as many times as necessary to remove all the gold from the microcentrifuge tube. The gold/plasmid DNA/ethanol mixture, known as slurry, was stored at -20°C in the 15 ml

tube, sealed with Parafilm® (Pechiney Plastic Packing, Menasha, WI, USA), until needed.

II.B.1.c. . Tubing Coating

The slurry was used to coat Tefzel tubing (Bio-Rad, Hercules, CA, USA). Initially, the Tefzel tubing was slid into the tube turner of a Tubing Prep Station (Bio-Rad, Hercules, CA, USA), which was attached to a nitrogen gas tank. One end of the Tefzel tubing was in contact with a hose that delivered nitrogen gas, while the other allowed evacuation of the gas. The tubing was cut leaving 2 extra inches on the side of nitrogen evacuation. The Tefzel tubing was purged with nitrogen gas for 15-20 min (0.4-0.5 liters per min) to remove moisture. Meanwhile, the slurry was vortexed and then sonicated for 5-10 sec or until gold clumps were no longer present. The Tefzel tubing was removed from the Tubing Prep Station and connected to a 10 ml syringe using a silicone adapter tube. Using the syringe, the votexed and sonicated slurry was drawn up into the Tefzel tubing and then slid back into the tube turner. The slurry was allowed to settle for 5 minutes. Following 5 min incubation, the ethanol was carefully drawn off by slowly pulling on the attached syringe (~1 inch per sec). The syringe silicone tube adapter was then removed and the Tefzel tubing was rotated in the tube turner (20 rpm) for ~45 sec, which allowed an even smear of the gold. The tubing was then dried for 5 min using nitrogen gas (0.35-0.4 liter per min), while still rotating. The tubing was inspected for even coating and then sectioned in ~1 cm (~0.4 inches) pieces (DNA bullets) using a tube cutter (Bio-Rad, Hercules, CA, USA). The areas of uneven coat were discarded. The DNA bullets were

stored at 4°C into tightly closed and Parafilm® sealed 50 ml conical tubes containing desiccant capsules (IMPAK, Los Angeles, CA, USA). Approximately 60 bullets of 1 μg DNA per 0.5 mg gold were prepared for each 90 μg of initial DNA.

II.C. Cell Culture

II.C.1. Human Embryonic Kidney (HEK) 293T Cells

The human (*Homo sapiens)* embryonic kidney 293 (HEK 293) cell line is adherent. HEK 293 cells are transformed with adenovirus 5 DNA (39768) and require Biosafety Level 2 facilities. HEK 293T is a highly transfectable derivative of the 293 cell line (American Type Culture Collection, (ATCC), CRL-1573), in which cells the temperature sensitive gene for simian virus 40 (SV40) T-antigen was inserted (276, 277). The cells were maintained according to the American Type Culture Collection (ATCC) recommended growth conditions in complete Dulbecco's Modified Eagle Medium (DMEM) (cDMEM) [DMEM supplemented to contain 10% heat-inactivated fetal bovine serum (FBS) (1 h at 56° C) (Atlanta Biologicals, Atlanta, GA, USA), 4 mM L-glutamine (Invitrogen Life Technologies, Carlsbad, CA, USA), and 0.4 mg/L gentamicin (Gibco, Grand Island, NY, USA)] at 37° C in a humidified 5% CO_2 incubator.

II.C.2. Transfections

II.C.2.a. . Cell Viability

The efficient expression of each plasmid was determined by transient transfection of plasmid DNA into the HEK 293T cell line (described in Section II.C.1.). HEK 293T cells were plated at a concentration of approximately 5 X 10^5 cells per well in 6-well plates (35 mm^2) (Becton Dickinson, Piscataway, NJ, USA). Cell viability was determined by trypan blue exclusion cell counting. Cells were detached from 75 cm^2 tissue culture flasks (Corning Inc, Corning, NY, USA) using 3ml of 1X trypsin (0.05% trypsin, 0.4% EDTA•4Na) (Gibco, Grand Island, NY, USA) for 3-5 min at RT. The cells were triturated with 7 ml of cDMEM and placed in a 50 ml conical tube. Following careful mixing, 10 µl of the cell suspension was incubated for 3 min with 10 µl of 0.4% Trypan Blue solution (Gibco, Grand Island, NY, USA) and half of the solution was added to each side of a hemacytometer (VWR, Bridgeport, NJ, USA). Within the hemacytometer, the cells within the four outer quadrants were counted using a light microscope (100X power) (Fryer Company, Inc, Huntley, IL, USA). Only the cells that did not uptake the blue dye were counted and the average of the four quadrants was recorded. The number of viable cells was determined with the following equation: # viable cells (cells/ml) = (average number of cells in 4 quadrants) X dilution factor for size of quadrant (i.e. 10,000) X dilution factor for addition of trypan blue. Each sample was counted per duplicate to accurately determine the cell number. Cell counts within 10% of each other were considered accurate. The total number of cells within the flask was determined by the following equation: Total cells in flask = cells/ml X # ml in flask.

II.C.2.b. . DNA Transfection

After the cell count was determined, the cells were seeded (5 X 10^5 cells/well, 6-well plate), allowed to attach and acclimate for 24 hours at 37° C plus 5% CO_2. Cell health and confluency (~90%) were determined by inspection, using a light microscope (100X power) (Fryer Company, Inc, Huntley, IL, USA). The medium was then removed by aspiration. The cells were transfected with the plasmid DNA listed in Table 1. Three μg of plasmid DNA were mixed with 1990 μl of Opti-MEM I reduced serum media (Invitrogen Life Technologies, Carlsbad, CA, USA) in a 1.5ml microcentrifuge tube (Tube A). In a separate 1.5ml microcentrifuge tube (Tube B), 5 μl of Lipofectamine™ 2000 reagent (1 mg/ml) (Invitrogen Life Technologies, Carlsbad, CA, USA) were incubated with 190μl of Opti-MEM I reduced serum media and incubated at room temperature for 5 minutes. Tubes A and B were mixed (Master Mix) and incubated at room temperature for 30 minutes. 1.6 ml of warmed (37°C) Opti-MEM I reduced serum media were added to each well, followed by the Master Mix (~400 μl). Plates were carefully rocked back and forth to achieve an even distribution of the Master Mix and incubated for 72 h at 37°C in a humidified 5% CO_2 incubator (ThermoForma, Waltham, MA, USA). Seventy-two hours post-transfection, the supernatants were harvested and placed into 1.5 ml centrifuge tubes. The supernatant was clarified by centrifugation (1 min for 12,000 rpm) and transferred to a new 1.5 ml centrifuge tube and stored at -80° C. The cells were incubated with either 500 μl of distilled water or 1 % Triton-X 100 (Sigma, St. Louis, MO, USA) until the cells completely detached from the plate. The

samples were incubated at -80° C for 5-10 min and then thawed at 37°C 1 min. Freeze/thawing was repeated three times. Cell lysate samples were centrifuged (1 min at 12,000 rpm), the supernatants transferred to a new 1.5 ml centrifuge tube and stored at -80°C.

II.D. Protein Expression

II.D.1. Western Blot

Supernatants and cell lysates from transfected HEK 293T cells (Section II.C.2) were analyzed for protein expression by Western Blot. Briefly, cell lysates (2%, 10 µl) or supernatants (1%, 20 µl) were diluted (1:2) in sodium dodecyl sulfate (SDS) Laemmil sample buffer (1M Tris-Cl pH 6.8, 20% SDS, 3.3 % Glycerol, 0.006 M bromophenol blue and 0.05 M beta-mercaptoethanol) (Bio-Rad, Hercules, CA, USA) and the mixture was boiled for 5 min. The sample mixtures and a pre-stained molecular weight ladder (Bio-Rad, Hercules, CA, USA) were loaded onto a 5-10% SDS-polyacrylamide gel (Stacking Gel: 30% acrylamide:bis, 10% SDS, 10% ammonium persulfate, 1% TEMED, 0.5 M Tris-HCl pH 6.8 or Resolving Gel: 1.5 M Tris-HCl, pH 8.8). The proteins were separated by electrophoresis at 100 V for 20 min followed by 200 V for 1-2 h in running buffer (25 M Tris base, 192 M glycine, 0.1% SDS, pH 8.3). The resolved proteins from the SDS-PAGE were transfered to a Immobilon™ nitrocellulose membrane (Millipore, Bedford, MA, USA) in transfer buffer (25 mM Tris, 192 mM glycine, and 20% v/v methanol, pH 8.3) using the transfer apparatus (Mini Trans-Blot, Bio-Rad, Hercules, CA, USA) for 1 h at 200 mA. The nitrocellulose membrane was then removed and placed in

blocking solution (5% dry non-fat milk, 0.05% Tween 20, PBS) overnight at 4° C with gentle rocking.

The blocking solution was removed and the transferred proteins were detected either with human polyclonal antiserum (HIV Ig) (NIH ARRRP, (278) Germantown, MD, USA) (1:10,000 in PBS containing 0.05% Tween 20 and 5% non-fat dry milk) or rabbit polyclonal anti-influenza virus serum (1:5,000 in PBS containing 0.05% Tween 20 and 5% non-fat dry milk). The nitrocellulose membranes were incubated with the corresponding antisera for 1 hour at room temperature on a shaker. The nitrocellulose membrane was then washed 3 times for 10 min each in 15 ml of PBS supplemented with 0.05% Tween 20 (PBS-T) on a shaker at room temperature. The membranes were then incubated either with goat anti-human IgG conjugated to horseradish peroxidase (HRP) (Bio-Rad Laboratories, Hercules, CA, USA) (1:10,000 in PBS-T and 5% non-fat dry milk) or goat anti-rabbit IgG conjugated to HRP (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) (1:7,000 in PBS-T and 5% non-fat dry milk) on a shaker for 1 h. The membrane was then washed 3 times for 10 min each in 15 ml of PBS-T on a shaker at room temperature. The membranes were then incubated with 1 ml of stable peroxide solution and 1 ml of lumino/enhancer solution (Amersham, Buckinghamshire, UK) and manually rocked for 3 min. The membranes were briefly dried, wrapped in cellophane, and exposed to CL-X Posure™ film (Pierce, Rockford, IL, USA) for 3 sec to 1 min.

II.D.2. Protein Purification

II.D.2.a. . DNA Transfections in T-75 Flasks

In order to purify proteins, transfections of HEK 293T cells were up-scaled to 75 cm^2 tissue culture flask (Corning, Inc, Corning, NY, USA). Briefly, HEK 293T-cells were grown in cDMEM until 90% confluency. DNA plasmids containing a Histidine-Tag (**Table 4**) were then transfected using Lipofectamine™ 2000 reagent (1 mg/ml) (Invitrogen Life Technologies, Carlsbad, CA, USA) in Opti-MEM I reduced serum media (Invitrogen Life Technologies, Carlsbad, CA, USA). The transfection mixture was prepared by adding 500 µl of Opti-MEM I reduced serum media and 20 µl of Lipofectamine™ 2000 reagent (1 mg/ml) into a 1.5 ml microcentrifuge tube (Tube A) and incubated at room temperature for 5 min. In another microcentrifuge tube (Tube B), 500 µl of Opti-MEM I reduced serum media were mixed with 8µg of plasmid DNA. Tubes A and B were mixed (Master Mix) and incubated at 25 °C for 30 minutes. cDMEM was removed from HEK 293T cells by aspiration and 5 ml of warmed (37 °C) Opti-MEM I reduced serum media were added. Following 30 min incubation, the Master Mix was added to the HEK 293T cells and the flask was carefully rocked back and forth to evenly distribute the transfection mixture. The flaks were incubated at 37° C in a humidified 5% CO_2 incubator. Four hours later, 5 ml extra of warmed (37 °C) Opti-MEM I reduced serum media were added to the cells. The flasks were then incubated for 72 h (37 °C - 5% CO_2). Following 72 h incubation, supernatants were harvested and a protease inhibitor cocktail for purification of Histidine-tagged proteins was added (2.5 µl/ml)

(Sigma, St. Louis, MO, USA). Supernatants were stored at -80°C until protein purification.

II.D.2.b. Affinity Chromatography

Histidine-tagged proteins were purified by affinity chromatography, at 4°C, using 5 ml HiTrap chelating nickel columns (Amersham Biosciences, Piscataway, NJ, USA). A peristaltic pump (Econo-Pump) (Bio-Rad, Hercules, CA, USA) at a flow rate of 5ml/min was used for the procedure. A general over view of the protein purification method is outlined in **Figure 7**. The Storing Solution (20% ethanol in distilled water) of the HiTrap chelating nickel column was removed. The HiTrap column was then washed with 15 ml of distilled water. The column was loaded with 5 ml of 0.1 M $NiSO_4$ (Fisher Scientific, Fair Lawn, NJ, USA) and subsequently washed with 15 ml of distilled water. The HiTrap column was equilibrated with 30 ml of Binding Buffer (20 mM phosphate, 0.5 M NaCl and 10 mM imidazole (Sigma, St Louis, MO, USA)). Supernatants containing Histidine-tagged proteins were gently thawed, pooled and loaded in the equilibrated HiTrap column at a flow rate of 5 ml/min. The column was then washed with 30 ml of Binding Buffer. Subsequently, the proteins were eluted using 15 ml of Elution Buffer (20mM phosphate, 0.5 M NaCl and 500 mM imidazole). The eluted fraction, containing the purified protein, was concentrated immediately (see below). The column was washed with 30 ml of Binding Buffer and the nickel ions were removed using 25 ml of Cleaning Solution (20mM sodium phosphate, 0.5 M NaCl, 0.05 M EDTA (Merk, Darmstadt, Germany), pH 7.4). Finally, HiTrap columns were filled with Store Solution and kept at 4°C. Samples of

all the protein purification fractions were collected and analyzed by western blot as described in Section II.C.2

II.D.2.c. . Concentration

The fraction containing the purified protein was concentrated using 30,000 to 100,000 molecular weight cut-off columns (MWCO) (Vivascience, Hannover, Germany). Briefly, the sample was loaded in the concentration columns and centrifuged for 30-45 min (3,000 g, 14°C) (Sorvall, Asheville, NC, USA). The flow-trough was decanted and the protein was washed twice with 15 ml of PBS (buffer exchange). The final volume in which the protein was resuspended was ~1.5 ml. The purified, concentrated and buffer exchanged protein was stored at -80°C.

II.D.3. BCA Protein Assay

The protein concentration was determined using a Micro (Bicinchoninic acid) BCA™ Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). A protein standard (2 mg/ml of bovine serum albumin (BSA) in a solution of 0.9% saline and 0.05% sodium azide) was used to produce a standard curve. The assay was performed following the recommendations of the manufacturer. Briefly, standard protein concentrations or test samples (150 µl) and 150 µl of the working reagent [52% Micro BCA Reagent A (sodium carbonate, sodium bicarbonate and sodium tartrate in 0.2N NaOH), 48 % Micro BCA Reagent B (4% bicinchoninic acid in water), 2% Micro BCA Reagent C (4% cupric sulfate, pentahydrate in water)] were pipetted into the wells of a 96 well plate (Corning,

Corning, NY, USA). The plates were covered and placed on a shaker for 2 h at 37°C. The samples were analyzed for a colorimetric change measured as the optical density (O.D.) at 540nm by a spectrophotometer (Dynex Technologies, Chantilly, VA, USA). Tests were performed in duplicate at two different dilutions, whereas the standard samples were analyzed in triplicate. The O.D. of each sample was compared to the standard curve, and the concentration of the protein was extrapolated from the standard curve.

II.D.4. Silver Stain

The purified proteins (Section II.D.2.) were verified by silver staining (279) using a ProteoSilver Stain Kit (Sigma, St. Louis, MO, USA), following the manufacturer's protocol. Samples were prepared and electrophoresed as described in Section II.D.1. After electrophoresis of the proteins by SDS-PAGE, the gel was placed in a clean tray with 100 ml of Fixing Solution (50% ethanol, 10% acetic acid in ultrapure water) for 1h. The Fixing Solution was removed and the gel was washed with 100 ml of Ethanol Solution (30% ethanol in ultrapure water) for 10 min. The Ethanol Solution was decanted and the gel was washed twice (10 min) with 200 ml of ultrapure water. The gel was then incubated for 10 min with 100 ml of Sensitization Solution (1% ProteoSilver Sensitizer in ultrapure water). Following removal of the sensitizing solution, the gel was washed twice (10 min) with 200 ml of ultrapure water. The water was decanted and 100 ml of Silver Equilibration Solution (1% ProteoSilver Silver solution in ultrapure water) was added to the gel for 10 min. After the Silver Equilibration Solution was removed, the gel was washed for 1 min with 200 ml of ultrapure water. The water was decanted and 100 ml of Developer Solution (5% of Proteo Silver Developer 1, 0.1% of ProteoSilver Developer 2

in ultrapure water) were added to the gel. The gel was carefully rocked back and forth for 3-7 min until the desired staining intensity was observed. 5 ml of the ProteoSilver Stop Solution was added to the Developer Solution to stop the reaction (5 min). Finally, the Developer / ProteoSilver Stop solution was decanted and the gel was washed with 200 ml of ultrapure water for 15 minutes.

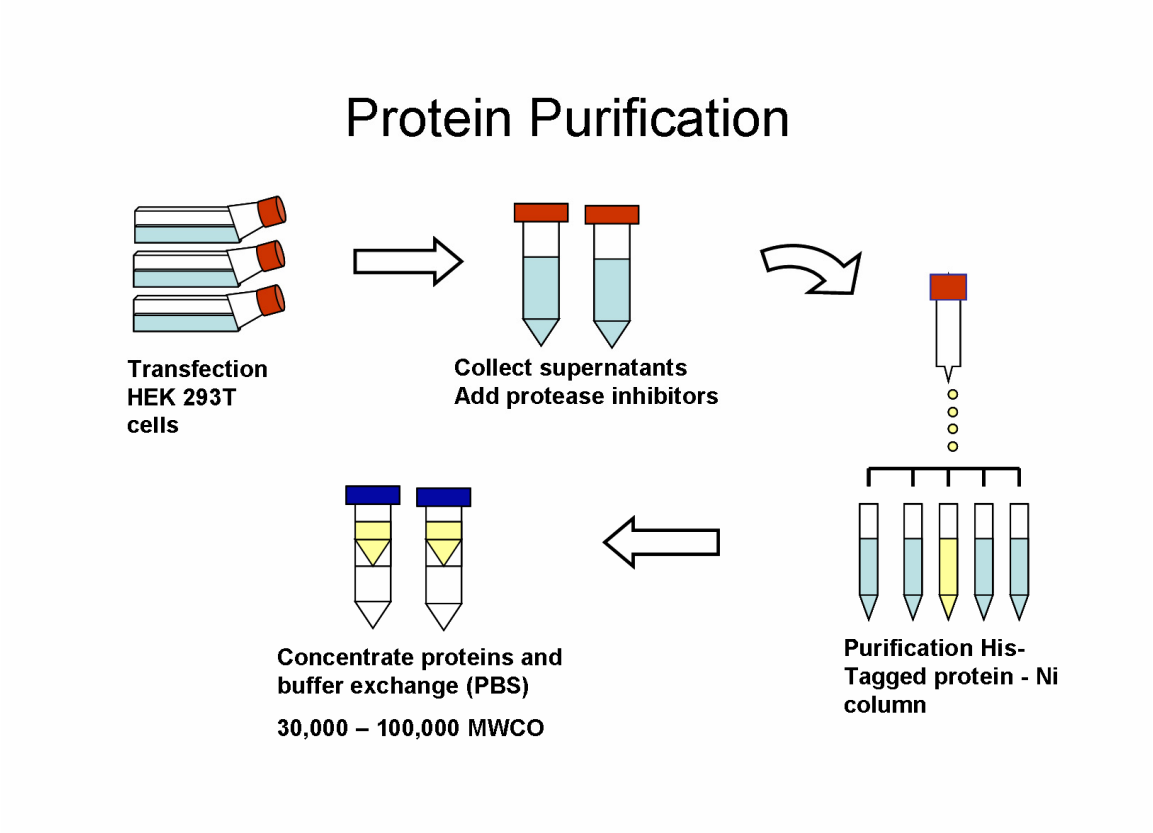

Figure 7. Histidine-Tagged Protein Purification.

HEK 293T cells were transferred using Lipofectamine 2000 reagent. The supernatants were harvested and clarified by centrifugation. Protease inhibitors were added. The proteins were purified by affinity chromatography using nickel columns. The fractions containing the purified protein were concentrated and buffer exchanged (PBS) using 30,000 to 100,000 MWCO columns

II.E. Mice

II.E.1. Husbandry and Anesthesia

Six to 8 weeks-old mice were used for vaccination studies. The mice were housed (5 mice per cage, free access to food and water, cages cleaned weekly) in compliance with the U.S. Department of Agriculture (USDA) regulations. Mice were anesthetized based on their weight with xylazine (Phoenix Pharmaceutical, Inc, St. Joseph, MO, USA) (20 mg/ml) and ketamine (Phoenix Pharmaceutical, Inc, St. Joseph, MO, USA) (100 mg/ml) administered subcutaneously (50 mg ketamine and 5 mg xylazine per kilogram of body weight, average 20 g) (Ketamine/Xylazine) in the abdomen prior to blood collection, intravenous immunization, or virus challenge. Mice were sacrificed by CO_2 (100%) inhalation.

II.E.2. Mouse Strains

II.E.2.a. . BALB/c Mice

BALB/c is a general purpose, white coat, inbred mouse strain. MacDonell started inbreeding these mice in 1923. The mice were then transferred to Snell in 1932, who transferred them to Andervont. Andervont, at F32, transferred the mice to the NIH in 1987 (280). The NIH has provided the different supplier companies with breeder mice that have been used to create colonies that are maintained in gnobiotic isolators. Coat

color loci include: A, b, c, D. Other loci of known importance include: Car-2^{b}, Ce-2^{a}, Cs-1^{a}, Es-1^{b}, Es-3^{a}, Es-10^{a}, Gpd-1^{b}, Gpi-1^{a}, Gus^{a}, Hba^{b}, Hbb^{d}, Hc^{l}, Idh-1^{a}, Lyb-2^{b}, Ly-1^{b}, Ly-2^{b}, Ly-3^{b}, Lv^{a}, Mod-1^{a}, Mup-1^{a}, Pep-3^{a}, Pgm-1^{a}, Thy-1^{b}, Tla^{c}, Trf^{b}, H-2D^{d} and H-2K^{d} (280). Six to 8 weeks-old BALB/c mice (Harlan Sprangue-Dawley, Indianapolis, IN, USA) were used in the vaccine studies and housed in compliance with the USDA regulations.

II.E.2.b. . C57BL/ 6 Mice

The black coated, inbred, C57BL/6 mouse strain is widely used as the genetic background for transgenic and mutant mice and is popular in the research applications of oncology, immunology and toxicology. This mouse strain was developed by C.C. Little in 1921, from a mating of Miss Abby Lathrop's stock that also gave rise to strains C57BR and C57L. Strains 6 and 10 were separated about 1937. These mice were transferred to Jackson laboratories in 1948 from Hall and then in 1951 to the NIH at F32. The coat color loci include a, B, C and D. Other loci described include: Car-2^{a}, Ce-2 ^{a}, Cs-1^{g}, Es-1^{a}, Es-3^{a}, Es-10 ^{a}, Gpd-1^{a}, Gpi-1 ^{b}, Gus^{b}, Hba ^{a}, Hbb^{s}, Hc^{l}, Idh-1^{a}, Lv^{b}, Lyb-2^{b}, Ly-1^{b}, Ly-2 ^{b}, Ly-3 ^{b},Mod-1 ^{b}, Mup-1^{b}, Pep-3 ^{a}, Pgm-1^{a}, Trf ^{b}, Thy-1^{b}, Tla^{c}, H-2D ^{b} and H-2K^{b} ((280, 281). C57BL/6 mice used in the vaccine studies were obtained from Harlan Sprangue-Dawley laboratories (Harlan Sprangue-Dawley, Indianapolis, IN, USA). All mice were housed in compliance with the USDA regulations.

II.E.2.c. . C3H/He Mice

The inbred, black agouti coated, C3H mouse strain is widely used as a model for drug treatments of neurologic disorders with applications in toxicology and oncology. This mouse line was established by Strong in 1920 by crossing a Bagg albino female with a Dilute Brown /Non-Agouti (DBA) male. The mouse line was transferred to Andervont in 1930, and then to Heston at F35. The NIH received the mouse strain in 1951 from Heston at F57. The coat color loci include: A, B, C, and D. Other loci described include: Car-2^{b}, Ce-2^{b}, Es-1^{b}, Es-3^{c}, Es-10^{b}, Gpd-1^{b}, Gpi-1^{b}, Gus ^{b}, Hc^{l}, Hbb^{d}, Idh-1^{a}, Lv^{a}, Lyb-2^{b}, Ly-1^{a}, Ly-2^{a}, Ly-3^{b}, Mod-1^{a}, Mup-1^{a}, Pep-3^{b}, Pgm-1^{b}, Trf ^{b}, Thy-1^{b}, Tla ^{b}, H-$2D^{k}$ and H-$2K^{k}$. Six to 8 weeks-old C3H/He mice used in the vaccine studies were acquired from Harlan Sprangue-Dawley laboratories and housed in compliance with the USDA regulations.

II.E.2.d. . CD1 Mice

The white coated, non-inbred (outbred) CD1 mouse strain was developed in the laboratory of Dr Coulon (Centre Anticancereux Romand, Lausanne, Switzerland). The original group of mice that served as progenitors consisted of two male and seven female albino mice. CD1 mice were imported to the USA by Dr. Clara Lynch (Rockefeller Institute) in 1926. The Hauschka Ha/ICR stock was initiated in 1948 at the Institute of Cancer Research (ICR) in Philadelphia from the “Swiss” mice of Rockefeller origin. The mice where then transferred to Dr. Edward Mirand of the Roswell Park Memorial Insitute. In 1959 the mice were acquired by Charles River Laboratories (281). CD1 mice

used in the vaccine studies were 6-8 weeks-old and acquired from Charles River Laboratories (Charles River Laboratories Inc, Wilmington, MD, USA). Mice were housed in compliance with the USAD regulations.

II.E.2.e. . CD21/CD35 Knock-out Mice

CD21/CD35 knock-out (CD21/CD35$^{-/-}$ or CR2$^{-/-}$) mice were developed by Haas, *et al*. (130) at Duke University. Briefly, CD21/35$^{-/-}$ mice were generated using a targeting construct with the 5′ promoter region of the *Cr2* gene deleted. Homologous recombination in embryonic stem (ES) cells replaced the *Cr2* promoter region and exon encoding the transcription initiation and signal sequence of the CD21/35 protein product with a Neomycin resistance cassette that terminated translation. Transmission of the mutation was verified by Southern blot and PCR analysis. Mice heterozygous for the targeted allele were mated to generate wild-type, CD21/35$^{+/-}$, and CD21/35$^{-/-}$ littermates. Mice were confirmed homozygous for the neo-disrupted *Cr2* locus by southernblot or PCR analysis of genomic tail DNA (130). CD21/CD35$^{-/-}$ mice used in the vaccine studies were housed in strict pathogen free conditions at the animal facility of Duke University and in compliance with the USDA regulations.

II.E.2.f. MHC class II Knock-out Mice

Six to eight weeks-old MHC class II Knock-out mice (MHCII$^{-/-}$) were acquired from Taconic (Taconic, Hudson, NY, USA). These mice exhibit a depletion of CD4^{+} T-cells

through the disruption of the *H2-Ab1* gene. The MHCII$^{-/-}$ mouse model is useful for research in transplantation, gene therapy and immunological diseases. This mouse model was developed by Grusby, *et al*. (282), at Harvard University. The mouse strain was received at Taconic in December 1991 from Tufts University (V. Papaioannou) at N4 onto the C57BL/6 from the (129/Sv x C57BL/6J) chimera. Male mutant mice were used in each backcross while at Tufts. At Taconic, the line was backcrossed 5 generations to C57BL/6NTac and cesarean derived in February 1992. Mice from the N5 colony were backcrossed 7 additional generations (N12) to C57BL/6NTac mice, and intercrossed to produce homozygous animals. The colony is maintained by brother x sister mating of homozygotes (280). MHCII$^{-/-}$ mice used in the vaccine studies were housed at the University of Pittsburgh animal facility in strict pathogen free conditions and in compliance with the USDA regulations.

II.E.3. Gene Gun Immunization

DNA immunizations were performed by intradermal bombardment of gold particles coated with plasmid DNA (DNA Bullets, described in Section II.B). DNA vaccinations were performed using a Bio-Rad handheld DNA deliver system (gene gun) (Bio-Rad, Hercules, CA, USA). Briefly, ~ 1 inch of abdominal fur of the animals to be immunized was shaved with a handheld Single-Speed clipper (Oster, McMinnville, TN, USA) using a size 40 blade. The gene gun was loaded with the corresponding DNA bullets and then connected to a Helium tank. The gas regulator pressure was adjusted at 400 psi and mice were immunized in two separate spots (2 μg total DNA per immunization) of the

abdominal shaved area. Mice were followed for tenderness or adverse reaction in the immunization area for three days.

II.E.4. Intravenous (I.V.) Immunization

Tail vein (I.V.) immunizations were performed in mice anesthetized with a mixture of Ketamine / Xylazine. Tail vessels were dilated using a heat lamp. A 1 ml tuberculin syringe with a 30G1/2 needle (Becton Dickinson and Co, Franklin Lakes, NJ, USA) was loaded with the injectate. Air bubbles were removed from the syringe and the mouse tail was inspected to verify dilation of the vessels. The mouse was placed on a restrainer (Braintree Scientific Inc., Braintree, MA, USA) and the tail swabbed with 70% ethanol. The tail was immobilized with gentle retraction. The lateral tail vein was visualized and the needle (with the bevel facing up) was inserted parallel to the vein 2 to 4 mm into the lumen. The fluid was injected slowly and when finished, the needle was gently withdrawn. Hemostasis was achieved applying digital pressure in the area of immunization. Immunizations were performed as close as possible to the tip of the tail.

II.E.5. Blood Sample Collection

Blood samples were collected by retro-orbital plexus puncture using a heparinized capillary tube (Drummond Scientific Company, Broomall, PA, USA) and a 1.5 ml centrifuge tube on anesthetized mice and incubated (4° C for 4 h) to allow coagulation of the red blood cells. Serum was separated from the red blood cells by centrifugation (5,000 rpm for 10 min). Collected serum was stored in at -20° C.

II.E.6. Cell Isolation from Spleens and Lungs

Spleens were exposed and removed by making an incision in the abdominal cavity from the rib cage to the tail using sterile scissors and tweezers. Similarly, lungs were exposed and removed by making an incision in the midline of the chest cavity using sterile scissors and tweezers. The lungs were carefully removed, avoiding contamination with thymic tissue. Both, lungs and spleens were carefully rinsed with sterile PBS. The removed spleens or lungs were then placed in a cell strainer (BD Biosciences, Bedford, MA, USA) over a 60 x 15 mm dish (U.S.A. Scientific, Ocala, FL, USA). The spleen or lungs were manipulated into a single cell suspension using the rubber stopper end of a 5 ml syringe plunger (Becton Dickinson & Co., Franklin Lakes, NJ, USA). The cell strainer was then rinsed with a total of 4 ml of sterile PBS and the cell suspension transferred to a 15 ml conical tube. The cells were centrifuged at 1200 rpm (5 min at 4°C) and the supernatants discarded. The cells were gently resuspended in 9 ml of distilled water, the tube was capped and carefully mixed for 3 sec. Immediately, 1ml of 10X PBS was added, the cells suspension gently mixed and centrifuged at 1200 rpm (5 min at 4°C). The supernatants were discarded, and the cells were then resuspended in 3 ml of PBS. In order to count the cells, 50 µl of the cell suspension were mixed with 450 µl of Trypan Blue solution (Gibco, Grand Island, NY, USA) in a microcentrifuge tube (1:10 dilution) and incubated for 3 min. 10 µl of this suspension was loaded in each side of a hematocytometer (VWR, Bridgeport, NJ, USA) and the cells within the four outer quadrants were counted using a light microscope (100X power) (Fryer Company, Inc,

Huntley, IL, USA). The number of viable cells (cells/ml) was determined using the same equation described in Section II.C.2.

II.E.7. Cell isolation from the bone marrow

The skin of the mouse was peeled from the top of each hind leg and down over the foot. The foot with the skin was cut off and discarded. The hind legs were then cut off and placed in a 60 x 15 mm dish (U.S.A. Scientific, Ocala, FL, USA) containing sterile PBS. The excess of muscle from the legs was removed by holding the end of bone with forceps. Following excess of muscle removal, the bones were severed between the joints. A 25 ml syringe was attached to a 26G needle (Becton Dickinson and Co, Franklin Lakes, NJ, USA) and filled with PBS. The needle was then inserted into the bone marrow cavity of femur or tibia and gently flushed with 2-5 ml of PBS or until bone cavity appeared white. The flushed PBS containing bone marrow cells was collected in a 15 ml conical tube (USA Scientific, Woodland, CA, USA) kept on ice. The 15 ml conical tube was centrifuged for 5 min, 4 °C, 1200 rpm (Sorvall Instruments, Newtown, CT, USA). The supernatant was decanted and the cell pellet was resuspended in 9 ml of distilled water. The tube was then capped, carefully mixed for 3 sec, and immediately 1ml of 10X PBS was added. The cells suspension was gently mixed and centrifuged at 1200 rpm (5 min at 4°C). The supernatants were discarded, and the cells were then resuspended in 3 ml of PBS 3. The cells were counted as described in Section II.E.6 and the number of viable cells determined as described in Section II.C.2. These cells were then resuspended at the appropriate concentration in RPMI [RPMI 1640 (Gibco, Grand Island, NY, USA), containing 10% FCS (Atlanta Biologicals, Atlanta, GA, USA), 10 mM glutamine

(Invitrogen Life Technologies, Carlsbad, CA, USA), 100 U/ml penicillin/streptomycin (Gibco, Grand Island, NY, USA) and 55 μM 2-ME)] and used in assays described in Section II.F.3.

II.E.8. Intranasal Influenza Virus Challenge

Different dilutions (1:1000, 1:500, 1:250 and 1:100) of live, mouse adapted influenza virus A/Puerto Rico /8/34 (A/PR/8/34) (H1N1) were tested in BALB/c and C57/BL/6 wild-type mice to determine the lethal dose 100 (LD_{100}). LD_{100} was defined as the viral dose able to cause morbidity in 100% of the mice challenged. Morbidity was defined as body weight loss of more than 20%. Mice with more than 25% of body weight loss were sacrificed for humanitarian reasons (mortality). Virus challenge was performed in mice anesthetized (Ketamine/Xylazine as described in Section II.E.1.) via intranasal instillation of 50 μl of allantoic fluid diluted in PBS to contain the above mentioned virus dilutions. Morbidity and mortality were recorded daily for two weeks in the virus challenged mice. LD_{100} for BALB/c mice was 1:1000, while for C57BL/6 mice was 1:100. The LD_{100} determined for each mouse strain was used in the subsequent vaccine/ challenge studies.

II.F. Immunological Assays

II.F.1. Enzyme-Linked Immunosorbent Assay (ELISA)

Sera samples were individually collected and tested for antibody responses to the immunizing antigens (HIV-1 $Env_{gp120\ (IIIB)}$, $Env_{gp120\ (YU2)}$ or sHA) by ELISA. Each well of

a 96-well plate (Corning, Corning, NY, USA) was coated either with either 1) recombinant HIV-1 Env $_{gp120\ (IIIB)}$ (NIH ARRRP, Germantown, MD, USA) (30-50 ng), 2) culture supernatant from HEK 293T cells transfected with DNA expressing Env$_{gp120\ (YU2)}$ from genes codon optimized for mammalian cells (pcoEnv$_{gp120(YU2)}$) (~50 ng) or 3) culture supernatant from HEK 293T cells transfected with DNA expressing sHA (psHA) (~100 ng). The coating was performed in a total volume of 100 µl / well of PBS and the 96-well plate was incubated overnight at 4°C in a humid chamber. Plates were blocked (25°C for 2 h) with PBS (200 µl) containing Tween 20 (0.05%) and non-fat dry milk (5%). The blocking buffer was removed and 100 µl of serially diluted sera samples were added to each well (25° C for 2 h). Following thorough washing (3X) in PBS-(0.05%) Tween 20 (PBS-T), samples were incubated (25° C for 1 h) with 100 µl of goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) (1:5,000) (Southern Biotechnology Associates, Inc., Birmingham, AL) diluted in PBS-T containing 5% non-fat dry milk. The unbound antibody was removed, and the wells were washed (3X) with PBS-T. 100 µl of TMB substrate (1 TMB tablet per 10 ml of phosphate-citrate pH 5.0 buffer; 2 µl 30% H_20_2) (Sigma, St Louis, MO, USA) were added to each well (25°C for 30 min). Following 30 min incubation, the reaction was stopped with 50 µl / well of 2N Sulfuric Acid. The colorimetric change was measured as the O.D. at 450 nm using a spectrophotometer (Dynex Technologies, Chantilly, VA, USA). Results were recorded as the arithmetic mean plus the standard deviation (S.D.) after the value of naïve sera was subtracted from the test samples.

In order to measure the levels of HIV-1 Env_{gp120} and sHA IgG subtypes, a modification to the above protocol included the use of biotinylated goat anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgM antibodies (1:5,000) (Southern Biotechnology Associates, Inc., Birmingham, AL) to detect antigen-antibody complexes in place of the goat anti-mouse IgG-HRP conjugated. Biotinylated IgG isotype antibodies were detected by Streptavidin-HRP conjugated (1:7000) in PBS-Tween 20 (1h, 25°C). Developing was performed as described above.

In order to detect specific anti-SA antibodies a similar ELISA protocol was used. The next modifications were included. Plates were coated with ~500 ng/well of purified SA in a total volume of 100 µl of PBS. IgM, IgG and IgG subtypes (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3) were detected using antibodies conjugated to alkaline phosphatase (AP) (Southern Biotechnology Associates, Inc., Birmingham, AL, USA). The substrate used was *p*-nitrophenyl phosphate (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) and the colorimetric change was measured as the O.D. at 405 nm.

II.F.2. Displacement (Avidity) ELISA

Antibody affinity maturation was assessed by disrupting the antigen-antibody interaction with increasing concentrations of the chaotropic agent sodium thiocyanate (NaSCN). A fresh 5M stock solution of NaSCN (Sigma, St. Louis, MO, USA) was prepared in PBS-T. This stock solution was used to prepare 5 ml the working dilutions: 0.5M, 1.0M, 1.5M, 2.0M, 2.5M, 3.0M and 3.5M. The sera sample dilution to be used was based on crossover O.D. readings (from ELISA results) between the samples to be compared. The

appropriate dilution of each sample was prepared in PBS-T and (5%) non-fat milk. The assay was performed on 96-well plates coated with 50 ng/well of purified recombinant HIV-1 $Env_{gp120\ (IIIB)}$ protein (NIH ARRRP, Germantown, MD, USA) in carbonate buffer (pH 9.6) similar to the procedure described in Section II.F.1. Unbound protein was removed from the 96-well plate followed by blocking (2 h, 25°C) with PBS-T with (5%) non-fat milk (200 µl/well). The blocking buffer was removed and the plate was then washed (4X) with PBS-T. 100 µl of each sera sample at the determined dilution was added in 8 wells. The samples were incubated for 1 h at 25°C. Unbound antibody was then removed from the plate and washed (4X) with PBS-T. 100 µl of the different NaSCN dilutions (0M, 0.5M, 1.0M, 1.5M, 2.0M, 2.5M, 3.0M and 3.5M) were added in the corresponding wells. In 0M well, 100 µl of PBS-T was added. The plate was incubated for 15 min at 25°C. Following the incubation time, NaSCN was removed and the plates washed (4X) with PBS-T. 100 µl of a 1:5000 dilution of goat anti-mouse IgG-HRP antibody (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) were added to each well (1h, 25°C). Unbound antibody was removed and washed (4X) with PBS-T. 100 µl of TMB substrate (1 TMB tablet per 10 ml of phosphate-citrate pH 5.0 buffer; 2 µl 30% H_20_2) (Sigma, St Louis, MO, USA) were added to each well (25°C for 30 min). The colorimetric change was measured as the O.D. at 450 nm by a spectrophotometer (Dynex Technologies, Chantilly, VA, USA). The concentration of NaSCN required to to disrupt 50% of the initial O.D. value for each sample was determined. The percent of initial IgG was calculated as the initial O.D.

II.F.3. Enzyme-Linked Immuno-Spot (ELISPOT) Assay for Detection of Antibody Forming Cell

The frequency of antibody-forming cells (AFC) was determined in cells isolated from the spleen (Section II.E.6) and bone marrow (Section II.E.7). The PVDF membrane on Immobilon-P Multiscreen 96-well plates (Millipore, Bedford, MA, USA) was activated with 50 µl per well of Methanol (1 min). The methanol was removed by flicking the 96-well plate and then it was washed once with 200 µl of sterile PBS. The plate was coated with 100 µl/well of the antigen of interest (5 µg/ml in PBS) and incubated overnight at 4 °C in a humid chamber. Next morning the plate was washed twice with 200 µl of sterile PBS. Following the wash with PBS, the plate was blocked for 1 h (25°C) with 200 ul/well of RPMI culture medium (RPMI 1640 containing 10% FCS, 10 mM glutamine, 100 U/ml penicillin/streptomycin, and 55 µM 2-ME). Bone marrow and spleen cells were plated at 10^4, 10^5, or 10^6 cells per well in 100 µl of RPMI culture medium for 18 h at 37°C in a CO_2 incubator. Following 18 h incubation, the plate was washed three times with Tris-Buffered Saline Tween-20 (20 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20) (TBS-T). Subsequently, the plate was incubated with 100 µl/well (1:1000 dilution) of polyclonal alkaline phosphatase-conjugated goat anti-mouse IgG antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) for 2 h at room temperature. The polyclonal antibody was removed by dumping the supernatant and the plate was washed (4X) with TBS-T. The plate was developed for 30 min using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (70 µl/well) (Sigma, St. Louis, MO, USA). Spots were counted by an ImmunoSpot ELISPOT reader (Cellular Technology Ltd., Cleveland, OH, USA). The number of spots per 1 X 10^6 cells was recorded after

subtracting the background values (naïve mice). The arithmetic mean ± the S.D. was determined for each group after the appropriate background values were subtracted from the test samples.

II.F.4. In vitro Cell Proliferation

Splenocytes from vaccinated mice were harvested (described in Section II.E.6) and resuspended (1 X 10^7 cells/ml) in FACs buffer [1X PBS (without calcium or magnesium), 3% newborn calf serum (Atlanta Biologicals, Atlanta, GA, USA), 0.02% Sodium Azide (Fisher Scientific, Fair Lawn, NJ, USA) and 1mM EDTA, pH 7.5]. The splenocytes (1 ml) were then incubated and stained with the lipophilic carbocyanine fluorescent dye dioctadecyl oxycarbocyanin (SP-$DiOC_{18}$, 2 μM) (Molecular Probes, Eugene, OR, USA) for 5 min at 37 °C. The samples were later incubated at 4 °C, protected from light, for 15 min. Following incubation at 4°C, the cells were gently centrifuged (1200 rpm, 5 min, 4°C) and resuspended in RPMI culture media. SP-$DiOC_{18}$ stained splenocytes (6 X 10^6 cells) were analyzed on a FACSVantage cytometer (Becton-Dickinson, Mountain View, CA, USA) and sorted by fluorescent intensity. The middle 10-15% of cell stained with the highest fluorescent intensity (~10^6 cells) were collected and resuspended in RPMI culture media. The sorted cells were then plated in 24-well plates (5 X 10^5 cells / ml) and incubated with the proliferation stimulant protein (4 μg/ml) for 5 days at 37°C, 5% CO_2. Stimulant protein was the same DNA product used for immunizations. For example, if mice were vaccinated with DNA encoding for HIV-1 $Env_{gp120\ IIIB}$, the stimulant was recombinant purified HIV-1 $Env_{gp120\ IIIB}$ protein. Control wells received PBS as stimulant. Following 5 days incubation, the cells were analyzed

for proliferative responses by measuring the fluorescent intensity of stained cells by FACScan and analyzed by Cell Quest software (Becton-Dickinson, Mountain View, CA, USA). SP-$DiOC_{18}$ is a lipohilic carbocyanine fluorescent dye that integrates into the cell membrane. Fluorescent intensity reduces as the cell divides as a part of the dye is passed to the cell membrane of the daughter cells. A reduction in the fluorescent peak intensity, following stimulation, was considered significant for proliferation.

II.F.5. Splenocyte cytokine secretion

Isolated splenocytes (Section II.E.6.), from vaccinated mice, were assessed for cytokine production (mIL-4 and mIFN-γ) by ELISA (Biosource International, Carmillo, CA, USA). Briefly, isolated splenocytes were resuspended RPMI culture media (1 X 10^7 cell / ml) and 1 X 10^6 cells / well (100 µl) were plated in a 24-wells plate, in a total volume of 1 ml. The cells were then stimulated with the appropriate stimulant protein (4 ug/ml) (i.e. recombinant HIV-1 $Env_{gp120\ IIIB}$ protein). Control wells received PBS. The plate was then incubated for 90 h at 37°C, 5% CO_2. Following 90 h incubation, supernatants were harvested and stored in a 1.5 ml microcentrifuge tube at -20 °C until assayed. The cell culture supernatants were assayed in 96-well plates pre-coated with monoclonal anti-mIL-4 or anti-mIFN-γ antibodies (Biosource International, Carmillo, CA, USA). Standard curves were prepared using the controls (recombinant mIL-4 and mIFN-γ) provided by the manufacturer (Biosource International, Carmillo, CA, USA). 100 ul / well of cell supernatant and standards were incubated for 1 h at room temperature. The supernatants were then removed and the plate was thoroughly washed with PBS-T. Captured cytokines were then detected using a 1:250 dilution (in PBS-T) of biotinylated

polyclonal anti-mIL-4 or anti-mIFN-γ antibodies (100µl /well) (1h, room temperature). The plate was washed 3 times with PBS-T and antigen-antibody complexes were detected by streptavidin-conjugated to horseradish peroxidase (HRP) (1:250 dilution) (1h, room temperature). The plate was then read on an Anthos Labtec 2001 ELISA reader at 405 nm. The O.D. of each sample was compared to the standard curve and the concentration of the cytokines was extrapolated from the standard curve.

II.G. Statistical Analysis

Sample size calculation for t test was determined using the following parameters: power = 90, alpha = 0.05 and confidence interval = 90 using the delta-sigma approach. All statistical analysis was done using the Student's *t* test in STATA (STATA Corp, College Station, TX, USA) or SPSS (SPSS Inc, Chicago, IL, USA) software packages.

III. Chapter 3: Specific Aims

III.A.Rationale of Research

The goal of a vaccine is to induce an immune response strong enough to prevent the development of an infectious disease. Live-attenuated vaccines, which use microorganisms with impaired virulence, remain as the gold standard in immune induction. Nevertheless, the important side effects, the difficulty in attenuation of the microorganisms, the limitations of their use in immune compromised patients and the need of a cold chain of these vaccines, have pushed the development of new kinds of vaccine technologies and adjuvants.

New vaccine technologies, such as protein (subunit), DNA, and virus like-particles (VLP) are safer than traditional live-attenuated vaccines; however, the immune responses induced are limited, requiring large doses and several boosts. In order to increase the immunogenicity of these vaccines and hence, reduce the dose and number boosts, different kinds of adjuvants have been developed. Adjuvants are substances that enhance the immune responses to different kinds of antigens. Several substances with adjuvant activity have been described (Section I.B) (Table 1) and despite the large number of experimental adjuvants alum remains as the primary licensed adjuvant used in humans. Some adjuvants are efficient inducers of the immune responses; however, their important side effects have limited their use.

III.A.1. Antigens and their immunization route

In order to study the properties of an adjuvant, it is necessary the use of antigens. Careful selection of antigens and the route of immunization is important as these can influence the type of immune responses elicited. For the study of the adjuvant properties of C3d (see Section III.A.3), HIV-1 Env_{gp120}, a soluble form of hemagglutinnin (sHA) from influenza virus (A/PR/8/34) and streptavidin were selected. The first two represent antigens from infectious agents that affect large portion of the population and thus, have been extensively studied. This has allowed the development of several assays and animal models to evaluate the immune response. Streptavidin, on the other hand, represents a novel, non-conventional and poorly immunogenic antigen used to evaluate the immune response. Different routes of immunization (*e.g.* intradermal, intramuscular or intravenous) stimulate different sets of immune cells, thus the immune response elicited can be different. DNA and protein immunizations were selected to evaluate the adjuvant effects of C3d.

DNA immunization is a novel vaccine technology that uses plasmids encoding for the antigen of interest. This vaccination strategy has the advantage of inducing both, humoral as well as cellular immune responses (See Section I.A.1.d). However, initial promising results have been limited, in some cases due to the poor immune properties of the antigens. Such is the case of wild-type gene sequences of HIV-1 Env_{gp120} from the various isolates (*e.g.* IIIB and YU2). The extensive study of HIV-1 and the efforts to develop a vaccine have helped to increase the methods to evaluate the immune response.

Furthermore, a large amount of reagents have become available, making this protein an ideal antigen for the study of adjuvants.

Protein immunizations, elicit mainly humoral immune responses. These immunizations, however, are usually more immunogenic than their DNA counterpart. This facilitated the study of adjuvants in immunodeficient mouse models. Furthermore, protein immunizations allow the normalization of the immunogenic molecule, which is difficult to achieve when using DNA vaccines.

Even though there is an influenza vaccine available (Trivalent inactivated influenza vaccine or TIV), this is not 100% efficient. Furthermore, due to the influenza antigenic drift (Section I.D.2.d), the vaccine needs to be updated continuously. Moreover, the continuous risk of a gene reassortment and the generation of a highly infectious influenza virus with the subsequent risk of a pandemia, has allowed the permanent development of influenza vaccines. As a consequence, several animal models (*e.g.* mice and ferrets) and species adapted influenza virus strains (*e.g*. mouse adapted A/PR/8/34 (H1N1)) have become available. sHA from influenza virus, which a poorly immunogenic molecule is a model antigen for the study of adjuvants, because 1) allows the evaluation of the adjuvant effect; and 2) the protective effect of the immune response elicited can be evaluated in live-virus challenge animal models.

III.A.2. Animal Models

Different animal models have become available for the study of the immune system. Inbred animals such as BALB/c and C57BL/6 have been widely used for vaccine studies; however, these are considered artificial systems because of the limited genetic variation (homozygocity - especially in MHC alleles) they posses. The limited genetic variation of inbred mouse strains is caused by many homozygous recessive mutations that may be detrimental to the organism and as a consequence have negative effects on the immune response to foreign antigens. Furthermore, the results obtained from vaccine studies in a particular inbred mouse strain may not be similar to those obtained from a different one due to the differences in MHC alleles. Moreover, some of the vaccine results obtained from inbred mouse strains may not translate into higher animals or humans, because these are not inbred populations. In most natural populations of animals, high levels of genetic variation are the norm and outbred mouse strains resemble this high level of genetic variation. Thus, outbred mice are considered a more "real life" animal model for initial evaluation of vaccines. In order to be potentially useful, an adjuvant has to enhance the immune responses similarly both in inbred as well as in outbred populations. Hence, one of the aims of this thesis was to evaluate the adjuvant properties of C3d in different inbred and outbred mouse strains (See Section III.B.).

The limited number of MHC alleles in inbred mice has some advantages for scientists. For example, inbred mice have made easier the characterization of the immune responses in specific mouse strains. Furthermore, inbred animals, especially C57BL/6 mice have become the base for the development of several knock-out and transgenic models, which

allowed dissecting the role of different cells and molecules in the immune response. A mouse strains that lacks CR2 is one example of these knock-out models. This model was used to explore the mechanism of enhancement of the immune response by C3d (See Section III.C.). Another mouse model which lacks MHC class II molecules ($MHCII^{-/-}$), and as a consequence CD4+ T-cells, was used to explore the adjuvant properties of C3d independently of T-cell help (Section III.D.).

In summary, both inbred and outbred animals are important for the study of vaccines and adjuvants. Each model contributes with different characteristics to the understanding of the immune response.

III.A.3. C3d as an adjuvants and its mechanism of work

C3d, which is the final degradation product of the third component of complement (C3), has been used as an adjuvant. In the development of a normal immune response, activation of the complement system (See Section I.C.1 and **Figure 1**) leads to the formation of C3d. This molecule is able to bind and coat the invading microorganisms. Once coated with C3d, the microorganisms are able to interact with immune cells that bear complement receptor 2 (CR2), such as follicular dendritic cells (FDCs) and B-cells. In this way, C3d enhances antigen uptake, processing and also induces B-cell activation (through CR2-CD19 signaling – Section I.C.5). Thus, C3d not only bridges the innate with the acquired immune responses but also acts as a natural adjuvant. CR2 has been considered the natural ligand of C3d and as consequence its signaling pathway has been implicated as the sole mechanism by which C3d performs its adjuvant effect. However,

the interaction between these molecules has remained conflictive and there is not consensus on the nature of the interaction and the interacting regions, and as a consequence the pathway(s) activated. This suggests that besides the assumed mechanism of enhancement of the immune response by the CR2 pathway, there could be other mechanisms by which this molecule could exert its job. Therefore, one aim of this thesis project was to explore the enhancement of the immune response in the absence of CR2, using a knock-out mouse model.

The classic mechanism by which C3d enhances the immune response involves co-binding of the surface IgM (sIgM) by an antigen in conjunction with C3d interacting with its receptor, CR2. Cross-linking of these molecules activates two pathways that cross-talk and result in the common endpoint of B-cell activation. As a consequence, ligation of multiple CR2 molecules by C3d allows for a reduction in the amount of antigen needed to activate B cells, because of redundant B-cell activation signaling. Most antigens require T-cell priming, activation and collaboration with B-cells in order to mount a proper humoral immune response (T-dependent antigens - TD). However, there is a group of antigens known as T-cell independent (TI), which can induce a proper immune response in the absence of T-cells. One of the main characteristics of these antigens is the redundancy of their structure, which allows them to bind simultaneously several sIgM molecules on the B-cells surface. The ligation of several sIgM molecules, redundantly signals through the pathway that activates B-cells and thus induces cell proliferation and antibody production. The ability of multiple copies of C3d fused to a T-cell dependent antigen (sHA) in reproducing the redundant signaling and thus activation

of B-cells in the absence of T-cells (resembling TI antigens) was explored in a T-cell knock-out mouse model ($MHCII^{-/-}$ – Section III.D.) (Section II.E.2.f)

III.B. Specific Aim I

Goal: To determine if C3d enhances immune responses in mice with different genetic backgrounds.

Hypothesis: C3d will similarly enhance the immune responses in mice from different genetic background

Summary: Previous studies have shown the ability of C3d to function as a molecular adjuvant with different antigens in BALB/c mice. However, it has been suggested that the immune responses to different immunogens can vary depending on the mouse strain used. Furthermore, in AIM 2 (see below) a mouse strain deficient in the CR2 receptor was proposed to be used. These mice have the C57BL/6 genetic background, thus it was important to determine the ability of C3d to enhance the immune response in mice with different H-2 haplotypes. It was hypothesized that C3d was able to enhance the immune response in mice from different genetic background. In order to test this hypothesis, three inbred [BALB/c (H2d), C57BL/6 (H2b), and C3H/He (H2k)] and one outbred (CD-1 Swiss) mouse strain were selected. Mice were intradermally immunized with 2 μg of DNA per immunization (Primed at day 1 and boosted at weeks 4 and 8) using a gene gun delivery system. Immunizations were performed with DNA constructs that expressed the HIV-1 envelope gp120 (Env_{gp120}) from the isolate IIIB alone ($pEnv_{gp120(IIIB)}$)or coupled to two or three copies of C3d ($pEnv_{gp120(IIIB)}$-$C3d_3$) . Blood samples were collected every

two weeks, serum isolated and stored at -80 ºC. Sera samples were used to determine total IgG antibody titers as well as the main IgG isotypes (IgG_1 and IgG_{2a}) by endpoint dilution ELISA. At week 12, splenocytes were harvested and used to perform proliferation and cytokine production assays upon specific protein stimulation. The results showed that after three intradermal DNA immunizations Env fused to various copies of C3d induced similar high titer anti-Env antibodies and enhanced affinity maturation in all mouse strain tested. The main IgG isotype class induced in inbred mice was IgG_1, in contrast to outbred mice which showed a mixed IgG class switch (IgG_1 and IgG_{2a}). Moreover, harvested splenocytes stimulated with recombinant Env_{gp120} showed that all mouse strains immunized with Envgp120-C3d2-3 had a high level of anti-Env specific proliferation. Cytokine assays demonstrated that inbred mice produced mainly IL-4 while outbred mice produced both IL-4 and IFN-γ, suggesting humoral and mixed (humoral and cellular) immune responses, respectively.

Conclusion: Three copies of C3d were able to similarly enhance the immune response both in inbred and outbred mouse strains. However, some minor differences between inbred and outbred mice were detected, the overall enhancement of the immune response was similar.

III.C. Specific Aim II

Goal: To determine if C3d can enhance the IR in the absence of CR2

Hypothesis: C3d will not enhance the immune responses in the absence of CR2

Summary: C3d is proposed to function as a molecular adjuvant by efficiently targeting antigens to CR2, which interacts with CD19 to regulate transmembrane signals during B

cell activation. However, a direct role for CR2 in this process has not been demonstrated, therefore, the importance of CR2 engagement in mediating the immunostimulatory effects of C3d was proposed for evaluation in mice completely deficient in CR2 expression. It has been reported that B-cells from $CR2^{-/-}$ are not able to bind streptavidin (rSA-C3dg) tetramers. However, C3dg tetramers bind efficiently to CR2 in wild-type mice and show functional activity on normal, but not in $CR2^{-/-}$ B-cells. In order to explore the role that C3d/CR2 interactions play in the adjuvant effect of C3d conjugated antigens, $CR2^{-/-}$ mice were immunized with recombinant (r)SA or HIV-1 $rEnv_{gp120(IIIB)}$ alone or coupled to C3d. Additionally, plasmid DNA immunizations using similar constructs to those described in AIM1 ($pEnv_{gp120(IIIB)}$±C3d2-3) were performed. Interestingly, both primary and secondary antibody responses to rSA and $rEnv_{gp120(IIIB)}$ were significantly higher in the $CR2^{-/-}$ mice when these proteins were complexed to C3d. Additionally, an ELISPOT assay to detect frequencies of antibody-forming cells (AFC) in isolated bone marrow and spleen cells corroborated that $CR2^{-/-}$ mice immunized with rSA-C3d had a significantly higher frequency of AFC than those immunized with rSA alone. Furthermore, the adjuvant properties of C3d were compared to a model adjuvant that does not require the CR2 pathway, the carrier protein chicken gamma globulin (CGG). These results showed that C3d and CGG induced similar IgM and IgG antibody titers in $CR2^{-/-}$ mice. On the other hand, $CR2^{-/-}$ mice that were DNA immunized with $pEnv_{gp120(IIIB)}$-$C3d_2$ or $pEnv_{gp120(IIIB)}$-$C3d_3$ did not show statistically significant differences compared to $Env_{gp120(IIIB)}$ alone, however, the tendency was to develop higher antibody titers in the presence of C3d. The inability of C3d to induce similar results between protein and DNA immunizations in $CR2^{-/-}$ mice could be explained by 1) the

low level of protein expression achieved by wild-type genes, like $Env_{gp120(IIIB)}$, in DNA immunizations; and 2) the fact that $CR2^{-/-}$ mice are an immunodeficient mouse model and require higher amounts of antibody to trigger an immune response.

Conclusion: C3d enhances the immune response even in the absence of its natural ligand (CR2), which suggest that additional mechanisms or receptors not yet described are involved in the adjuvant effect.

III.D. Specific Aim III

Goal: To determine if C3d enhances humoral immune responses independently of CD4+ T-cell help

Hypothesis: Antigens fused to C3d are able to mimic the antigenic redundancy of T-cell independent antigens and thus induce secondary humoral immune responses (IgG) in the absence of CD4+ T-cells.

Summary: Some viruses are able to induce T-cell independent (TI) immune responses (*e.g.* Polyomaviruses, Vesicular stomatitis virus, coxsackie virus) in animals deficient in CD4+ T-cells. Virus infection in these deficient mice leads not only to secretion of IgM, but also an immunoglobulin (Ig) class switch to IgG/IgA and in some models results in protection from lethal virus challenges. TI viral antigens have a repetitive, highly organized antigenic structure, which allows extensive cross-linking of B-cell receptors (surface immunoglobulin M –sIgM-) thereby delivering a strong activating signal to B-cells. During influenza virus infections, TI humoral immune responses are important in resolving primary infections and prevention of re-infections in mice deficient in CD4+ T-cells. Live, as well as inactivated influenza viral particles, induce TI immune responses,

induce secretion of IgM, and stimulate an immunoglobulin class switch to IgG and IgA in the absence of CD4+ T-cells. One C3d-adjuvant mechanism involves the cross-linking of the sIgM to an antigen in conjunction with C3d binding to its receptor, CR2. Cross-linking of these molecules activates two pathways that cross-talk and result in the common endpoint of B-cell activation. As a consequence, ligation of multiple CR2 molecules by C3d allows for a reduction in the amount of antigen needed to activate B cells. In order to investigate the ability of antigens fused to $C3d_3$ to mimic the redundancy of TI antigens and thus induce Ig class switch in the absence of CD4+ T-cells, a soluble form of recombinant hemagglutinin (sHA) was fused to three tandem copies of C3d and used to immunize animals deficient in CD4+ T-cells (MHC $II^{-/-}$). MHC $II^{-/-}$ as well as C57/BL6 wild type mice were immunized (I.V.) with 20 μg of sHA alone or fused to $C3d_3$. Immunizations were normalized for the immunogenic portion (sHA) and performed at weeks 0, 5 and 9. Mice were challenged with a lethal dose of the mouse adapted influenza virus strain A/PR/8/34 at week 10. Morbidity (weight loss) and mortality were monitored for 2 weeks. The results in wild type mice showed that, C3d induced a significant enhancement of the secondary humoral immune responses after the second and third immunizations ($P<0.05$). This correlated with a reduced morbidity (weight loss) and higher survival in the sHA-C3d3 vaccinated group, following virus challenge. The IgG isotype profile induced pre and post- virus challenge was similar, with IgG_1 as the main isotype, suggesting a dominant Th2 immune response. The results in MHC $II^{-/-}$ mice showed twenty percent of mice immunized with sHA-$C3d_3$ developed a significant IgG class switch, while none of the mice that received sHA. The main isotype induced by C3d was IgG_{2b}, suggesting an environment rich in TGF-β. Following

virus challenge IgG was detected (mainly IgG_3) in all MHC $II^{-/-}$ mice challenged with A/PR/8/34; however, sHA-$C3d_3$ mice had a higher, but not statistically significant, titer. Despite this, weight loss was delayed and mortality was lower in mice vaccinated with sHA-$C3d_3$ compared to mice that received sHA alone. This suggests that C3d stimulates other immune factors (*e.g.* innate cells) besides B-cell and the enhanced survival is the result of collaboration between all these factors.

Conclusions: sHA-$C3d_3$ vaccinations induced mild secondary immune responses in the absence of CD4+ T-cells. However, these weak secondary immune responses, possibly in conjunction with other innate immune responses stimulated by C3d enhanced protection in mice to the virus challenge.

IV. Chapter 4: Specific Aim I

Mouse strain-dependent differences in enhancement of immune responses by C3d

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Franklin R Toapanta and Ted M. Ross

Mouse strain-dependent differences in enhancement of immune responses by C3d

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IV.A. Introduction

Previous studies have shown that DNA vaccinations effectively induce both humoral and cellular immune responses to immunogens from diverse infectious agents (for reviews, see (283-287)). DNA vaccines provide a promising future for the development of new vaccination strategies. These genetic vaccinations consist of eukaryotic expression plasmids that are inoculated into target cells and translated into proteins (283, 284). Animal studies have shown that DNA vaccinations induce protective immunity against a variety of infectious agents. DNA vaccines are comparatively easy to develop and manufacture and are likely to not require a cold chain for worldwide distribution. However, DNA vaccines have been less effective at eliciting immune responses to the gp120 subunit of human immunodeficiency virus (HIV)-1 envelope (Env) (33, 288-290). In addition, these vaccines have been less successful at generating neutralizing antibodies against HIV-1 (16, 33, 289, 291, 292). This inability to elicit high titer, cross-clade antibodies may be due to the long period of maturation that is required for Env-specific antibodies (292).

Recent studies in our laboratory have utilized a component of the complement system, the murine C3d (mC3d), conjugated to viral immunogens to enhance the immune responses directed against the fused antigen (84, 85, 127, 132, 138). Using C3d as a molecular adjuvant provides promising alternatives to administration of exogenous adjuvants. Mice vaccinated with DNA expressing a soluble form of the poorly

immunogenic gp120 fused to three copies of mC3d accelerated both the onset and the avidity maturation of antibody and enhanced neutralizing antibody titers compared to mice vaccinated with antigen alone (85, 132). In the natural immune response, C3d serves as a regulator of several B cell functions, including antibody production, antigen uptake, processing and presentation, and inducing B cell memory response (120, 124, 293, 294). The most likely mechanisms by which C3d serves as a molecular adjuvant is by binding of C3d to CD21 (CR2) on the surface of B cells and thereby enhancing signaling through CD19 and the IgG binding receptor CD32 (FcγRIIb). C3d bound antigens can directly stimulate antibody producing B cells, leading to the expansion of antigen specific B cells (120, 294).

Our laboratory and others have demonstrated that C3d enhances the immunogenicity to a variety of other antigens, however the level of immune responsiveness varies (84, 85, 126-128, 132, 135, 138, 156). These variable responses may be dependent on the antigen, the dose of inoculum, the route of inoculation, the method of vaccination, or the mouse strain used for the study. In order to examine the effects of MHC haplotype on immunoresponsiveness by DNA plasmids expressing C3d conjugated antigens, three inbred strains, BALB/c (H-2^d), C57BL/6 (H-2^b) and C3H/He (H-2^k), and one outbred strain, CD-1 Swiss, were vaccinated with DNA expressing Env gp120 (Env_{gp120}) (isolate IIIB) fused to multiple copies of murine C3d (mC3d). All $Env_{gp120(IIIB)}$-C3d-DNA vaccinated mice had an enhanced anti-Env antibodies compared to mice vaccinated with DNA expressing $Env_{gp120(IIIB)}$. However, $Env_{gp120(IIIB)}$-C3d-DNA elicited both Th1- and

Th2-mediated responses in outbred mice that correlated with enhanced avidity maturation of the anti-Env IgG response.

IV.B. Materials and Methods

IV.B.1. Plasmid DNA

pTR600, a eukaryotic expression vector, has been described previously (138). Briefly, the vector was constructed to contain the cytomegalovirus immediate-early promoter (CMV-IE) plus intron A (IA) for initiating transcription of eukaryotic inserts and the bovine growth hormone polyadenylation signal (BGH poly A) for termination of transcription. The vector contains the Col E1 origin of replication for prokaryotic replication and the kanamycin resistance gene (Kanr) for selection in antibiotic media (**Figure 8**A).

A soluble for of $Env_{gp120(IIIB)}$ was constructed by PCR amplification of a fragment representing the gp120 portion from the gene that encodes for $Env_{gp160(IIIB)}$ (**Figure 8**B). Each construct has the first 32 amino acids deleted from the N-terminus of each IIIB Env and replaced with a leader sequence from the trypsin plasminogen activator (tpA). The $Env_{gp120(IIIB)}$ gene was subsequently fused to two or three copies of murine C3d (**Figure 8**B). Linkers composed of two repeats of four glycines and a serine [(G4S)2] were fused at the junctures of Env and C3d and between each C3d repeat.

The plasmids were amplified in Escherichia coli strain-DH5α, purified using an endotoxin-free, anion-exchange resin columns (Qiagen, Valencia, CA) and stored at

−20 °C in dH2O. Plasmids were verified by appropriate restriction enzyme digestion and gel electrophoresis. Purity of DNA preparations was determined by optical density reading at 260 and 280 nm.

IV.B.2. In vitro Expression of DNA Vaccines

The human embryonic kidney cell line, 293T (HEK 293T), (5×10^5 cells/transfection) was transfected with 2 μg of DNA using 12% lipofectamine (Life Technologies, Grand Island, NY) according to the manufacture's guidelines. Supernatant was collected and 1.5% of supernatant was diluted 1:2 in SDS sample buffer (Bio-Rad, Hercules, CA) and loaded onto a 10% polyacrylamide/SDS gel. The resolved proteins were transferred onto a nitrocellulose membrane (Bio-Rad) and incubated with a 1:3000 dilution of polyclonal human HIV-infected patient antisera in PBS containing 0.05% Tween-20 and 5% fetal calf serum. After extensive washing, bound human antibodies were detected using a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-human antiserum and enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

IV.B.3.Animals and Immunizations

Three inbred strains of 6–8-week-old female mice [C57BL/6 (H-2^b), BALB/c (H-2^d), and C3H/He (H-2^k)] (Harlan Sprague–Dawley, Indianapolis, IN) and one outbred strain of female [CD-1 Swiss] (Charles River) were used for inoculations. Mice, housed with free access to food and water, were cared for under USDA guidelines for laboratory animals. Mice were anesthetized with 0.03–0.04 ml of a mixture of 5 ml ketamine HCl

(100 mg/ml) and 1 ml xylazine (20 mg/ml). Gene gun (G.G.) immunizations were performed on shaved abdominal skin using the hand held Bio-Rad® gene delivery system as described previously (295-297). Mice were immunized with two gene gun doses containing 1 μg of DNA per 0.5 mg of approximately 1 μm gold beads (Bio-Rad, Hercules, CA) at a helium pressure setting of 400 psi.

IV.B.4.Immunological Assays

IV.B.4.a. ELISA for Detection of Anti-Env Antibodies

An endpoint ELISA was performed to assess the titers of anti-Env IgG in immune serum using recombinant HIV-1 $Env_{gp120(IIIB)}$ to coat plates as described (298). Briefly, plates were coated with recombinant $Env_{gp120(IIIB)}$ (over night) and then probed with antisera from vaccinated mice (1 h). Subsequently, the primary antiserum was detected by anti-mouse IgG conjugated to horseradish peroxidase. The reaction was detected using TMB substrate and measured on a Anthos Labtec 2001 ELISA reader at 405 nm. Endpoint titers that were two fold higher than age-matched, naïve sera were considered positive.

Avidity ELISAs were performed similarly to serum antibody determination ELISAs up to the addition of samples and standards (292, 299-303). Samples were diluted to give similar concentrations of specific IgG by O.D. Plates were washed three times with 0.05% PBS–Tween-20. Different concentrations of the chaotropic agent, sodium thiocyanate (NaSCN) in PBS, were then added (0, 1, 1.5, 2, 2.5, 3, and 3.5 M NaSCN). Plates were allowed to stand at room temperature for 15 min and then washed six times

with PBS–Tween (0.05%)-20 (PBS-T). Subsequent steps were performed similarly to the serum antibody determination ELISA. Percent of initial IgG calculated as a percent of the initial O.D. All assays were done in triplicate.

IV.B.4.b. In vitro proliferation and FACS analysis

Splenocytes from vaccinated mice were harvested and resuspended (10^7 cells/ml) in FACS Buffer (2% FCS in PBS). In order to determine anti-Env specific immune cells elicited in vaccinated mice, splenocytes were incubated and stained with dioctadecyl oxycarbocyanin (SP-DiOC18, 2 μM) (Molecular Probes, Eugene, OR, USA) for 5 min at 37 °C, followed by incubating at 4 °C for 15 min. Cells were gently centrifuged (2000 rpm) and resuspended in RPMI with 10% FCS plus supplements. SP-DiOC18 stained splenocytes (6×106 cells) were analyzed on a FACSVantage cytometer (Becton-Dickinson, Mountain View, CA, USA) and sorted by fluorescent intensity. The middle 15% of cell stained with the highest fluorescent intensity (~10^6 cells) were collected, resuspended in RPMI with 10% FCS plus supplements and were plated in 24-well plates (5×10^5 cells/ml). Sorted splenocytes were immediately incubated with recombinant HIV-1 $Env_{gp120(IIIB)}$ (4 μg/ml) for 5 days and then analyzed for proliferative responses by measuring the fluorescent intensity of stained cells from $Env_{gp120(IIIB)}$-stimulated and unstimulated cells by FACScan and analyzed by Cell Quest software (Becton-Dickinson). An induction of proliferation was determined by measuring the peak intensity of stained cells before and after stimulation of cells.

IV.B.4.c. Splenocyte cytokine secretion

In order to determine the cytokine secretion by $Env_{gp120(IIIB)}$-stimulated cells, aliquots of cell culture supernatant was collected and analyzed for murine interleukin-4 (mIL-4) or murine interferon-gamma (mINF-γ) by ELISA (Biosource International, Carmillo, CA, USA) and by ELISpot (R&D Systems, Minneapolis, MN, USA). Harvested splenocytes from vaccinated mice were stimulated with recombinant HIV-1 $Env_{gp120(IIIB)}$ (4 μg/ml) for 90 h. Cell culture supernatants were incubated onto pre-coated monoclonal anti-mIL-4 or anti-mINF-γ ELISA wells for 1 h. Captured cytokines were detected using a biotinylated polyclonal anti-mIL-4 or anti-mINF-γ. Antigen-antibody complex was detected by streptavidin-conjugated horseradish peroxidase and read on an Anthos Labtec 2001 ELISA reader at 405 nm according to the manufacture's directions.

IV.B.5. Statistics

For statistical analysis, a Student's *t* test was employed. The difference between DNA vaccines expressing $Env_{gp120(IIIB)}$ fused to multiple copies of murine C3d was compared to DNA expressing $Env_{gp120(IIIB)}$ alone. Differences in titer were considered statistically significant when *p*-value was less than 0.05.

IV.C. Results

IV.C.1.. Expression of plasmids

Vaccine plasmids, expressing a secreted form of Env ($Env_{gp120(IIIB)}$), were constructed using the previously described pTR600 vector (85). Vaccine plasmids expressing the soluble gp120 from the HIV-1 isolate IIIB ($Env_{gp120(IIIB)}$) Env or fused to two ($Env_{gp120(IIIB)}$-$C3d_2$), or three ($Env_{gp120(IIIB)}$-$C3d_3$) copies of the murine homologue of C3d have been previously described (**Figure 8**B). The molecularly cloned gp120 region represented the entire surface domain of Env, but excluded the oligomerization and transmembrane domains and the cytoplasmic regions. The $Env_{gp120(IIIB)}$-C3d fusion proteins were generated by cloning tandem repeats of the murine C3d (293) in frame with the soluble gp120 gene. The proteolytic cleavage sites, found at the junction between each C3d molecule as well as the junction between the gp120 protein and the first C3d coding region, were destroyed by mutagenesis.

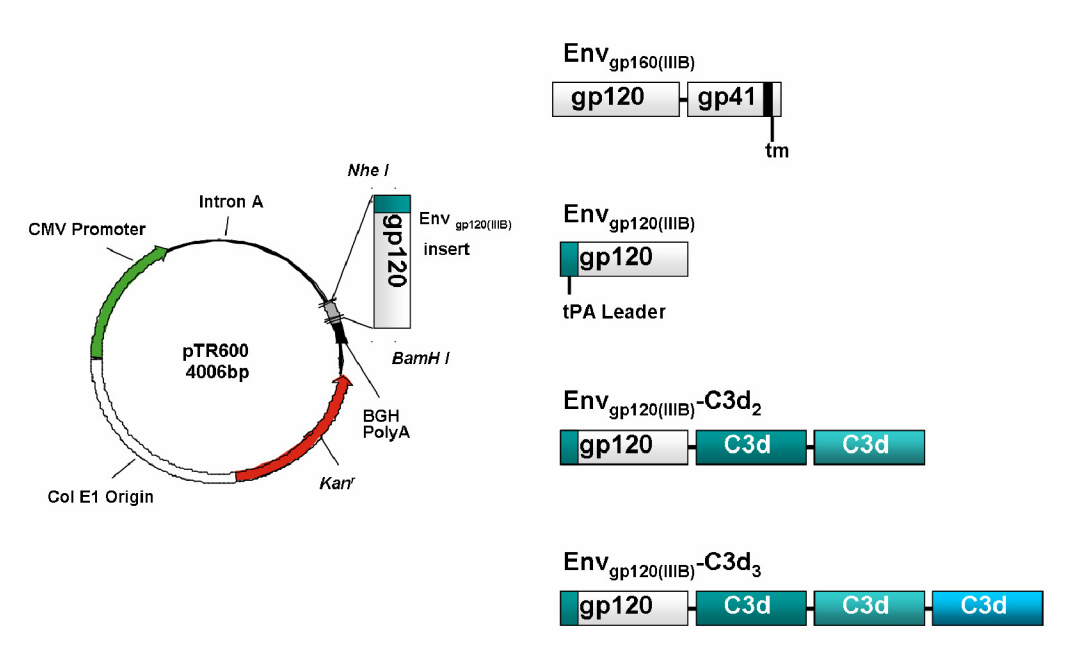
Overall, Env was expressed at similar levels by plasmids containing each secreted form of the antigen, however expression was lowered by two- to four-fold with plasmids expressing C3d fused forms of Env (data not shown). HEK 293T cells were transiently transfected with 2 μg of plasmid and both supernatants and cell lysates were assayed for $Env_{gp120(IIIB)}$ using an antigen capture ELISA. As observed previously (85), approximately 90% of the Env protein was present in the supernatant for both $Env_{gp120(IIIB)}$ and $Env_{gp120(IIIB)}$-C3d3-DNA transfected cells.

Western blot analyses revealed $Env_{gp120(IIIB)}$ and $Env_{gp120(IIIB)}$-C3d proteins of the expected sizes (Fig. IV.1.C). Using human patient polyclonal antisera (HIV-Ig), western blot analysis showed the expected broad band of 115–120 kDa corresponding to $Env_{gp120(IIIB)}$ (Fig. IV.1.C). A higher molecular weight band was seen for each of the sgp120-mC3d fusion proteins (Fig. IV.1.C). Consistent with the antigen-capture assay, intense protein bands were present in the supernatants of cells transfected with $Env_{gp120(IIIB)}$-DNA. No evidence for the proteolytic cleavage of the $Env_{gp120(IIIB)}$-C3d fusion protein was seen by western analysis.

IV.C.2. Antibody response to Env gp120 fused with mC3d DNA immunizations

Three different inbred strains of female mice [C57BL/6 (H-2^b), BALB/c (H-2^d), and C3H/H3 (H-2^k) and one outbred strain [CD-1 Swiss] were vaccinated with DNA coated gold particles via gene gun with a 1 μg dose per inoculum. Mice received a total of 2 μg of DNA per immunization. Mice were vaccinated at day 1 and then boosted at week 4 and 8. Specific anti-Env antibodies were elicited in all strains of DNA immunized mice (Fig IV.2). DNA plasmids expressing $Env_{gp120(IIIB)}$ fused to multiple copies of murine C3d ($Env_{gp120(IIIB)}$-$C3d_2$ or $Env_{gp120(IIIB)}$-$C3d_3$) raised higher titers of ELISA antibody than the $Env_{gp120(IIIB)}$ (Fig. IV.2). In addition, the titers of elicited anti-Env antibodies were similar in all four mice strains. As observed in our previous mouse studies using BALB/c mice (85, 132), the temporal pattern for the appearance of anti-Env antibodies in C57BL/6 and CD-1 Swiss mice revealed titers that were boosted after each inoculation with DNA expressing $Env_{gp120(IIIB)}$-$C3d_2$ or $Env_{gp120(IIIB)}$-$mC3d_3$.

Antisera were typed for both the immunoglobulin class and isotype of IgG elicited by vaccination (**Table 6**). Our previous studies using BALB/c mice indicated that the predominant class of immunoglobulin after three DNA inoculations was IgG and to a lesser extent IgE in mice vaccinated with $Env_{gp120(IIIB)}$ fused to two or three copies of murine C3d (132). In contrast to the overall antibody titer, the isotype of elicited antibody differed between the inbred and outbred strains. Inbred mice vaccinated via gene gun with DNA expressing $Env_{gp120(IIIB)}$-$C3d_2$ or $Env_{gp120(IIIB)}$-$C3d_3$ had an almost exclusive IgG_1 bias. However, outbred CD-1 Swiss mice vaccinated with the same DNA plasmids elicited a more mixed response with similar titers of IgG_{2a} and IgG_1 isotypes (**Table 6**). There was no detectable IgA or IgM after the third DNA vaccination at week 10, however, low levels of anti-Env IgM antibodies were observed early in the inoculation schedule (week 4) (data not shown). These same three mouse strains were vaccinated with the same DNA vaccines via intramuscular (I.M.) injection and similar IgG isotype responses were observed as in mice vaccinated via gene gun. (data not shown). Therefore, the three inbred mice strains elicited a predominately IgG_1 anti-Env antibody after vaccination with $Env_{gp120(IIIB)}$-C3d-DNA, however, outbred mice elicited a more mixed response with a significant anti-Env IgG_{2a} antibody titer.



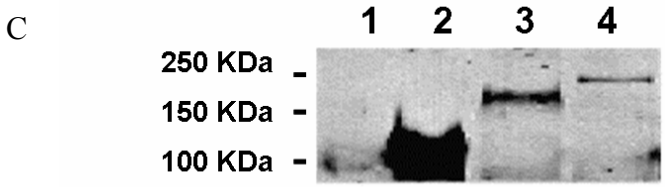


Figure 8. Schematic representation of vector DNA vaccine constructs and expression of vaccine constructs in vitro.

A) Schematic representation of TR600. This expression vector was constructed to contain the cytomegalovirus immediate-early promoter (CMV-IE) plus intron A (IA) for initiating transcription of eukaryotic inserts and the bovine growth hormone polyadenylation signal (BGH poly A) for termination of transcription. The vector contains the Col E1 origin of replication for prokaryotic replication and the kanamycin resistance gene (Kanr) for selection in antibiotic media.

B) HIV-1 Env gene inserts from the isolate IIIB were cloned into pTR600 using the NheI and BamHI restriction endonuclease sites directly 3′ to the tissue plasminogen activator (tpA) sequence. The first schematic on the right represents the wild-type, transmembrane form of the Env protein ($Env_{gp160(IIIB)}$). The second schematic represents the secreted gp120 form of the Env ($Env_{gp120(IIIB)}$). The third schematic represents the secreted $Env_{gp120(IIIB)}$-$C3d_2$ construct used as a vaccine insert. The fourth schematic represents the secreted $Env_{gp120(IIIB)}$-$C3d_3$ construct used as a vaccine insert. Linkers composed of two repeats of four glycines and a serine [(G4S)2] were fused at the junctures of Env and C3d and between each C3d repeat.

C) HEK 293T cells, were transfected with 2 µg of each vaccine plasmid. Supernatant was collected and 1.5% of total volume was electrophoresed on a 10% polyacrylamide gel. Lane 1: molecular weight marker; lane 2: secreted $Env_{gp120(IIIB)}$; lane 3: secreted $Env_{gp120(IIIB)}$-$C3d_2$; and lane 4: secreted $Env_{gp120(IIIB)}$-$C3d_3$.

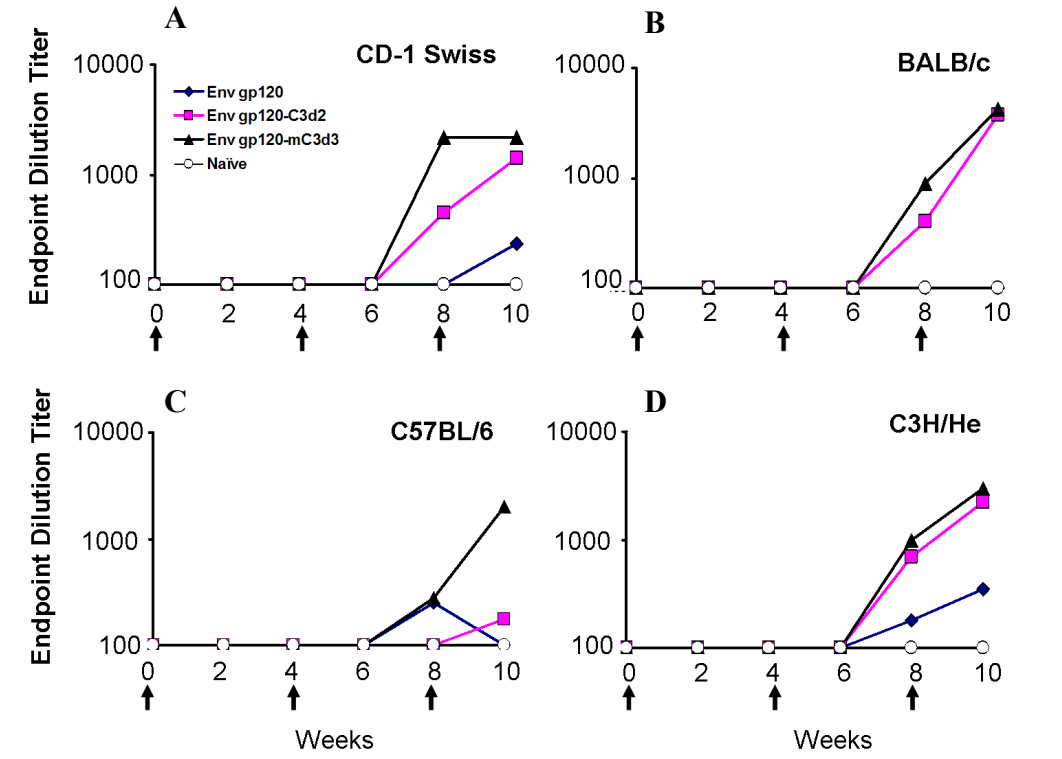


Figure 9. Anti-Env IgG Raised by Gene Gun DNA Immunizations

Mice were intradermally immunized with 2µg of DNA (arrows) at weeks 0, 4 and 8. Immunizations were performed using a gene gun handheld delivery system. Sera samples were collected from mice at weeks 0, 4, 6, 8 and 10. Mice (CD-1Swiss, BALB/c, C57BL/6 and C3H/He) received plasmid DNA encoding eirther Env $_{gp120(IIIB)}$ (Red rhomboid symbol), Env $_{gp120(IIIB)}$-C3d_2 (Blue Square), Env $_{gp120(IIIB)}$-C3d3 (Black triangle) or an empty vector (Naïve) (White circle). Sera collected a the indicated times from each mouse was assayed for specific IgG levels by ELISA. 96-well plates were coated with recombinant gp120 protein derived from Chinese haster ovary (CHO) cells expressing HIV-1 isolate IIIB. Data are presented as the average of five mice. Preimmune sera has not detectable specific IgG. Endpoint dilution titers were conducted by diluting the sera until O.D. valued reached background.

IV.C.3. Avidity of anti-Env antiserum

Sodium thiocyanate (NaSCN) displacement ELISAs demonstrated that the avidity of the antibody generated with $Env_{gp120(IIIB)}$-$C3d_3$ expressing DNA was consistently higher than that from $Env_{gp120(IIIB)}$-DNA vaccinated mice (**Table 7**). The avidity of specific antibodies to Env was compared by using graded concentrations NaSCN, a chaotropic agent, to disrupt antigen-antibody interaction (304). The binding of antibodies with less avidity to the antigen is disrupted at lower concentrations of NaSCN than that of antibodies with greater avidity to the antigen. In vaccinated BALB/c mice, the effective concentration of NaSCN required to release 50% of antiserum (ED_{50}) collected at week 10, after three inoculations of the $Env_{gp120(IIIB)}$-C3d3-DNA vaccine, was ~2.2 M (**Table 7**) and was significantly higher than C57BL/6 or C3H/HE mice vaccinated with $Env_{gp120(IIIB)}$-$C3d_3$-DNA (~0.8–1.25 M). Inbred mice vaccinated with $Env_{gp120(IIIB)}$-$C3d_2$-DNA had lower ED_{50} values (~0.5–0.75 M). In contrast, outbred CD-1 mice vaccinated with DNA expressing $Env_{gp120(IIIB)}$ conjugated to two or three copies of murine C3d elicited high avidity antibodies after three inoculations. The effective concentration of NaSCN required to release 50% of antiserum collected from mice vaccinated with $Env_{gp120(IIIB)}$-$C3d_3$-DNA was >3.5 M (**Table 7**). These results indicate that the antibody from $Env_{gp120(IIIB)}$-C3d3-DNA vaccinated outbred CD-1 mice had undergone more rapid avidity maturation than antibody from vaccinated inbred mice strains.

Table 6. Anti-Env antibody titers and induction of T-cell cytokine profiles

Vaccine†	CD-1 Swiss		BALB/c		C57BL/6		C3H/He	
	IgG isotype	Cytokine	IgG isotype	Cytokine	IgG isotype	Cytokine	IgG isotype	Cytokine
$Env_{gp120(IIIB)}$	IgG_1	IL-4	IgG_1	IL-4	IgG_1	IL-4	IgG_1	N.D.
$Env_{gp120(IIIB)}$-$C3d_2$	IgG_1/IgG_{2a}	IL-4/IFN-γ	IgG_1	IL-4	IgG_1	IL-4	IgG_1	N.D.
$Env_{gp120(IIIB)}$-$C3d_3$	IgG_1/IgG_{2a}	IL-4/IFN-γ	IgG_1	IL-4	IgG_1	IL-4	IgG_1	N.D.

†Mice were unvaccinated or vaccinated with DNA expressing the indicated vaccine

Table 7. Affinity Maturation of Anti-Env Antibody

Vaccine†	CD1-1 Swiss	BALB/c	C57BL/6	C3H/He
$Env_{gp120(IIIB)}$-$C3d_2$	2.50*	0.75	NT**	0.50
$Env_{gp120(IIIB)}$-$C3d_3$	3.50	2.25	0.80	1.25

† Assays used polled serum samples from each mouse group at a dilution with similar O.D. Data are representative of two independent experiments.

* Activity of the anti-Env IgG raised by the Env DNA vaccines (isolate IIIB). Sera were analyzed from week 10 collection in an Env-specific NaSCN-displacement ELISA. Plates were coated with recombinant $Env_{gp120(IIIB)}$.

** NT: Not tested.

Table 8. Splenocyte Proliferation

Vaccine†	CD-1 Swiss		BALB/c		C57BL/6	
	Unstim	Stim*	Unstim	Stim	Unstim	Stim
Naive	27.0	29.5	34.1	38.4	33.2	33.2
$Env_{gp120(IIIB)}$	28.2	45.2	35.6	50.2	33.1	50.6
$Env_{gp120(IIIB)}$-$C3d_2$	30.8	58.1	39.9	62.9	36.3	59.4
$Env_{gp120(IIIB)}$-$C3d_3$	35.9	72.6	45.7	78.0	40.2	68.9

† Mice were unvaccinated to vaccinated with DNA expressing the indicated vaccine.

* Splenocytes were stimulated with 4 μg/ml of ecombinant $Env_{gp120(IIIB)}$ for 5 days

IV.C.4. Splenocyte proliferation

All strains of mice vaccinated with DNA expressing $Env_{gp120(IIIB)}$-C3d had significantly more anti-Env splenocytes than mice vaccinated with DNA expressing $Env_{gp120(IIIB)}$ (**Table 8**). After three inoculations (week 10), mice vaccinated with DNA expressing $Env_{gp120(IIIB)}$-$C3d_3$ had ~40% reduction of fluorescent intensity after 5 days for in vitro recombinant $Env_{gp120(IIIB)}$ stimulation indicating a high level of anti-Env specific splenocyte proliferation (**Table 8**). In contrast, splenocytes from mice vaccinated with $Env_{gp120(IIIB)}$-DNA had significantly fewer cells proliferate in response to Env stimulation. Unstimulated splenocytes or splenocytes stimulated with influenza HA had little to no proliferation.

Supernatants were collected from each set of Env-stimulated splenocytes and were examined for the secretion of IL-4 (an indicator of a Th2 response) and INF-γ (an indicator of a Th1 response). Splenocytes from inbred mice vaccinated with $Env_{gp120(IIIB)}$ conjugated to two or three copies of C3d elicited higher levels IL-4 compared to inbred mice vaccinated with $Env_{gp120(IIIB)}$-DNA (**Table 6**). Little, if any, INF-γ was detected in the supernatant from Env-stimulated splenocytes collected from inbred mice vaccinated with any of the DNA vaccines. In contrast, splenocytes from outbred mice, vaccinated with $Env_{gp120(IIIB)}$-C3d-DNA, secreted both IL-4 and INF-γ (**Table 6**). However, the splenocytes from outbred mice vaccinated with $Env_{gp120(IIIB)}$-DNA secreted predominately IL-4. Similar to the immunoglobulin subclass, inbred mice vaccinated with these $Env_{gp120(IIIB)}$-DNA vaccines elicited primarily a Th2 response and that was enhanced by conjugating C3d to $Env_{gp120(IIIB)}$. In contrast, outbred mice vaccinated with $Env_{gp120(IIIB)}$-C3d-DNA elicited a mixed T helper response.

IV.D. Discussion

The use of DNA vaccines has the potential to revolutionize commercial vaccinology. However in several cases, DNA vaccines have not been as effective as live-attenuated vaccines (283-285). In order to improve the efficacy of DNA vaccination, previous studies from our laboratory have shown that the complement protein, C3d, when conjugated to an immunogen, can elicit high titer antibodies (84, 85, 127, 132, 138). Mice (BALB/c) vaccinated with DNA expressing $Env_{gp120(IIIB)}$ coupled to multiple copies of murine or human C3d elicit specific antibody titers 1–2 logs higher than mice vaccinated

with $Env_{gp120(IIIB)}$-DNA (124, 132). Recently, Suradhat et al. (156) reported that two copies of murine C3d fused to bovine rotavirus (BRV) VP7 or bovine herpesvirus type 1 (BHV-1) glycoprotein D (gD) did not enhance antibody titers to either antigen in C57BL/6 mice. Previously, our laboratory and others have used BALB/c mice to test the ability of C3d, conjugated to an antigen, to enhance of immune responses (84, 85, 128, 135). Therefore, we tested whether mice with different H-2 haplotypes affected the ability of C3d to enhance immune responses.

In this study, one outbred and three inbred strains of mice were vaccinated with DNA plasmids expressing the sgp120 protein of HIV-1 fused to murine C3d. All four mouse strains that were inoculated with DNA plasmids expressing $Env_{gp120(IIIB)}$-$C3d_2$ or $Env_{gp120(IIIB)}$-$C3d_3$ elicited similar high titer anti-Env antibodies (**Figure 9**). After three inoculations (week 10), the anti-Env titers ranged between 1:800 and 1:6400. Mice vaccinated with DNA plasmids expressing $Env_{gp120(IIIB)}$ had significantly lower anti-Env titers (<1:100 to 1:400).

Even though the titer of anti-Env antibodies elicited by $Env_{gp120(IIIB)}$-C3d-DNA in each of the four mouse strains tested was similar, IgG_1 was the primary antibody isotype elicited by DNA expressing C3d conjugated vaccines in the inbred mice (**Table 6**). IgM was detected after the first vaccination, however, by week 6, after the second inoculation, the immunoglobulin class switched to almost exclusively IgG_1 (data not shown). A similar immunoglobulin class switch was observed using vaccines expressing C3d conjugated to PPS14 from serotype 14 Streptococcus pneumoniae (128). In contrast to inbred mice,

outbred CD-1 mice vaccinated with $Env_{gp120(IIIB)}$-C3d-DNA elicited similar levels of IgG_1 and IgG_{2a} antibodies, indicating a mixed Th1/Th2 response (**Table 6**).

In most natural populations of animals, high levels of genetic variation are the norm. Outbred populations have advantages over inbred organisms due to the heterozygosity of immune alleles. The genetic variation of inbred mouse strains is caused by many homozygous recessive mutations that may be detrimental to the organism, but do little harm when in an organism with heterozygous alleles. Therefore, mice with homozygous recessive alleles can have a detrimental effect on the immune response to a foreign antigen. Outbred mice vaccinated with DNA expressing $Env_{gp120(IIIB)}$ conjugated to multiple copies of C3d elicited higher avidity and different IgG isotypes compared to inbred mice. Previous reports have demonstrated that differences in antibody response induced by DNA vaccination depends on the H-2 haplotype of the mouse strain (305). In addition, antibody responses to vaccination are differentially regulated in aged mice from different genetic backgrounds (306). Also, outbred mice vaccinated with $Env_{gp120(IIIB)}$-C3d-DNA had splenocytes that proliferated more robustly and secreted both IL-4 and INF-γ in response to Env stimulation. Splenocytes from vaccinated inbred mice secreted primarily IL-4 and had a reduced proliferative response to in vitro Env stimulation. These results were consistent with higher levels of IL-4 observed in mice vaccinated with sHA-C3d and *S. pneumoniae* PPS14-C3d (127, 128).

The C3d receptor, CD21 or CR2, is located primarily on B-cells and follicular dendritic cells, however, reports have indicated that this receptor can be expressed on thymocytes,

subsets of CD4+ and CD8+ peripheral T cells, basophils, mast cells, keratinocytes, and epithelial cells (149). CR2 acts as a co-receptor for antigen receptor mediated signal transduction. On the surface of B-cells, binding of CR2 covalently links complexes of antigen with C3d ligand and results in a variety of enhanced immune outcomes, including the release of intracellular calcium and the proliferation and/or activation of mitogen-activated protein kinases (120, 126, 307-309). Direct stimulation of B-cells by C3d fused antigens may lead to antigen-specific cellular proliferation and thereby directly leads to the enhancement of antibody production (120, 126, 307-309). In this study, DNA expressing $Env_{gp120(IIIB)}$-$C3d_3$ is likely to have supported the height of antibody responses by stimulating antibody production by B cells and expanding the pool of anti-Env specific B-cells (309). In addition, C3d conjugated antigen could lower both the concentration threshold and the affinity threshold for B-cell activation (120, 122) or C3d could have reduced B-cell apoptosis (121, 123).

One of the more interesting results from this study was the enhancement of avidity maturation of anti-Env specific antibody by C3d in outbred mice compared to inbred mice (**Table 7**). Immunization with DNA expressing $Env_{gp120(IIIB)}$ coupled to multiple copies of murine C3d resulted in enhanced avidity maturation of anti-Env antibody. Previous studies have described the lengthy evolution of antibody responses to HIV infection (292, 310). Antibodies gradually mature over a period of months from low avidity to a higher avidity levels (292, 310). Avidity maturation occurs in germinal centers where the somatic hypermutation of immunoglobulin results in a large repertoire of Ag-specific B cells that undergo selection for high affinity B cell receptors. CR2-null

mice have impaired humoral immunity, including decreased affinity/avidity maturation and germinal center development (304). C3d may enhance the avidity of the anti-Env response by binding to CR2 on follicular dendritic cells (FDCs) and thereby aiding the entry of B cells producing anti-Env antibody into germinal centers. Prior studies on the avidity of DNA-raised, anti-Env antibodies have also revealed low avidity anti-Env antibodies (292). The heavily glycosylated Env_{gp120} protein allows for the protein to remain "non-immunogenic" in response to immune pressure during chronic HIV infection (311, 312). The formation of germinal centers, which are critical for antibody maturation, could be impeded by the glycosylation of Env (312). C3d may help Env overcome this limitation by directing $Env_{gp120(IIIB)}$-C3d immunogens to FDC and enhance germinal center formation leading to enhanced avidity maturation. Outbred mice may have a genetic background that allows for C3d to enhance the maturation of antibodies elicited to C3d conjugated antigens compared to inbred mice. C3d may also assist in trafficking fused antigens to sites in the spleen where germinal centers form by binding to CD21 on FDC. Additional studies will be required to address the mechanisms of C3d enhancement and determine if C3d is advancing germinal center formation in these vaccinated mice.

Recently, our laboratory and others have demonstrated that C3d can enhance antibody responses directed towards a specific antigen by a DNA vaccine (84, 85, 126-128, 132, 135, 138, 156). DNA vaccines expressing secreted HA molecules from either influenza or measles virus or the HIV-1 envelope fused murine $C3d_3$ achieved an earlier and more efficient immune response compared to non-C3d fused antigens (84, 127). In this report,

we demonstrated that the outbred mouse strain, CD-1 Swiss, vaccinated with DNA expressing C3d conjugated to sgp120 elicited similar titers of anti-Env antibodies as vaccinated inbred strains. However, a mixed T helper immune response was elicited in the outbred mice vaccinated with DNA expressing C3d conjugated to $Env_{gp120(IIIB)}$ and a Th2 biased response was elicited in all three inbred strains. In addition, the avidity of the elicited antibody in the outbred strain was enhanced compared to inbred strains. These results may have implications for the use C3d conjugated vaccines in outbred primate populations.

IV.E. Acknowledgements

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V. Chapter 5: Specific Aim II

C3d functions as a molecular adjuvant in the absence of CD21/35 expression

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Cutting Edge: C3d functions as a molecular adjuvant in the absence of CD21/35 expression

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V.A. Introduction

Complement is a key component of the innate immune system that can also influence humoral immune responses. Upon activation, C3 cleavage products form covalent bonds with foreign antigens, thereby generating ligands such as C3dg and C3d (a proteolytic fragment of C3dg) that engage CD21/CD35 complement receptors expressed by mature B-cells and follicular dendritic cells (FDCs). Deficiencies in either C3 or the common gene that generates leukocyte complement receptors 1 (CD35) and 2 (CD21) result in impaired antibody responses in mice (130, 313-318). Moreover, covalently linking C3d fragments to antigens results in a potent adjuvant effect. In the first demonstration of this, fusing multiple copies of C3d to hen egg lysozyme (HEL) lowered the dose of antigen required for antibody responses by at least 1000-fold in transgenic mice expressing B-cell antigen receptors (BCR) specific for HEL (126). Likewise, immunization of wild-type mice with DNA-based vaccines encoding HIV-1 Env_{gp120} fused to multiple copies of C3d results in higher antibody responses with enhanced avidity maturation when compared with Env_{gp120} immunization alone (85, 132). Mice immunized with either influenza or measles virus hemagglutinin fused to multiple copies of C3d also generate more rapid antibody responses and higher neutralizing titers (as high as 8-fold greater) than mice immunized with either antigen alone (84, 127, 138). Finally, antibody titers and isotype switching in response to pneumococcal capsular polysaccharide type 14 are enhanced when pneumococcal capsular polysaccharide type 14 is conjugated to C3d (128). C3d is

therefore an effective molecular adjuvant that appears safe and acceptable for use in vaccines.

C3d is proposed to function as a molecular adjuvant by efficiently targeting antigens to CR2 (CD21), which interacts with CD19 to regulate transmembrane signals during B cell activation (48, 319). Because a direct role for CR2 in this process has never been demonstrated, the importance of CR2 receptor engagement in mediating the immunostimulatory effects of C3d was assessed in mice completely deficient in CR2 ($CR2^{-/-}$) expression (130, 320). Notably, B cells from $CR2^{-/-}$ mice do not bind streptavidin (SA)-C3dg tetramers, which are formed by the attachment of four mono-biotinylated C3dg molecules to SA (129, 130). However, C3dg tetramers effectively reveal CR2 ligand binding in wild-type mice and exhibit functional activity on normal, but not $CR2^{-/-}$ B-cells, including augmentation of anti-IgM-mediated intracellular Ca^{2+} flux and activation of p38 mitogen-activated protein kinase (129, 130). To investigate the role that C3d-CR2 interactions play in humoral responses to C3d-antigen conjugates, $CR2^{-/-}$ mice were immunized with SA and recombinant HIV-1 envelope glycoprotein gp120 (isolate IIIB) ($Env_{gp120(IIIB)}$), either alone or complexed to C3d. Unexpectedly, antibody responses to SA and $Env_{gp120(IIIB)}$ were significantly augmented in $CR2^{-/-}$ mice when these proteins were complexed with C3d in comparison to antigen alone. Thus, C3d can function as a molecular adjuvant through CR2 receptor-independent pathways.

V.B. Materials and Methods

V.B.1. SA-C3dg and SA-Chicken γ-Globulin (CGG) Formation

Biotinylated C3dg was produced and purified as described (129). Purified C3dg was treated with polymyxin B-agarose (Sigma-Aldrich, St. Louis, MO). Endotoxin contamination was determined to be <0.028 endotoxin U/μg of C3dg (Pyrogent Plus; BioWhittaker, Walkersville, MD; Lineberger Comprehensive Cancer Center Cell Culture Facility, Chapel Hill, NC). To form SA-C3dg tetramers and SA-CGG complexes for injections, 40 μg of biotinylated C3dg or CGG (Sigma-Aldrich) was incubated with 10 μg of SA (Sigma-Aldrich) in 200 μl of PBS for 45 min at room temperature.

V.B.2. $Env_{gp120(IIIB)}$ DNA Constructs, Protein Expression, and Purification

DNA plasmids encoding soluble $Env_{gp120(IIIB)}$ and $Env_{gp120(IIIB)}$ fused to three copies of murine C3d ($Env_{gp120(IIIB)}$-$C3d_3$) were expressed in HEK 293T cells as described (85). Recombinant Env_{gp120} proteins were purified using a HiTrap chelating nickel column using the N-terminal (6X)-Histidine tag (Amersham Biosciences, Piscataway, NJ). DNA vaccine plasmids encoding soluble $Env_{gp120(IIIB)}$ alone or fused to two or three copies of murine C3d ($Env_{gp120(IIIB)}$-$C3d_{2–3}$) for use in DNA immunizations were as described (85).

V.B.3. Mice

$CR2^{-/-}$ ($CD21/35^{-/-}$) mice were as described (130). Eight- to 10-week-old $CR2^{-/-}$ and wild-type littermates on a mixed B6/129 background were used in SA immunizations.

C57BL/6 wild-type mice and $CR2^{-/-}$ mice backcrossed six to seven times onto the C57BL/6J background were used in $Env_{gp120(IIIB)}$ protein and DNA immunizations. Mice were housed under specific pathogen-free conditions. All procedures conformed to Duke University Animal Care, Use Committee guidelines and USDA.

V.B.4. Immunizations

SA (10 μg) was administered alone or complexed with either biotinylated C3dg (129) or biotinylated CGG intravenously (IV) in 200 μl of PBS. For Env_{gp120} protein immunizations, recombinant Env_{gp120} (50 μg) was administered alone or fused with murine $C3d_3$ IV in 200 μl of PBS. DNA immunizations were performed on shaved abdominal skin using the hand-held Bio-Rad (Hercules, CA) Gene Delivery System as described (85). Mice received two immunizations at each time point, each containing 1 μg of DNA plasmid encoding soluble $Env_{gp120(IIIB)}$, $Env_{gp120(IIIB)}$-$C3d_2$, or $Env_{gp120(IIIB)}$-$C3d_3$ per 0.5 mg of 1-μm gold beads (Bio-Rad) at a helium pressure setting of 400 psi.

V.B.5. ELISAs

SA or $Env_{gp120(IIIB)}$-specific antibodies were quantified by coating 96-well plates with SA (5 μg/ml; 100 μl/well) or recombinant $Env_{gp120(IIIB)}$ (0.3 μg/ml; 100 μl/well) in 0.1 M borate buffered saline overnight at 4°C. Plates were washed in TBS and blocked with TBS containing 1% gelatin/2% BSA for 90 min at 37°C. Sera were diluted 1/250 in TBS containing 1% BSA and incubated in duplicate wells at room temperature for 90 min. Plates were washed using TBST and incubated with alkaline phosphatase-conjugated

polyclonal goat anti-mouse IgG, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, or IgM antibodies (Southern Biotechnology Associates, Birmingham, AL) for 1 h at room temperature. Plates were developed using p-nitrophenyl phosphate substrate (Southern Biotechnology Associates) with O.D. 405 values determined.

End-point titers of anti-SA IgG antibodies were determined using 3-fold serial dilutions of serum samples. End-point titers were determined as the reciprocal dilution of sera yielding an O.D. 405 value that was 3-fold higher than background O.D. values where sera was omitted. End-point anti- Env_{gp120} IgG titers were assessed using recombinant Env_{gp120} as described (85, 132). Briefly, plates were coated with $Env_{gp120(IIIB)}$ (0.3 μg/ml) overnight at 4°C, blocked with 5% non-fat dry milk in PBS containing 0.05% Tween 20 for 1 h at 25°C, and washed with PBS containing 0.05% Tween 20. Plates were incubated with 2-fold serial dilutions of sera for 1 h, washed, and incubated with biotinylated anti-mouse IgG antibodies followed by SA-conjugated HRP (Southern Biotechnology Associates). Plates were developed using tetramethylbenzidine substrate (Sigma-Aldrich). End-point titers were determined as the reciprocal dilution of sera yielding an O.D. value that was 2-fold higher than O.D. values measured for serum samples from control mice immunized with vector alone.

V.B.6. ELISPOT Assays

Immobilon-P Multiscreen 96-well plates (Millipore, Bedford, MA) were precoated with SA (5 μg/ml). Bone marrow and spleen cells were plated at 10^4, 10^5, or 10^6 cells per well in 100 μl of culture medium (RPMI 1640 containing 10% FCS, 10 mM glutamine, 100

U/ml penicillin/streptomycin, and 55 μM 2-ME) for 18 h at 37°C in a CO2 incubator. The plates were washed three times with TBST, incubated with polyclonal alkaline phosphatase-conjugated goat anti-mouse IgG antibodies for 2 h at room temperature, washed, and developed for 30 min using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (Sigma-Aldrich).

V.B.7. Statistical Analysis

Data are shown as means ± SEM. Student's *t* test was used to identify significant differences between sample means.

V.C. Results

V.C.1. C3d Augments Primary and Secondary Antibody Responses to SA in $CR2^{-/-}$ Mice

$CR2^{-/-}$ and wild-type littermates were immunized with 10 μg of SA protein, either alone or complexed with biotinylated C3dg. SA immunization resulted in modest IgM and IgG responses in both $CR2^{-/-}$ and wild-type littermates (**Figure 10**A). By comparison, SA-C3dg induced significant IgM and IgG anti-SA antibody responses in both $CR2^{-/-}$ and wild-type littermates (Fig. 1A), without inducing anti-C3dg antibody responses (data not shown). Seven days after SA-C3dg immunization, SA-specific IgM responses were significantly higher in both $CR2^{-/-}$ and wild-type mice ($p < 0.05$). IgG responses were also significantly higher in both $CR2^{-/-}$ and wild-type littermates at days 14–28 following SA-C3dg immunization relative to SA-immunized mice ($p < 0.05$). An analysis of serum

antibody titers generated similar conclusions: SA-specific IgG responses were ≥50-fold higher in $CR2^{-/-}$ and wild-type littermates receiving SA-C3dg compared with SA alone (**Figure 10**A). Although $CR2^{-/-}$ mice responded well to SA-C3dg immunization, they had lower mean SA-specific IgM and IgG titers than wild-type littermates (**Figure 10**A). Thus, CD21/35 deficiency impairs humoral immune responses to soluble protein Ags as described (130, 313, 314). Despite this, C3dg functioned as a molecular adjuvant in the absence of $CR2^{-/-}$ expression.

Whether SA-C3dg immunization augmented secondary anti-SA antibody responses was assessed in $CR2^{-/-}$ and wild-type littermates that had been immunized with SA alone or SA-C3dg on day 0. Mice immunized and boosted with SA on days 0 and 120, respectively, did not generate significant IgM or IgG responses (**Figure 10**B). By contrast, both $CR2^{-/-}$ and wild-type littermates first immunized with SA-C3dg generated significantly higher secondary IgM and IgG responses following the SA boost ($p < 0.05$) than mice first immunized with SA alone. IgM antibody responses following SA boosting were lower ($p < 0.05$) in $CR2^{-/-}$ mice compared with their wild-type littermates, although IgG responses were similar. In fact, $CR2^{-/-}$ and wild-type littermates immunized with SA-C3dg at day 0 had IgG titers on day 128 that were >2,500-fold higher than those of mice immunized and boosted with SA alone (**Figure 10**). Likewise, the frequencies of SA-specific IgG-secreting cells were 10-fold higher in spleens of $CR2^{-/-}$ and wild-type littermates immunized with SA-C3dg compared with mice receiving SA alone (**Figure 10**C). SA-specific IgG-secreting cell frequencies were also 3- to 6-fold higher in the bone marrow of SA-C3dg-immunized $CR2^{-/-}$ and wild-type littermates, respectively. In

summary, administration of C3dg-antigen complexes during primary immunization elicited long-lasting antigen-specific IgG production during the primary response and significantly enhanced the secondary antibody response to antigen alone. However, C3d enhancement of the antibody response to SA occurred through a pathway that was largely independent of CR2 expression.

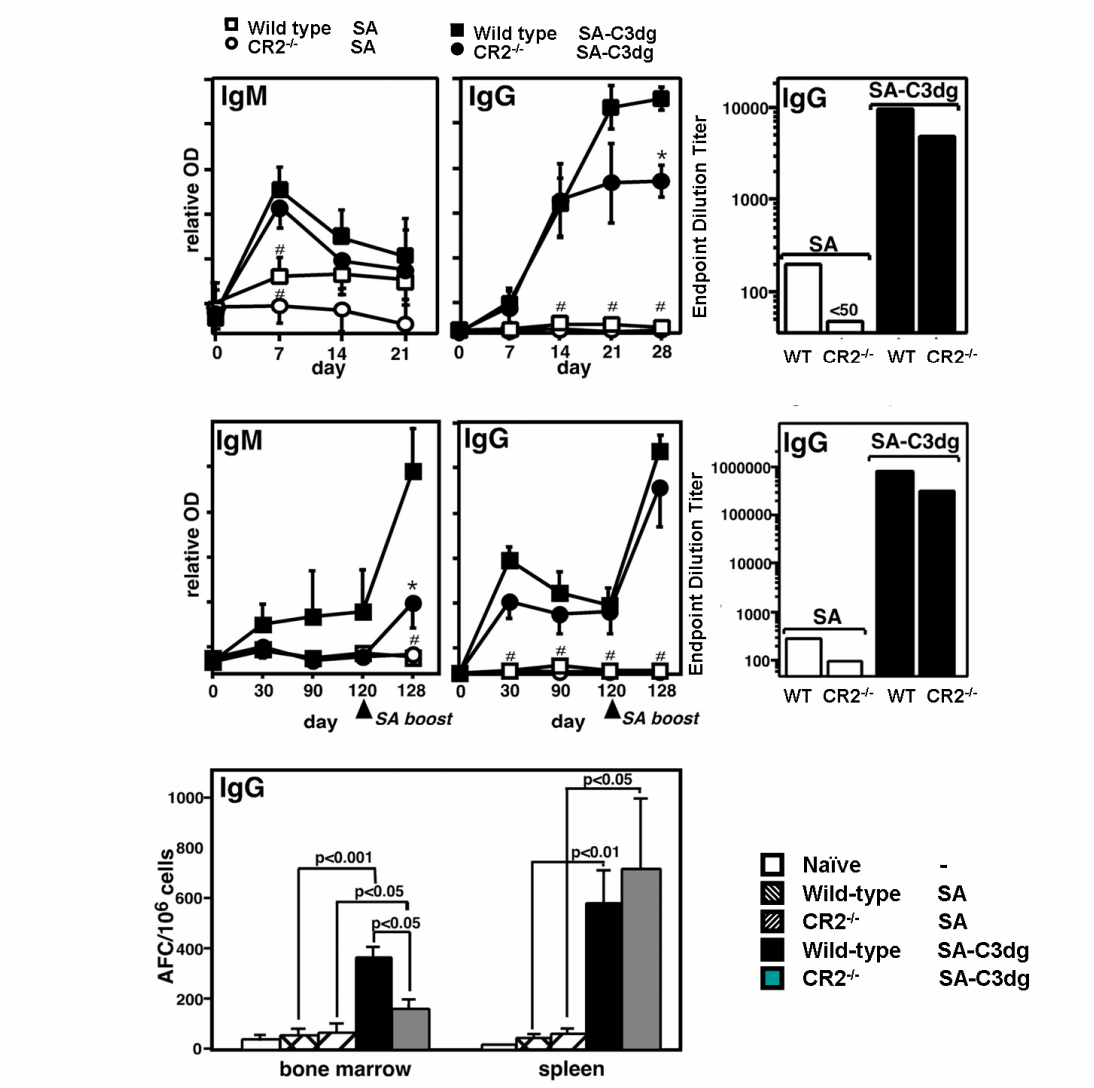


Figure 10. C3d Enhances Humoral Responses in CD21/35$^{-/-}$ and Wild-Type Mice.

A) CR2$^{-/-}$ and wild-type (WT) littermates were immunized with SA protein (10 µg) either alone or complexed with biotinylated C3dg (SA-C3dg) on day 0, with serum SA-specific IgM and IgG antibody levels quantified by ELISA. In the right panel, SA-specific IgG titers are shown for pooled sera harvested 21 days postimmunization.
B) Secondary SA-specific antibody responses by CR2$^{-/-}$ and wild-type littermates. Mice immunized with SA alone or SA-C3dg on day 0, were boosted at day 120 with 10 µg of SA alone. SA-specific IgG titers are shown for pooled serum samples harvested 7 days after the SA boost. A and B values represent the mean O.D. (±SEM) from four to five mice per group. *, Significant differences ($p < 0.05$) between CR2$^{-/-}$ and wild-type littermates immunized with SA-C3dg. #, Significant differences ($p < 0.05$) between mice of the same genotype immunized with SA-C3dg compared with SA alone.
C) Antibody-forming cell (AFC) frequencies in naive and immunized CR2$^{-/-}$ and wild-type littermates 7 days after a SA boost on day 120.

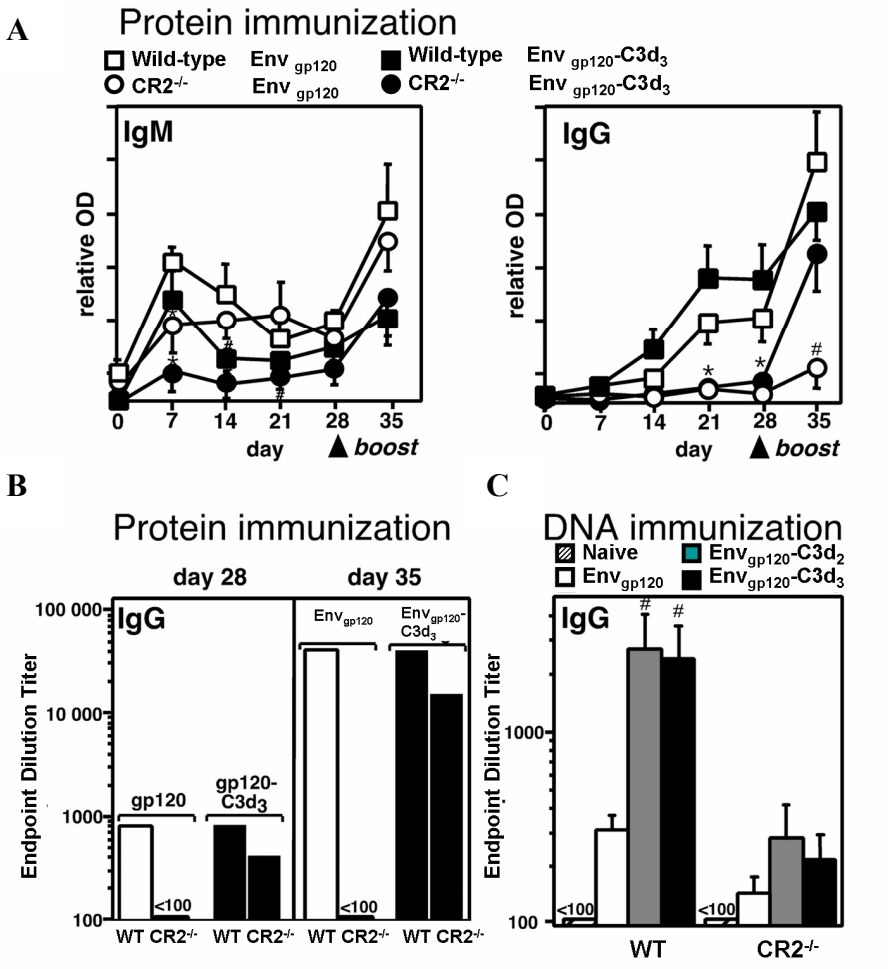


Figure 11. HIV $Env_{gp120(IIIB)}$-specific Antibody Responses of $CR2^{-/-}$ and Wild-Type (WT) Mice Following Immunization with $Env_{gp120(IIIB)}$ Alone or $Env_{gp120(IIIB)}$-$C3d_{2–3}$.

A) Mice were immunized on days 0 and 28 with $Env_{gp120(IIIB)}$ protein (50 μg) either alone or with three attached copies of C3d. Serum $Env_{gp120(IIIB)}$-specific IgM and IgG antibodies were quantified by ELISA. Values represent the mean O.D. (±SEM) from four to five mice per group.
B) IgG titers of serum from the $CR2^{-/-}$ and wild-type mice shown in A.
C) HIV $Env_{gp120(IIIB)}$-specific antibody responses of $CR2^{-/-}$ and wild-type mice following immunization with DNA encoding $Env_{gp120(IIIB)}$ alone, $Env_{gp120(IIIB)}$-$C3d_2$, or $Env_{gp120(IIIB)}$-$C3d_3$. Mice were immunized with plasmid DNA at week 0, 4, and 8. Values represent mean IgG titers (±SEM) obtained for four to five mice per group at week 10. Titers determined to be at or below a dilution of 1/100 are indicated as <100. *, Significant differences ($p < 0.05$) between means of CD21/35–/– and wild-type mice immunized with gp120-C3d3. #, Significant differences ($p < 0.05$) between means for mice of the same genotype immunized with gp120-C3d2–3 compared with gp120 alone.

V.C.2. C3d Augments Humoral Responses to $Env_{gp120(IIIB)}$ in $CR2^{-/-}$ Mice

$CR2^{-/-}$ and wild-type mice were immunized with 50 μg of $Env_{gp120(IIIB)}$ protein, either alone or fused with three copies of murine C3d in tandem at the C terminus (85, 132). Wild-type mice immunized with either $Env_{gp120(IIIB)}$-$C3d_3$ or $Env_{gp120(IIIB)}$ generated similar primary and secondary IgM and IgG responses (**Figure 11** A and B). Because the effectiveness of C3d as a molecular adjuvant is dependent on the nature of the antigen itself, the dose of antigen, and the route of immunization (84, 127), it was not surprising that $Env_{gp120(IIIB)}$-specific responses were similar in wild-type mice immunized with $Env_{gp120(IIIB)}$-$C3d_3$ and $Env_{gp120(IIIB)}$ proteins. By contrast, $Env_{gp120(IIIB)}$ immunization generated low primary IgM and IgG responses in $CR2^{-/-}$ mice, although $Env_{gp120(IIIB)}$-$C3d_3$ immunization generated elevated titers of gp120-specific IgG in $CR2^{-/-}$ mice by day 28 (**Figure 11** B). However, secondary $Env_{gp120(IIIB)}$-specific IgG responses were near wild-type levels in $CR2^{-/-}$ mice immunized with $Env_{gp120(IIIB)}$-C3d3 (**Figure 11** A). In fact, secondary $Env_{gp120(IIIB)}$-specific IgG end-point titers were at least 150-fold higher in $CR2^{-/-}$ mice immunized with $Env_{gp120(IIIB)}$-$C3d_3$ compared with $Env_{gp120(IIIB)}$ alone (**Figure 11** B). Anti-C3d antibody responses were not detected in $Env_{gp120(IIIB)}$-$C3d_3$-immunized mice (data not shown). Immunization of mice with DNA-based vaccines encoding $Env_{gp120(IIIB)}$-$C3d_2$ or $Env_{gp120(IIIB)}$-$C3d_3$ also resulted in increased $Env_{gp120(IIIB)}$-specific antibody responses compared with immunization with $Env_{gp120(IIIB)}$ alone in both wild-type (by ~8-fold; $p < 0.05$) and $CR2^{-/-}$ (by ~1.5- to 2-fold; NS) mice (**Figure 11** C). However, $Env_{gp120(IIIB)}$-specific responses were much weaker than those obtained with direct protein immunizations (**Figure 11** B vs C). Nonetheless, C3d enhanced

$Env_{gp120(IIIB)}$-specific IgG responses in $CR2^{-/-}$ mice despite their impaired primary and secondary antibody responses to $Env_{gp120(IIIB)}$.

V.C.3. CGG Augments Humoral Responses to SA in $CR2^{-/-}$ Mice

To compare the effect of C3dg on the immune response to SA to that resulting from complexing SA to a well-characterized immunogenic carrier protein, $CR2^{-/-}$ and wild-type mice were immunized with SA-CGG complexes. SA-C3d tetramers significantly augmented SA-specific IgM and IgG antibody responses in both wild-type and $CR2^{-/-}$ mice to levels similar to those elicited by SA-CGG complexes (**Figure 12**), although differences in anti-SA-specific antibody isotypes were observed. Thus, C3dg and CGG similarly augment the overall magnitude of antibody responses in both the presence and absence of CR2 expression.

V.D. Discussion

This study confirms that C3d can function as a molecular adjuvant during humoral immune responses to Ags administered either directly as proteins (SA and Env_{gp120}) or as DNA vaccines (Env_{gp120}). Unexpectedly, C3d could also function as an effective adjuvant in the absence of CR2 expression. Antibody responses to SA and Env_{gp120} were significantly impaired in $CR2^{-/-}$ mice, confirming the importance of CR2 expression in antibody responses to antigens administered in the absence of adjuvants (130, 313, 314). However, IgG antibody responses to SA-C3dg and $Env_{gp120(IIIB)}$-C3d were significantly augmented in $CR2^{-/-}$ mice in comparison to these antigens given without C3d. These

effects were also reflected in the enhanced frequencies of SA-specific antibody-producing cells in $CR2^{-/-}$ mice immunized with SA-C3dg tetramers compared with mice immunized with SA alone (Fig. 1C). Thus, C3d can function as an adjuvant through pathways that are independent of CR2 receptor expression.

C3d can function as a natural adjuvant for a number of physiologically important immunogens, including HIV Env_{gp120}, viral hemagglutinin, and pneumococcal polysaccharide (84, 85, 127, 128, 132, 138). In all cases, C3d has been postulated to augment humoral responses by targeting antigen complexes to B cells and follicular dendritic cells that express $CR2^{-/-}$. On B cells, coligation of the BCR and the CD19/CR2 (CD21) complex by C3d-antigen complexes is proposed to lower the signaling threshold required for B cell activation and expansion (120, 321, 322). Although Dempsey et al. (126) originally proposed that the adjuvant effect of C3d bound to antigen was mediated through coligation of the CD19/CR2 complex with a HEL-specific BCR, the only direct evidence supporting this conclusion was that pretreatment of mice with an antibody against CR2 suppressed the effect elicited by C3d. However, although anti-CR2 mAb treatment is known to inhibit humoral immune responses to a variety of Ags (323-325), anti-CR2 monoclonal antibody treatment may have effects on B-cell function beyond blocking C3d binding (326). In the current study, the importance of CR2 expression in mediating C3d effects was examined using $CR2^{-/-}$ mice (130), whereas the study by Dempsey et al. (126) used transgenic mice where all B-cells expressed high-affinity antigen-specific BCRs. Thus, differences in conclusions between our current study and those of Dempsey et al. may be explained by differences in experimental approach.

Moreover, our data do not discount the model first proposed by van Noesel et al. (327) and evoked by Dempsey et al. (126) for CR2 function, because CR2 expression is important for optimal humoral immune responses, and immunization with C3d-conjugated antigens did not always restore immune responses of $CR2^{-/-}$ mice to wild-type levels (**Figure 10** and **Figure 11**). Thus, there may be CR2-dependent pathways through which C3d functions, in addition to the CR2-independent pathways revealed in the current study.

Although the precise mechanisms through which C3d functions as a molecular adjuvant remain to be elucidated, several hypotheses can be offered. First, C3d interacts with numerous serum proteins, cell surface receptors, and membrane-associated regulatory proteins (328-332). Thus, C3d aggregates may bind antigen complexes to proteins that are distinct from CR2 to enhance humoral responses. Alternatively, attachment of C3d to antigens could prolong the *in vivo* half-life of antigen, perhaps by forming molecular aggregates or facilitating molecular interactions. Finally, C3d could function as a simple protein carrier. In support of this, OVA functions as an adjuvant for pneumococcal polysaccharide in a manner similar to C3d (128). Similarly, SA-C3d tetramers or SA-CGG complexes significantly augmented anti-SA antibody responses in both wild-type and $CR2^{-/-}$ mice to similar levels (**Figure 12**). However, C3d was not immunogenic and did not elicit anti-C3d antibody production, unlike CGG. Thus, although both C3dg and CGG were effective adjuvants in $CR2^{-/-}$ and wild-type mice, they may function through distinct pathways. Given the unexpected finding that C3d augments humoral immune responses through CR2-independent pathways, understanding the mechanisms of C3d

action may provide important insight into the identity of other molecules with adjuvant activity that will allow the design of even more potent vaccines.

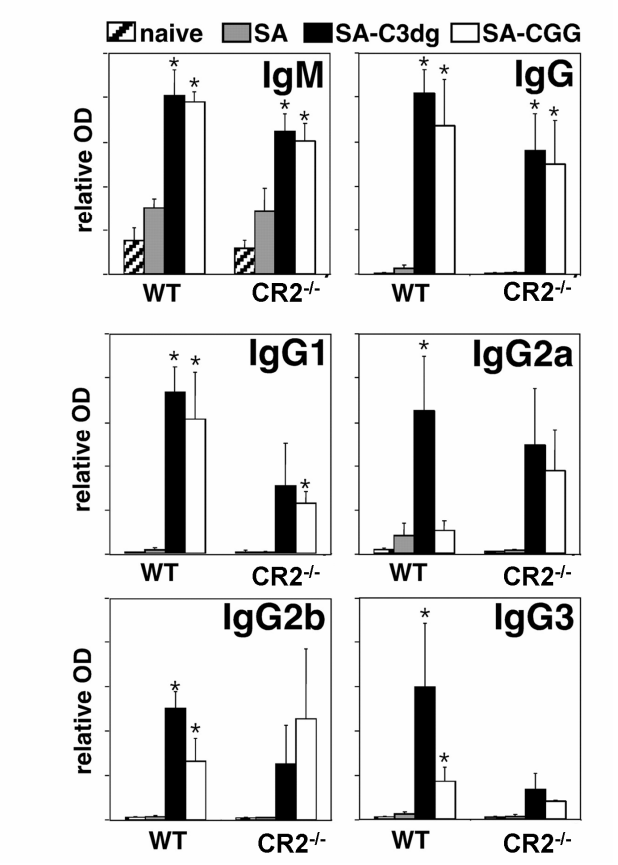


Figure 12. C3d and CGG Enhance Humoral Responses in CR2$^{-/-}$ and Wild-Type Mice.

CR2$^{-/-}$ and wild-type (WT) littermates were immunized with SA protein (10 μg) either alone or as SA-C3dg or SA-CGG, with serum SA-specific IgM (day 7) and IgG or IgG isotype (day 21) antibody levels quantified by ELISA. Values represent the mean O.D. (±SEM) from three to five mice per group. *, Significant differences ($p < 0.05$) between mice of the same genotype immunized with SA-C3dg or SA-CGG compared with SA alone.

VI. Chapter 6: Specific Aim III

Mild enhancement of secondary humoral immune responses by C3d reduces morbidity and mortality in CD4+ T-cell deficient mice

VI.A. Introduction

It is generally accepted that T cell-dependent (TD) humoral immune responses play an important role in the clearance of several virus infections. Furthermore, these immune responses are important for preventing disease upon secondary infection. In contrast, the role of T-cell independent (TI) immune responses has not been clearly explored. TD immune responses require the critical interaction of antigen-specific TRCαβ+ CD4+ T-cells [CD4+ helper T-cells (Th)] with activated B-cells (333). The "help" provided by CD4+ helper T-cell includes signaling through surface molecules (CD40 ligand and CD28, on the surface of the T-cell; CD40 and B7 on the surface of B-cells) and the production of multiple cytokines (*e.g.* IL-4, IFN-γ, TGF-β, TNF-α, IL-5, IL-13, etc) (334). The consequences of this "help" include antibody production, induction of class switch recombination (IgG, IgA and IgE) and differentiation into plasma B-cells. On the other hand, T-cell independent (TI) antigens can stimulate B-cells in the absence of CD4+ helper T-cells (335, 336). These antigens are large molecules with repetitive antigenic structures that bind and stimulate several surface immunoglobulin (Ig) M (sIgM) molecules, which redundantly trigger the B-cell activation. Consequently, TI antigens induce Ig production, class switch and secretion in the absence of CD4+ T-cell help. The antibodies produced by TI are not as efficient as those induced by TD antigens; however, their importance for prevention of primary viral infection and resolution has been proven in various animal models (337, 338). TI immune response can be optimized by T-cells and other immune cells, such as NK, NK-T cells or γ/δ T-cells, which produce

cytokines (*e.g.* IFN-γ, TGF-β, IL-5) in the presence of TI antigens (339, 340). The cytokines produced by the latter cells may be the dominant ones in TI immune response induction.

It has been reported that some viruses induce TI immune responses (*e.g.* polyomavirus, rotavirus, vesicular stomatitis virus) in animal models deficient in CD4+ T-cells. Virus infection of these mice has lead not only to secretion of IgM, but also to Ig class switch (IgG and IgA). Furthermore, in some models of disease these antibodies mediate protection from lethal virus challenge (337, 341, 342). The structure of these viral antigens shows high repetitiveness, which resembles that of classic bacterial TI antigens. However, it seems that the whole viral structure is needed to induce class switch recombination as protein (subunit) or virus-like particles immunizations using these viral antigens did not induce class switch (342). Recent studies demonstrated the importance of TI humoral immune responses in influenza virus infections since these immune responses helped in the resolution of primary infections and prevented reinfections (338). In the case of influenza, live as well as inactivated viral particles, are able to induce TI immune responses. Both were able to induce IgM secretion and Ig class switch recombination to IgG and IgA (338, 343). Furthermore, infection of CD4+ T-cell deficient mice with a sublethal dose of influenza virus (A/PR/8/34 –H1N1) induced the production of not only specific IgG antibodies, but also protected mice from a subsequent lethal challenge (344).

The final degradation product of the third component of complement, C3d, acts as an adjuvant to enhance immune responses to several viral antigens (*e.g.* hemagglutinin from influenza virus and envelope from HIV-1). Chimera proteins expressing viral antigens to three tandem copies of C3d have been generated and used in DNA or protein immunizations (84, 85, 127, 132, 133, 135, 138, 275). The receptor for C3d, complement receptor 2 (CR2/CD21), is present on the surface of follicular dendritic cells (FDC), B-cells and in some subsets of T-cells (108, 109, 115, 119, 322). C3d conjugated antigens stimulate B-cells by co-ligating sIgM and CR2 resulting in activation of two cross-talking signaling pathways that synergize cell activation (46, 48, 105, 119, 124, 308, 345). As a consequence, C3d reduces the amount of antigen needed to activate the B-cell and enhances the amount of antibodies secreted (90).

TI antigens, with their repetitive antigenic structure, stimulate several sIgM molecules and thus redundantly trigger B-cell activation. In contrast, antigens fused to C3d co-ligate the sIgM and several CR2 molecules, which activate signaling pathways that cross-talk and activate the B-cell. Therefore, in the present study, a soluble form of hemagglutinin from influenza virus was fused to three tandem copies of C3d (sHA-$C3d_3$). These proteins were evaluated in their ability to 1) mimic the repetitiveness of TI antigens and thus induce an Ig class switch, and 2) protect mice from a lethal virus challenge, in the absence of CD4+ T-cells. The results indicated that sHA-C3d3 induced IgG class switch only in 20% of the immunized animals. However, these weak secondary humoral immune responses, possibly in conjunction with other innate immune responses stimulated by C3d, reduced morbidity and mortality following lethal virus challenge.

VI.B. Materials and Methods

VI.B.1. Plasmids

A soluble form of hemagglutinin (sHA) (A/PR/8/34 –H1N1) with a 6X(His) tag at the 3' end was engineered by PRC amplification from a DNA copy of this gene. The PCR products were cloned in a transition vector, Topo 2.1 (Invitrogen Life Technologies, Carlsbad, CA, USA) following the recommendations of the manufacturer (Invitrogen Life Technologies, Carlsbad, CA, USA). Later, the gene *sHA-6X(His)* was cloned into the expression vector TR600 (84, 133), in frame with its tPA leader sequence, using the unique restriction sites NheI/BamHI, generating the plasmid psHA-6(X)-His (**Table 4**).

A second Topo 2.1 plasmid encoding *sHA-10X(His)* was generated as described above. This gene was later cloned in front of 3 copies of C3d and in frame with the tPA leader sequence of a TR600 vector that encodes for these elements. The unique restriction sites used were NheI/BamHI and this generated the plasmid psHA-10(X)-His-$C3d_3$ (**Table 4**).

DNA Plasmids were amplified in *Escherichia coli* DH5-α; purified by using endotoxin-free, anion-exchange resin columns (Qiagen, Valencia, CA, USA); resuspended in distilled water and stored at -20 C. Purity of DNA preparations were determined based on the optical density (O.D.) using 260 and 280 nm ultraviolet wavelength.

psHA-6(X)-His and psHA-10(X)-His-C3d$_3$ were assayed for expression by transfecting HEK 293T cells. Transfections were performed in six-well plates (~ 500,000 cells/well) using Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and Opti-MEM I reduced serum media (Gibco, Grand Island, NY, USA), following the recommendations of the manufacturers. Supernatants and cells lysates were collected 72 hours later and analyzed by westernblot.

Supernatants (1%) and cell lysates (2%) were diluted in SDS sample buffer and loaded onto SDS-polyacrylamide gels (5% stacking; 10% resolving) and run for 2 hours (100 Volts). The resolved proteins were transferred onto PVDF membranes (Millipore, Bedford, MA, USA) (0.2 mA, 2 hours) and incubated with rabbit anti-A/PR/8/34 sera 1:5000 (2 hours). Bound rabbit antibodies were detected using goat anti-rabbit IgG antisera conjugated to HRP (1:7000) (1hour) (Southern Biotechnology, Birmingham, AL, USA), followed by enhanced chemiluminiscence (Pierce Biotechnology, Rockford, IL, USA).

VI.B.2.Protein Purification

In order to purify proteins, transfections of HEK 293T cells were up-scaled to T-75 flasks. Briefly, HEK 293T-cells were grown in complete Dubelcco's Modified Media (cDMEM) [DMEM supplemented to contain 10% heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA, USA), 4 mM L-glutamine (Invitrogen Life Technologies, Carlsbad, CA, USA), and 0.4 mg/L gentamicine (Gibco, Grand Island, NY, USA)] until 90% confluency. psHA-6(X)-His and psHA-10(X)-His-C3d$_3$ were then

transfected using Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and Opti-MEM I reduced serum media (Gibco, Grand Island, NY, USA). In one reaction (Tube A), 500 ul of Opti-MEM I reduced serum media were mixed with 20 µl of Lipofectamine 2000 and incubated for 5 minutes at room temperature. In a second reaction (Tube B), 500 µl of Opti-MEM I reduced serum media were mixed with 8 µg of plasmid DNA. Tubes A and B were mixed (Master Mix) and incubated at room temperature for 25 minutes. Media was removed from HEK 293T cells and 5 ml of fresh, warmed Opti-MEM I reduced serum media were added, followed by the Master Mix. Cells were incubated for 6 hours at 37 C/ 5% CO2 and 5 ml extra of fresh, warmed Opti-MEM I reduced serum media were added. Flasks were incubated for 72 hours (37 C/ 5% CO2) and supernatants were harvested. A protease inhibitor cocktail for purification of His-tagged proteins was added (2.5 µl/ml) (Sigma, St. Louis, MO, USA). Supernatants were stored at -80 °C until protein purification.

Supernatants were pooled and proteins (sHA and sHA-C3d3) were purified at 4 °C using a 5 ml HiTrap chelating nickel column (Amersham Biosciences, Piscataway, NJ, USA). A peristaltic pump (Econo-Pump) (Bio-Rad, Hercules, CA, USA) at a flow rate of 5 ml/min was used for the procedure. The Storing Solution (20% ethanol in distilled water) of the HiTrap column was removed. The column was loaded with 5 ml of 0.1 M $NiSO_4$ (Fisher Scientific, Fair Lawn, NJ, USA) and subsequently washed with 15 ml of distilled water. The HiTrap column was equilibrated with 30 ml of Binding Buffer (20mM phosphate, 0.5 M NaCl and 10 mM imidazole (Sigma, St. Louis, MO, USA)). Supernatants containing His-tagged proteins were gently thawed, pooled and loaded in th

equilibrated HiTrap column at a rate of 5ml/min. The column was then washed with 30 ml of Binding Buffer. Subsequently the proteins were eluted using 15 ml of Elution Buffer (20 mM phosphate, 0.5 M NaCl and 500 mM imidazole). The eluted fraction, containing the purified protein, was concentrated immediately. The column was washed with 30 ml of Binding Buffer and the nickel ions were removed using 25 ml of Cleaning Solution (20mM sodium phosphate, 0.5 M NaCl, 0.05 M EDTA (Merk, Darmstadt, Germany), pH 7.4). Finally, HiTrap columns were filled with Store Solution and kept at 4°C. Samples of all the protein purification fractions were collected and analyzed by western blot.

The fraction containing the purified protein was concentrated using 30,000 to 100,000 molecular weight cut-off columns (MWCO) (Vivascience, Hannover, Germany). Briefly, the sample was loaded in the concentration columns and centrifuged for 30-45 min (3,000 g, 14°C) (Sorvall, Asheville, NC, USA). The flow-trough was decanted and the protein was washed twice with 15 ml of PBS (buffer exchange). The final volume in which the protein was resuspended was ~1.5 ml. The purified, concentrated and buffer exchanged protein was stored at -80°C.

VI.B.3. Animals and immunizations

A MHC class II knock-out (MHC $II^{-/-}$) mouse model that results in absence of CD4+ T-cells was selected (282) (Taconic, Hudson, NY, USA). Eight to ten week-old females $MHC^{-/-}$ mice were immunized by tail vein (I.V.) injection with a 20 μg of sHA in a total volume of 100 μl. sHA-C3d3 immunizations were normalized for the immunogenic

portion (20 µg of sHA). Animals were primed at day 0 and boosted at weeks 4 and 9. Mice were housed according to the regulation of the IACUC of the University of Pittsburgh and the USDA. Food and water were provided *ad lividum*. Sera samples were collected every two weeks by retro-orbital plexus puncture in mice anesthetized with 0.05-0.08 ml of a mixture of ketamine-HCl (100 mg/ ml) and xylazine (20 mg/ ml) [ketamine/xylazine]. Collected samples were stored at -20 ºC.

VI.B.4. ELISA

Antigen-specific antibody titers were determined by endpoint dilution ELISA. To determine anti-sHA antibodies, microtiter plates (96-well) were coated with sHA (A/PR/8/34) containing supernatants from transiently transfected HEK 293T cells. Plates were incubated overnight at 4 ºC and then blocked with 5 % nonfat dry milk in 1X PBS/0.05% Tween (PBS-T) (2 h). After extensive washing with PBS-T, antiserum collected from vaccinated mice was serially diluted (initial dilution 1:50) in 5 % nonfat dry milk in PBS-T. Serum was allowed to bind to antigen coated plates (2 h), followed by thorough washing with PBS-T. The plates were then incubated (25° C for 1 h) with 100 µl of goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) (1:5,000) (Southern Biotechnology Associates, Inc., Birmingham, AL) diluted in PBS-T containing 5% non-fat dry milk. The unbound antibody was removed, and the wells were washed (3X) with PBS-T. 100 µl of TMB substrate (1 TMB tablet per 10 ml of phosphate-citrate pH 5.0 buffer; 2 µl 30% H_20_2) (Sigma, St Louis, MO, USA) were added to each well (25°C for 30 min). Following 30 min incubation, the reaction was stopped with 50 µl /

well of 2N Sulfuric Acid. The colorimetric change was measured as the O.D. at 450 nm using a spectrophotometer (Dynex Technologies, Chantilly, VA, USA).

In order to determine anti-sHA IgG subtypes and IgM, a modification to the above protocol included the use of biotinylated goat anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgM antibodies (1:5,000) (Southern Biotechnology Associates, Inc., Birmingham, AL) to detect antigen-antibody complexes in place of the goat anti-mouse IgG-HRP conjugated. Biotinylated IgG isotype antibodies were detected by Streptavidin-HRP conjugated (1:7000) in PBS-Tween 20 (1h, 25°C). Developing was performed as described above.

VI.B.5. Virus Challenge

Different dilutions (1:1000, 1:500, 1:250 and 1:100) of live, mouse adapted influenza virus A/PR/8/34 were tested in C57/BL/6 wild type mice to determine the lethal dose 100 (LD_{100}). Challenge was performed in mice anesthetized (ketamine/xylazine) via intranasal instillation of 50ul of allantoic fluid diluted in 1X PBS to contain the above mentioned dilutions. Following administration, mice were monitored for weight loss (morbidity) and survival for 2 weeks. LD_{100} induced 20% or more weight loss in all mice/group by days 8-10 post-challenge. Mice that lost more than 25% of body weight were sacrificed (mortality). The determined LD_{100} (1:250), was used to challenge wild-type and $MHCII^{-/-}$ mice 4-5 days after the second boost (week 10). Virus challenge, as well as morbidity and mortality were monitored as described above. Following sacrifice, lungs were harvested, rinsed with 1X PBS, finely chopped, and transferred to a fresh 1.5 ml centrifuge tube. Two hundred ul of 1X PBS were added to the samples and spin down

(12,500 rpm / 3 min). The supernatants were transferred to a fresh centrifuge tube, stored at -80 C and used to determine IgG antibody titers.

VI.C. Results

VI.C.1. C3d enhances secondary humoral immune responses in wild-type mice and protects from lethal virus challenge

Wild-type mice (C57BL/6) were immunized (I.V.) with either purified sHA or sHA-$C3d_3$ at day 0 and boosted at weeks 4 and 9. Mice that received sHA-$C3d_3$ developed higher anti-HA antibody titers than mice immunized with sHA alone. Futhermore, sHA-$C3d_3$ vaccinated mice required only two vaccinations to elicit high sustained anti-HA antibody titers, while sHA required three vaccinations (**Figure 13** A). Even though, the antibody titer difference between sHA-$C3d_3$ and sHA immunized mice was reduced following the third immunization, sHA-$C3d_3$ mice consistently had at least 1 log higher antibody titers of anti-HA and this difference was statistically significant ($p<0.05$) (**Figure 13** B). Furthermore, anti-sHA antibody titers were higher in the lungs of sHA-$C3d_3$ vaccinated mice, following virus challenge (**Figure 13** C).

Following the third immunization, wild-type mice were challenged with influenza virus. Mice were monitored for weight loss as a sign of morbidity (**Figure 14** A) and survival (**Figure 14** B). Unvaccinated mice challenged with influenza [Naïve (A/PR)] had a steady decline in weight (**Figure 14** A) and between days 8-10 post-challenge, all mice had lost greater than 20-25% of their original body and were sacrificed (0% survival)

(**Figure 14** A and B). On the other hand, mock challenged naïve mice [Naïve (PBS)] did not lose weight (100% survival) (**Figure 14** A and B). Mice immunized with sHA alone and virus challenged [sHA (A/PR)], had a similar weight loss as Naïve (A/PR) at day 4 post-challenge and on day 6, ~20% of the mice had died (81% survival). The surviving mice continued to lose weight and by day 8, 45% of the mice had died (55% survival). Mice that did not lose more than 25% of their original body weight, started to recover (**Figure 14** A). In contrast, mice vaccinated with sHA-$C3d_3$ and challenged with influenza virus [sHA-$C3d_3$ (A/PR)], had minimum loss of body weight and by day 10, only 12% of the mice had died (88% survival) (**Figure 14** A and B). These mice began to recover weight by day 10 and at the end of the experiment had recovered their original weight (**Figure 14** A).

VI.C.2. C3d induced low titer secondary humoral immune responses in the absence of CD4+ T-cells and reduces morbidity in mice challenged with a lethal dose of influenza virus

The ability of C3d to stimulate Ig class switch in the absence of CD4+ T-cells was examined in $MHCII^{-/-}$ mice. We hypothesized that C3d would mimic the structural antigenic redundancy of TI antigens when fused to TD antigens. Following the second immunization (week 6), the only mice that developed a class switch from IgM to IgG, were those vaccinated with sHA-$C3d_3$ (20%) (**Figure 15** A and C). None of the sHA vaccinated mice switched to IgG. However, in contrast to wild-type mice, these anti-HA antibody titers did not sustain and by week 8 (4 weeks post boost) had dropped below the level of detection (**Figure 13** A vs **Figure 15** A). Following the third immunization, mice were challenged with influenza. Virus challenge was effective at inducing class switch to

IgG in mice previously vaccinated with sHA or sHA-C3d$_3$ and in naïve mice (**Figure 16** D). Development of an immunoglobulin class switch by live influenza virus has previously been reported (338, 343, 344). However, mice that received previously sHA-C3d3 had higher, even though not significant, titers (**Figure 15** D). sHA-C3d$_3$ induced IgG titers were significant only compared to naïve mice that received a mock (PBS) [Naïve (PBS)] challenge ($p<0.05$). sHA-C3d$_3$ immunized mice also had higher IgG titers in the lungs; however, not statistically significant compared to the other mouse groups (**Figure 15** B).

MHC II$^{-/-}$ mice were challenged 5 days after the third immunization. sHA and naïve challenged mice [Naïve(A/PR)] started to lose weight and between day 8-10 100% of the mice had died. Mice immunized with sHA-C3d3 also started to lose weight by day 4; however, the weight loss was delayed and less extreme than sHA or Naïve(A/PR) groups. Furthermore, 40% of these mice survived four days longer than Naïve (A/PR) mice and 20% survived until the end of the experiment (day 14). Control mice negative for infection [Naïve (PBS)] did not loose weight and 100% of them survived (**Figure 16** A and B).

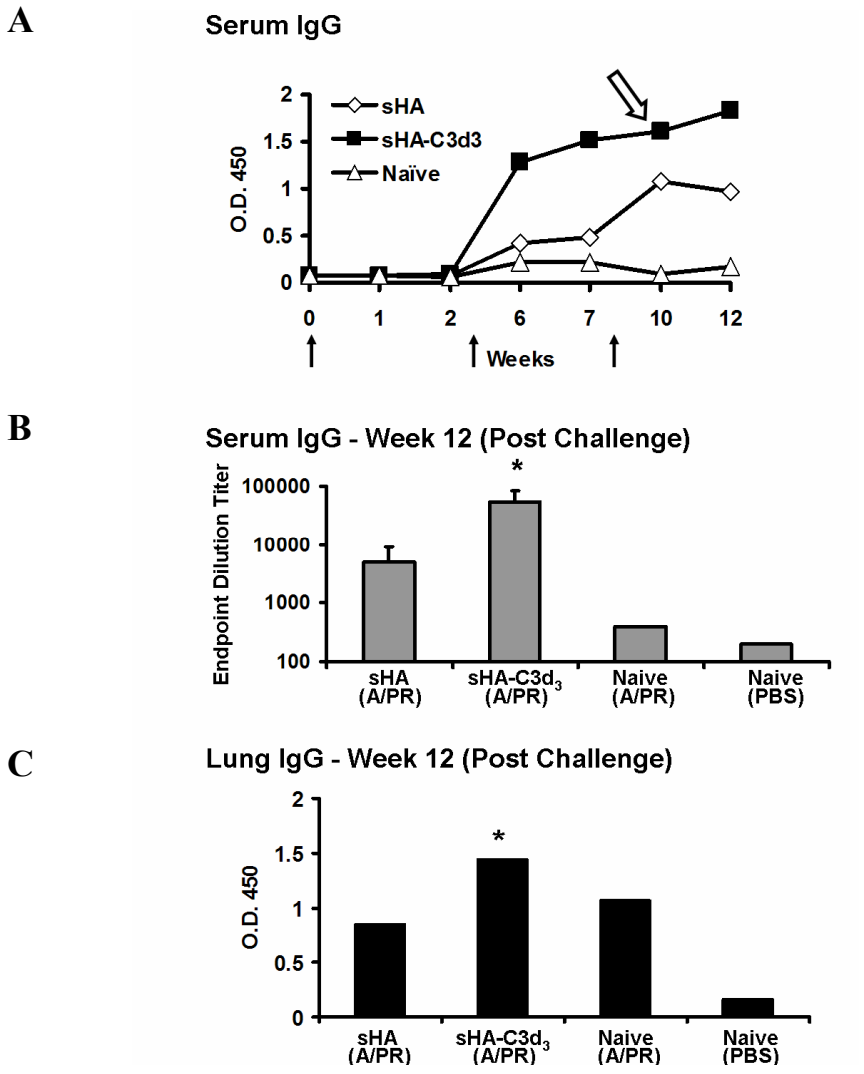


Figure 13. Anti-HA Antibody Titer in Wild-Type Mice

A) Time course of anti-sHA antibody titer development. Wild-type mice were IV immunized (black arrows) with sHA (white rhomboid), sHA-C3d3 (black square) or PBS (Naïve) (white triangle) at day 0 and weeks 4 and 9. Virus challenge [A/PR/8/34 (H1N1)] was performed at week 10 (white arrow). Blood samples were collected every two weeks by retroorbital puncture, the sera (1:400) isolated and used anti-sHA antibodies were detected by ELISA.
B) Anti-sHA endpoint dilution titer. Total anti-sHA IgG was detected post-virus challenge. Mouse serum was diluted until it reached the same O.D. reading that naïve mice. sHA-C3d3 mice developed higher total anti-sHA antibodies than sHA or naïve mice and this difference was statistically significant (* $p<0.05$).
C) Anti-sHA IgG in lungs. Supernatants of harvested lungs (1:400 dilution) were tested for the presence of anti-sHA IgG. sHA-C3d3 mice consistently had higher anti-sHA antibodies in the lungs ($p<0.05$). The type of challenge (live A/PR/8/34 or PBS) is indicated in parenthesis.

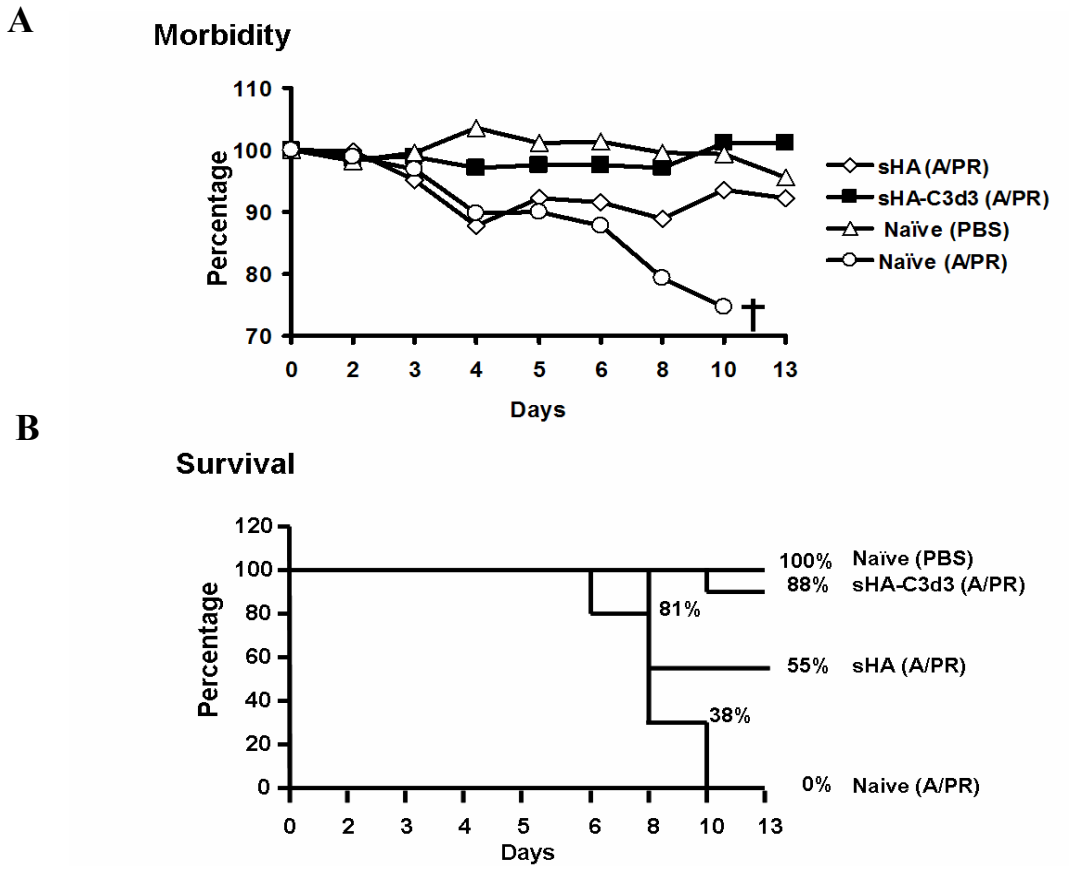


Figure 14. Virus Challenge in Wild-Type Mice

A) Wild-type mice were challenged (week 10) with a LD_{100} of the mouse adapted strain of influenza virus A/PR/8/34 (H1N1), following the last immunization. Positive and negative control groups for infection included naïve mice challenged with PBS (negative control) (white triangles) and naïve mice challenges with A/PR/8/34 (positive control) (white circles). sHA (white rhomboid) and sHA-C3d3 (black square) mice received A/PR/8/34. Weight loss (morbidity) is reported as percentage of original weight. The cross (┼) indicates that 100% mice died from that group.

B) Survival following virus challenge in the groups is reported as percentage. The type of challenge received (A/PR/8/34 or PBS) is indicated in parenthesis.

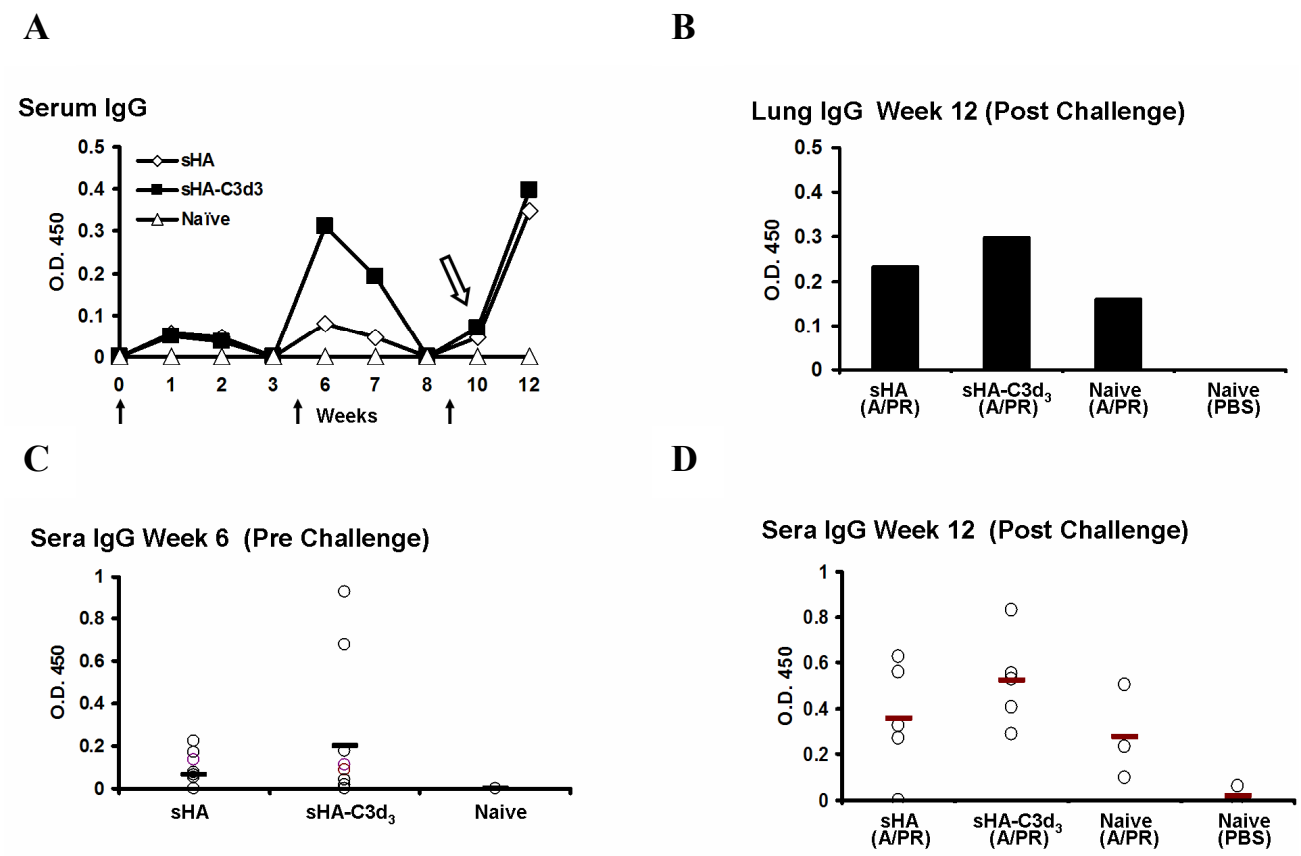


Figure 15. Secondary Humoral Immune Responses in MHC II$^{-/-}$ Mice

A) Anti-sHA IgG titer was determined by ELISA in sera (1:50 dilution) from MHCII-/- mice. Immunizations (I.V.) are indicated by the black arrows. Blood samples from sHA (white romboinds), sHA-C3d3 (black squares) and Naïve mice (white triangle) were collected every two weeks by retroorbital puncture. Panel A shows a time course of the development of anti-IgG following each immunization. Five days after the final immunization, mice were virus challenged (white arrow).
B) Anti-sHA IgG was determined in the supernatants (1:50 dilution) of lungs harvested at the end of the virus challenge. The type of challenge received (A/PR/8/34 or PBS) is indicated in parenthesis.
C) Anti-sHA IgG determined in sera (1:50 dilution) by ELISA in individual mice at week six, following the second immunization and previous to virus challenge. Each white dot represents an individual mouse and the bar indicated the arithmetic mean in each group.
D) Anti-sHA IgG determined in sera (1:50 dilution) by ELISA in individual mice at week 12, following the third immunization and virus challenge. Each dot represents an individual mouse and the bar indicates the arithmetic mean in each group. The type of challenge in indicated in parenthesis.

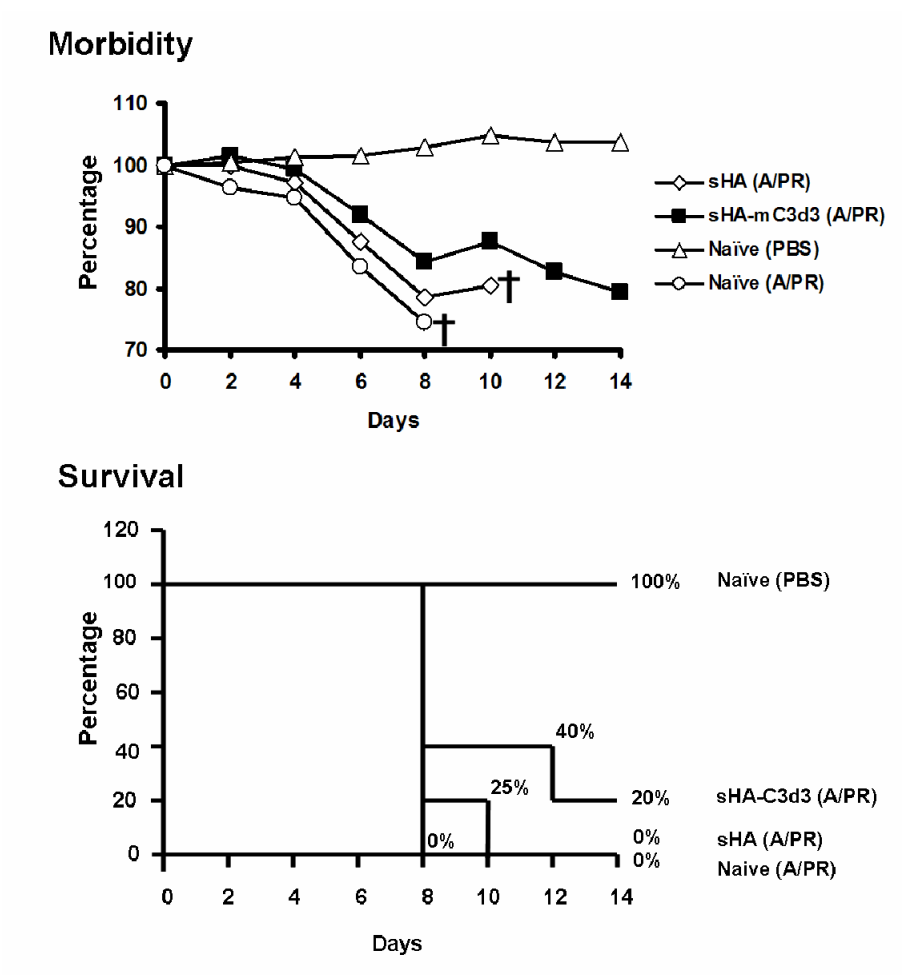


Figure 16. Virus Challenge in MHCII-/- mice

A) MHCII-/- mice were challenged (week 10) with a LD_{100} of the mouse adapted strain of influenza virus A/PR/8/34 (H1N1), 5 days following the last immunization. Positive and negative control groups for infection included naïve mice challenged with PBS (negative control) (white triangles) and naïve mice challenges with A/PR/8/34 (positive control) (white circles). sHA (white rhomboid) and sHA-C3d3 (black square) mice received A/PR/8/34. Weight loss (morbidity) is reported as percentage of original weight. The cross (┼) indicates that 100% mice died from that group.

B) Survival following virus challenge in the groups is reported as percentage. The type of challenge received (A/PR/8/34 or PBS) is indicated in parenthesis.

VI.C.3. Different IgG isotype class switch following virus challenge in wild-type and MHCII$^{-/-}$ mice

Once B-cells are activated and start to secrete antibodies, these Igs class switch, from IgM to IgG, IgE or IgA. Several isotypes (subtypes) of IgG have been described, each one with a different function(s), the result of a different cytokine environment and consequently the result of different cell priming or activation. Thus, analysis of the different IgG class switch helps to understand if the same set of cells are stimulated by the vaccines (pre-challenge) and live virus (post-challenge) in wild-type and MHCII$^{-/-}$ mice.

IgG isotype class switch was analyzed in wild-type and MHCII$^{-/-}$ mice previous to virus challenge and post-challenge (weeks 6 and 12, respectively). Wild-type mice vaccinated with sHA-C3d$_3$, had significantly higher IgG$_1$, IgG$_{2a}$ and IgG$_{2b}$ than sHA vaccinated mice ($p<0.001$), previous to the virus challenge (**Figure 17** A). IgG1 was significantly higher that IgG$_{2b}$ and IgG$_{2a}$ ($p<0.001$ and $p<0.0001$, respectively), in the sHA-C3d$_3$ mice, suggesting a dominant Th2 (humoral) immune response and thus an environment rich in IL-4 (346). Finally, IgG$_{2b}$ was significantly higher that IgG$_{2a}$ ($p<0.005$). Following virus challenge, sHA, as well as sHA-C3d$_3$ vaccinated mice, developed higher titers of anti-HA antibody; however, sHA-C3d$_3$ mice still showed a more robust immune response ($p<0.05$) (**Figure 17** B). The IgG class switch profile developed by sHA mice was similar to sHA-C3d$_3$ with IgG$_1$ as the main isotype induced. Anti-HA IgG$_1$ in the sHA-C3d$_3$-vaccinated group was significantly higher than IgG$_{2a}$ ($p<0.001$) and IgG$_{2b}$ ($P<0.05$). The same isotype was also significantly higher in the sHA-vaccinated group when compared

to IgG_{2a} ($p<0.05$) and IgG_{2b} ($p<0.001$). IgG_{2a} and IgG_{2b} were not statistically different in sHA-$C3d_3$ ($p=0.09$) or sHA ($p=0.06$) vaccinated groups (**Figure 17** B).

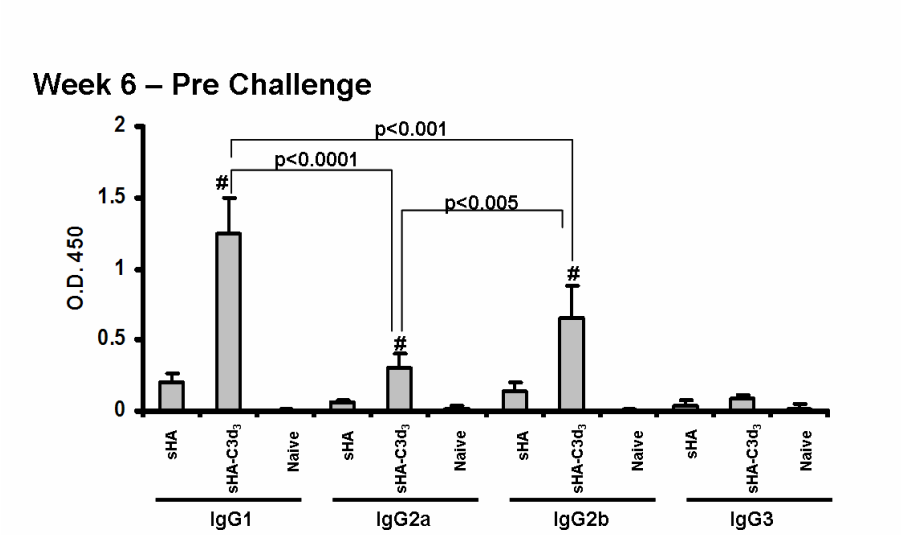
Only MHC $II^{-/-}$ mice that received sHA-$C3d_3$ (20%) developed IgG class switch. The main isotype developed in these mice was IgG2b, suggesting the presence of TGF-β (347) (**Figure 8** A). As suggested by Snapper, this class switch may indicate B-cell activation and autocrine production of TGF-β (348), which might be the predominant cytokine in these mice. Interestingly, also lower titers of anti-HA specific IgG_1 and IgG_{2a} were detected, suggesting the presence of lower concentrations of IL-4 and IFN-γ. The IgG_{2b} class switch was significantly higher than IgG_1 ($p<0.05$) and IgG_{2a} ($p<0.001$). Following virus challenge, however, there was a shift in the IgG isotype profile and IgG_3 became the dominant isotype (**Figure 18** B). sHA-vaccinated mice had higher IgG_3 titers than sHA-$C3d_3$ immunized animals ($p<0.001$) or Naïve(A/PR) mice ($p<0.0001$). Immunoglobulin class switch to IgG_3 has been suggested to be independent of cytokines, since B-cells stimulated only with LPS (T-cell independent B-cell stimulator) induce γ3 germline transcripts and the cells switch to this isotype (349-351). However, a different T-cell independent B-cell stimulant (dextran conjugated anti-IgD antibody - αδ-dex -) is also able to induce this class switch; nevertheless, requires IFN-γ and IL-5 (352). Thus, either no cytokine or a combination of IFN-γ and IL-5 may be responsible for the presence of IgG_3. Since IgG isotype class switch is dependent on the cytokine environment and this depends on the cells stimulated, the change in isotype profile in MHC $II^{-/-}$ mice from pre- to post-virus challenge suggests different mechanisms by which sHA-$C3d_3$ and live influenza virus stimulate B-cells and other immune cells.

Furthermore, despite, the higher IgG_3 levels on sHA vaccinated mice, the total IgG titer was higher in sHA-$C3d_3$ mice; although not statistically significant. Thus, the less pronounced weight loss and enhanced survival are probably the result of the interaction of various immune mechanisms stimulated by C3d.

VI.D. Discussion

The current study intended to evaluate the ability of C3d to induce a secondary humoral immune response in the absence of CD4+ T-cell. Only few mice (20%) deficient in CD4+ T-cell (MHC $II^{-/-}$) class switched to IgG, following immunizations with sHA-$C3d_3$. However, after virus challenge, IgG was detected in all groups that received live influenza virus [A/PR/8/34 (H1N1)] (**Figure 16**). The IgG levels were higher in sHA-C3d3 vaccinated mice than in mice vaccinated with sHA; although, not statistically significant. Despite the small difference in IgG titers, mice vaccinated with sHA-$C3d_3$ had a less pronounced decline in weight and a prolonged survival compared to sHA-vaccinated mice. Analysis of the elicited IgG isotype pre- and post-challenge suggested that different pools of immune cells were stimulated. Thus, C3d, delayed morbidity and enhanced survival of CD4+ T-cell deficient mice by directly stimulating B-cells and possibly in collaboration with other immune cells.

A



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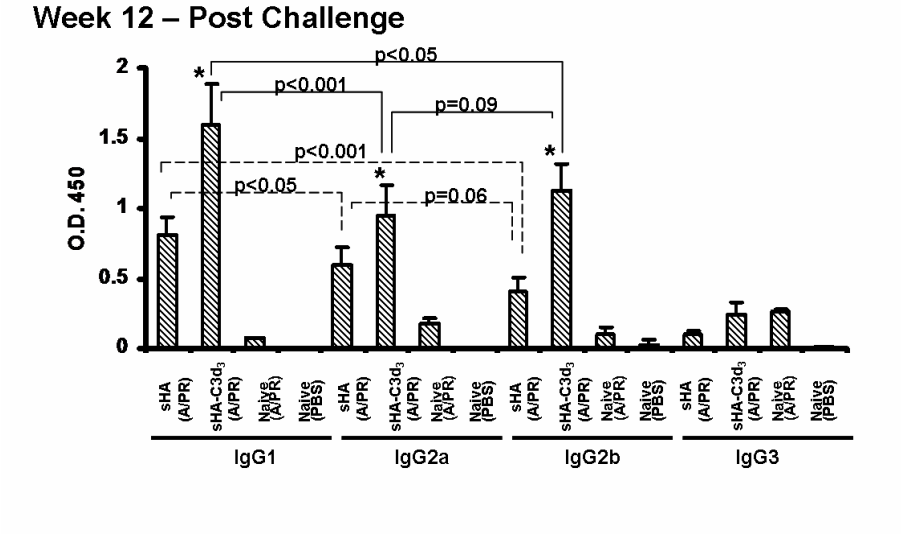


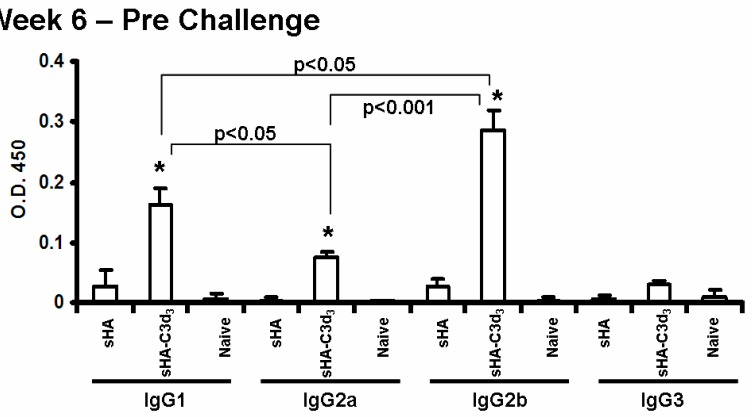
Figure 17. IgG Isotype Class Switch in Wild-Type Mice

A) IgG isotype class switch was determined by ELISA in pooled sera (1:400) from wild-type mice, following the second immunization (week 6 - pre-challenge). The bars represent the summary of three individual experiments. From higher to lower, the IgG isotypes induced by sHA-C3d3 were IgG1, IgG2b and IgG2a.

B) The IgG isotypes induced following virus challenge were determined by ELISA in pooled sera (1:400 dilution). From higher to lower the IgG isotypes induced by C3d were IgG1, IgG2b and IgG2a, which is the same profile induced pre-challenge. On the other hand, in the sHA group, from higher to lower, virus challenge induced IgG1, IgG2a and IgG2b.

Statistical significance ($p<0.001$) when sHA-C3d3 vs sHA groups compared
* Statistical significance ($p<0.05$) when sHA-C3d3 vs sHA groups compared

A



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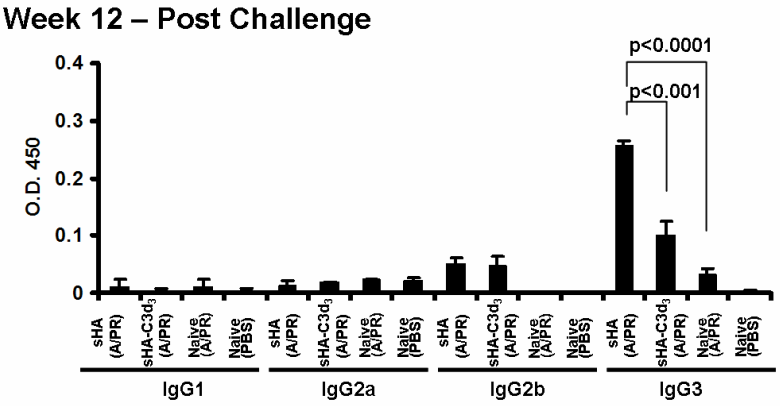

Figure 18. IgG Isotype Class switch in MHC$^{-/-}$ Mice

A) IgG isotype class switch was determined by ELISA in pooled sera (1:50) from MHCII-/- mice, following the second immunization (week 6 - pre-challenge). From higher to lower, the IgG isotypes induced by sHA-C3d3 were IgG2b, IgG1 and IgG2a. The bars represent the summary of three individual experiments.

B) The IgG isotypes induced following virus challenge (week 12) were determined by ELISA in pooled sera (1:50 dilution). From higher to lower the IgG isotypes induced by sHA-C3d were IgG3 and IgG2b. In the sHA group, from higher to lower, virus challenge induced the same profile; however, IgG3 was significantly higher than in sHA-C3d vaccinated mice.

* Statistical significance ($p<0.001$) when sHA-C3d3 vs sHA groups compared

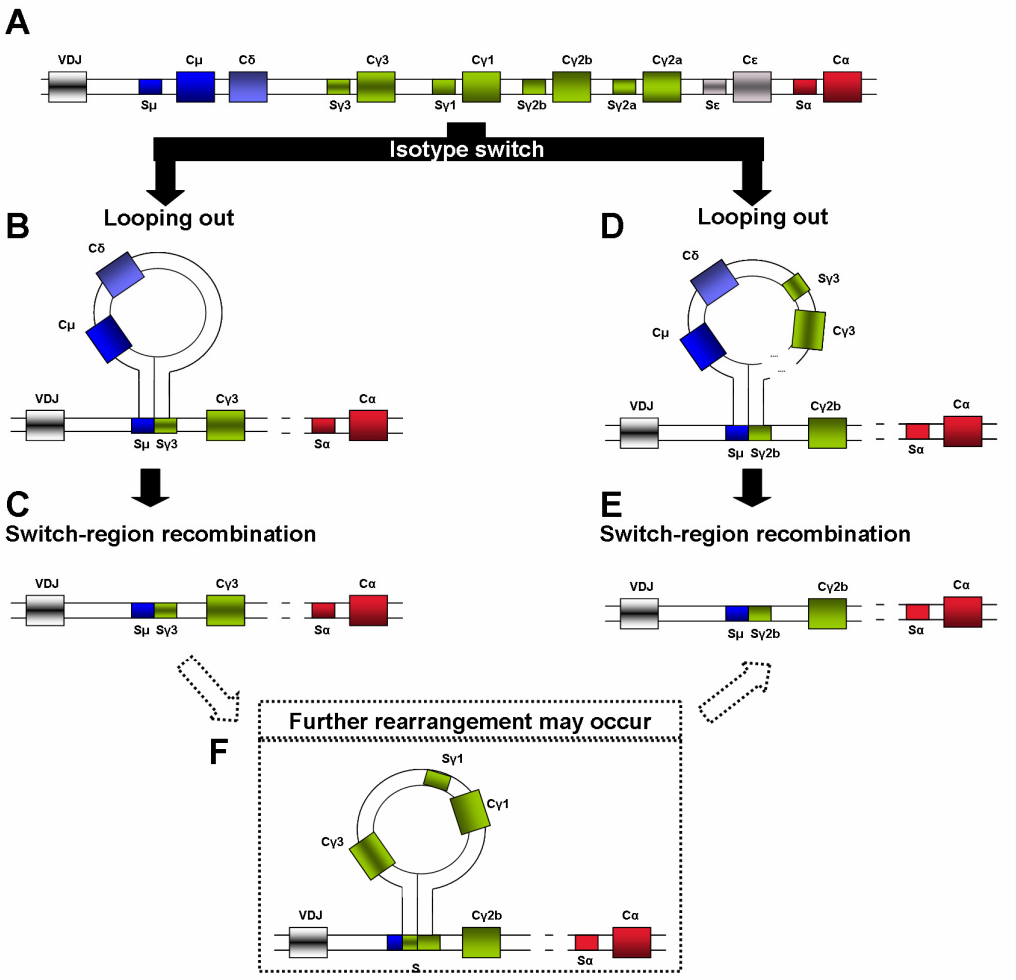

Figure 19. Isotype Class Switch Recombinantion

Isotype switching involves recombination between specific switching signals.
A) Illustration of the heavy chain (C_H) genes segments. Each C_H gene segment (*e.g.* Cμ or Cγ1) have a switching (S) region (*e.g.* Sμ or Sγ1), which is immediately in front of the CH gene, except for Cδ.
B) Under the proper cytokine stimulation, two S regions come in close proximity, looping out DNA segment between them. In the illustration, the cytokine environment has stimulated Sμ and Sγ3 to come in close proximity and loop out the DNA segment containing the C_H genes Cμ and Cδ.
C) Recombination of the Sμ or Sγ3 gene regions causes deletion of the previously looped DNA segment. Thus this cell is unable to produce IgM or IgD anymore.
D and F) Represent looping out and class switch from IgM to IgG2b. In this case a larger DNA segments has been deleted and the cell has lost its ability to produce IgM, IgD, IgG3 and IgG1.
F) A cell that has class switched to IgG3 still can produce IgG2b under the proper stimulus as new looping and recombination events can occur.

Wild-type (C57BL/6) mice that received sHA-C3d3 developed an enhanced anti-HA antibody titer compared to mice vaccinated with sHA alone. Furthermore, only two vaccinations of sHA-C3d$_3$ were required to induce significant anti-HA antibody titers, while three immunizations were necessary to elicit similar anti-HA titers using sHA. (**Figure 13** A and B). The enhancement of the immune response by C3d involves CR2-dependent and –independent mechanisms (275). The CR2 dependent mechanism has been more widely studied, especially in B-cells. Co-binding of the sIgM and CR2 by the antigen and C3d triggers signaling pathways that synergize and lead to B-cell activation. Furthermore, reducing the antigenic threshold required to activate the cell (47, 105, 293, 308, 353). In contrast, CR2-independent mechanisms are largely unknown and may involve interaction(s) with other receptor(s) on non-B-cells (329-331).

There is a direct correlation between anti-flu antibody titers and protection against influenza virus infection (248, 264, 265). In this, study, wild-type mice vaccinated with sHA-C3d$_3$ had high titer anti-HA antibodies and a reduced morbidity and mortality following a lethal virus challenge (**Figure 14** .A and B). The virus challenge did not further increase the anti-sHA antibody titers levels, suggesting that the B-cells in these mice were fully activated. The enhanced antibody titers by sHA-C3d$_3$ were not only detected in the sera, but also in the supernants of harvested lungs following virus challenge (**Figure 13** C).

Recombinantion of Ig variable gene segments (VDJ) results in expression of a new downstream heavy chain constant region (C_H) gene (*e.g.* Cγ1, Cγ2b, Cε) (**Figure 19**A). Ig

class switching is effected by a deletional recombination that occurs between switching region (S) sequences (*e.g.* Sγ1, Sγ2b, Sε) located upstream of each one of the C_H genes, except Cδ (**Figure 19** A). Class switch recombinantion is directed to a particular C_H gene by cytokines that induce transcription from germ-line C_H genes before switch recombinantion to the same C_H gene. This recombination occurs via deletional recombinantion and excision of the intervening DNA between the two S regions as a switch circle (loop) (**Figure 19** B and C). In summary, class switch recombination is the result of the cytokine environment that surrounds the B-cell. The cytokine environment at the same time, depends on the immune cells stimulated by the antigen.

As previously reported, in wild-type mice, C3d enhances both Th1 and Th2 cytokines (133), but usually there is an enhanced development of T helper 2 (Th2) cells (secrete IL4, IL-5 and IL-13), which stimulate class switch to the IgG_1 isotype. Consistent with this, IgG1 was the main isotype induced in sHA-C3d3 wild-type vaccinated mice previous to virus challenge (**Figure 17** A). IgG_1 was significantly higher that IgG_{2b} and IgG_{2a} ($p<0.001$ and $p<0.0001$, respectively). Furthermore, IgG_{2b} was significantly higher that IgG_{2a} ($p<0.005$). This suggests that the cytokine environment was principally composed of IL-4 (induce IgG_1); however TGF-β (induce IgG_{2b}) was also present and this was higher than IFN-γ (induce IgG_{2a}). Virus challenge induced important anti-HA titers in sHA-vaccinated mice; however the immune response in sHA-$C3d_3$ mice remained significantly higher ($p<0.05$). The IgG isotype profile induced in sHA and sHA-$C3d_3$ was similar with IgG_1 as the main statistically significant isotype, followed by IgG_{2a} and IgG_{2b}. These last isotypes were not significantly different. Hence, in the sHA-

$C3d_3$ group from pre- to post-challenge there is a difference as IgG_{2b} is no longer higher than IgG_{2a}, suggesting that the environment pre-challenge was richer in TGF-β. Therefore, C3d induces an environment richer in TGF-β previous to virus challenge (347). The sources of TGF-β include T-cells, B-cells, macrophages, and platelets (354-358), all of which could potentially interact with C3d through CR2 or non-CR2 receptors (329). However, as suggested by the data from MHC $II^{-/-}$ mice a good portion of this TGF-β may be B-cell derived (348). Considering that natural infections with influenza virus induce significant amounts of IFN-γ (267, 333, 359), it is not surprising that live virus challenge increased IFN-γ levels and enhanced class switch to IgG_{2a}, reducing the gap with IgG_{2b}.

Only a few MHC $II^{-/-}$ mice vaccinated with sHA-C3d3 developed an IgG class switch following the second immunization (pre-virus challenge) (**Figure 15** A and C). However, in those mice that did class switch to IgG, C3d most likely activated B-cells. The chimera proteins used in this study comprise three tandem copies of C3d fused to sHA, which enables the binding of three CR2 molecules and one sIgM on the surface of the B-cell. Thus, there is redundant triggering in the pathways that activate B-cells. This redundant activation may resemble that induced by TI antigens, which are large molecules with repetitive antigenic subunits that bind several sIgM molecules and trigger B-cell activation. One of the molecules activated and involved in the cross-talk of these signaling pathways is the Bruton's tyrosine kinase (Btk) (**Figure 3**). Interestingly, as recently described, Btk is also activated in the CD40 signaling pathway through the protein kinase μ (PKD) / tumor necrosis factor receptor-associated factor (TRAF)-2

molecules (360). Furthermore, co-engagement of sIgM and CD40 is also implicated in a synergistic effect in the activation of the B-cells (360) and the CD40L/CD40 pathway is known for its importance in class switching from IgM to IgG, IgA and IgE (361-363). Thus, the signaling triggered by C3d can cross-talk with the CD40 pathway, through Btk, and in this way activate B-cells and induce IgG class switch without CD4+ T-cell help. Further evidence of a direct activation of B-cells by C3d comes from the elicited IgG isotype induced prior to virus challenge in MHC $II^{-/-}$ mice. The induction of IgG_{2a} suggests an environment rich in TGF-β and possibly the result of autocrine production by the B-cell.

Following virus challenge all MHC $II^{-/-}$ mice that were infected with influenza developed different degrees of IgG class switch (**Figure 15** D). This is concordant with previous studies that have demonstrated that live (338, 342), as well inactivated influenza viruses (343) induce IgG class switch in the absence of CD4+ T cells. However, mice previously vaccinated with sHA-$C3d_3$ had slightly higher anti-HA IgG antibody titers in sera, as well as in lungs, compared to sHA-vaccinated mice (**Figure 15** B and C). This enhanced anti-HA antibody titer may indicate that C3d primed B-cells and thus when these cells encountered a stronger stimulus, such as live virus, cells that already initiated class switching (cytokine expression induced without deletion recombination) could continue (deletion recombination). The enhanced IgG antibody titers could also be a consequence of a richer cytokine environment. As previously described, C3d is able to interact primarily with B-cell, follicular dendritic cells and T-cells (CD4+ and CD8+), through CR2 (105, 106, 111, 115, 364). Using other surface molecules, not yet completely

characterized, C3d is able to interact with monocytes, neutrophils and platelets (328-331). All these cells have the potential to produce cytokines and thus may induce a richer and more varied cytokine profile that may enable a stronger IgG class switch.

One of the most interesting findings of this study was the difference in the IgG isotype profile pre- and post-virus challenge in MHC $II^{-/-}$ mice (**Figure 18** A and B). Previous to virus challenge, only mice vaccinated with sHA-C3d3 class switched to produce IgG and even though not all mice class switched, the mice that did, had IgG_{2b} as the predominant isotype ($p<0.05$ compared to IgG1; $p<0.001$ compared to IgG2a). The presence of this isotype (IgG_{2b}) suggests a microenvironment rich in TGF-β. TGF-β is released by numerous cell types including B-cells, T-cells, macrophages and platelets (354-358). Of these cells, B-cells are the most likely producers of this cytokine because co-interaction of sHA-$C3d_3$ with sIgM and CR2 on the surface can activate B-cells and induce release of TGF-β (355, 365). Furthermore, B-cell production of TGF-β has been shown to have an autocrine role (348, 366, 367). The possibility that CD8+ T-cells could be involved in the production of TGF-β is low as 90% of this cytokine produced in T-cells comes from CD4+ T-cells (368), which are absent in $MHCII^{-/-}$ mice.

Virus challenge was more effective at inducing an isotype class switch from IgM to IgG in MHC $II^{-/-}$ mice (**Figure 15** D). sHA-$C3d_3$ mice had slightly higher total anti-HA IgG titer than sHA alone vaccinated mice, both in sera and lungs (**Figure 15** B and D). However, when the IgG isotype profile was compared to pre-challenge, there where noticeable differences. Post-challenge, IgG_3 was the predominant isotype in both sHA

and sHA-C3d$_3$ vaccinated mice. IgG$_3$ is an unusual isotype, which is mainly induced by TI type 2 antigens (352, 369). Ig class switch to IgG$_3$ was initially suggested to be independent of cytokines, as B-cells stimulated with LPS (TI B-cell stimulant) alone produced γ3 germline transcripts and switched to this isotype (349-351). More recent studies have provided evidence suggesting that IFN-γ (along with IL-5) may also be responsible for this kind of Ig class switch (352). However, the latter experiments utilized a different type of model antigen, dextran-conjugated anti-IgD antibody (αδ-dex)(model TI type 2 B-cell stimulant). Thus, the nature of the stimulant or B-cell activator (*e.g.* LPS or αδ-dex) and the mechanism of interaction with the B-cell has an important role in determining the cytokine directed-isotype profile (*e.g.* LPS + no cytokine induces = IgG$_3$ or αδ-dex + IFN-γ (IL-5) = IgG$_3$). IFN-γ usually stimulates IgG$_{2a}$ isotype in the presence of LPS; however, in these experiments IgG$_3$ class switch was inhibited (370). In the presence of αδ-dex and IL-5, IFN-γ, induces IgG$_3$ (352), reinforcing the idea that the nature of the B-cell activator later defines the cytokine profile and thus the isotype class switch. The concentration of the cytokines induced also plays an important role as αδ-dex + IL-5 + IFN-γ (1 U/ml) induced significant IgG$_3$ without IgG$_{2a}$. However, when the IFN-γ concentration was increased to 10 U/ml, IgG$_3$, as well as IgG$_{2a}$, were induced (352). Hence, in this study post-virus challenge, there are probably moderate amounts of IFN-γ and IL-5. The most likely candidate cells to produce these cytokines are CD8+ T-cells (371), which are present in MHC II$^{-/-}$ mice. Furthermore, these cells alone have an important role in clearing influenza virus infection (372); however, their function is not optimal in the absence of CD4+ T-cells (373). The non-optimal function of CD8+ T-cells may result in moderate levels of cytokines production, thus inducing only IgG3 (but not

IgG_{2a}) class switch. Other candidates to consider are γ/δ T-cells, which are also able to produce these cytokines. Moreover, γ/δ T-cells are mainly confined (but not solely) to the CD8+ subset and present in epithelial and mucosal surfaces (340), which are the primary site of encounter with influenza virus. Finally, cell combinations, such as natural killer (NK) (374), which produce IFN-γ, but not IL-5, could be potential candidates along with CD8+ T-cells, which may secrete the IL-5.

Post-challenge the total IgG levels in sHA-$C3d_3$ MHC $II^{-/-}$ vaccinated mice were slightly higher than in sHA mice; however not statistically significant. Nevertheless, when the IgG isotype was analyzed, the level of IgG_3 seemed to be higher in sHA mice than in the sHA-$C3d_3$ group. This may be explained by the class switch induced by sHA-$C3d_3$ vaccinations prior to challenge. The C_H gene segment that encodes for Cγ3 is located upstream of Cγ2b (**Figure 19** A). Initial class switch does not require deletion of the gene segments, but only transcription induction of determined C_H gene (*e.g.* Cγ2b), by cytokines (*e.g.* TGF-β). However, at a later time, there is going to be excision of the intervening DNA between the two switching regions (Sμ and Sγ2b in the example) (**Figure 19** D and E). As the Cγ2b region is located downstream of Cγ3, once the gene segment has been deleted, there is not possibility that the cells can produce IgG_3 again. On the other hand, if a cell class switches initially to IgG3 (**Figure 19** B and C), the Cγ2b region is still present and consequently the cell still has the potential to produce IgG2b, under the proper cytokine stimulation (**Figure 19** F and E). Five days after the third immunization, MHC $II^{-/-}$ mice were virus challenged. If the main isotype induced by sHA-$C3d_3$ (following vaccination) was IgG2b and the pool of B-cells is limited in MHC

$II^{-/-}$ mice (without proper stimulation, B-cells do not proliferate), then the cells that are producing IgG_{2b} would be unable to produce IgG_3, even under the proper cytokine stimulus. Furthermore, the pool of naïve B-cell in these mice may be smaller because some cells already class switched to IgG_{2b}. On the other hand, it seems that sHA vaccination induced low levels of B-cell activation, as the total IgG titer is similar to naïve(A/PR) mice (**Figure 15** D). Thus, when sHA vaccinated mice encountered the strong viral stimulus, the naïve B-cell population was probably a larger percentage of the total B lymphocyte pool compared to sHA-$C3d_3$ vaccinated mice and predisposed to IgG_3 class switch (probable effect of sHA alone vaccination). So, even if the virus challenge induced a similar cytokine profile in sHA- and sHA-$C3d_3$-vaccinated mice, the larger naïve population in the sHA group may be responsible for the higher IgG_3 titers. In contrast, the reduced naïve population is responsible for lower IgG3 in sHA-$C3d_3$ mice. Furthermore, the previously class switched IgG_{2b} B-cell population may stop producing antibodies due to the absence of the proper cytokine environment.

Despite of the higher IgG_3 class switch (post-challenge) in MHC $II^{-/-}$ mice vaccinated with sHA ($p<0.001$ compared to sHA-$C3d_3$), the total IgG titer was slightly higher in the sHA-$C3d_3$ group (**Figure 17** B). This slightly higher total IgG titer was not statistically significant; however, the weight loss was reduced and the survival was better in this group (**Figure 16** B). This suggests that protection against live virus was not only dependent on anti-HA antibodies, but is also on other immune cells or factors, which were possibly activated by C3d. C3d not only stimulates B-cells, but also collaborates in the activation of non-B-cells such as monocytes, neutrophils and platelets, which bear

non-CR2 C3d-binding proteins on their surface (328-331, 375). All these cells produce cytokines when activated and thus collaborate in the development on immune responses. Furthermore, it has been known for years that T-cells bear CR2 on their surface (106, 107, 376-378); however, the role of this receptor on these cells has been elusive. Recent data demonstrate that C3 derivatives promote migration and priming of T-cells (CD4+ and CD8+) into the lungs during influenza infection (379, 380), thus it can be speculated that in MHC $II^{-/-}$ mice, C3d helped in CD8+ T-cell migration and activation. The reduced weight loss and enhanced survival on MHC $II^{-/-}$ is probably the result of the addition of various immune factors stimulated by C3d and may involve 1) activation, antibody production and cytokine secretion of B-cells (CR2 dependent and independent); 2) activation, migration and cytokine production of CD8+ T-cells (CR2 dependent and possibly CR2 independent); and 3) activation and cytokine production of innate cells, such as neutrophis and monocytes (CR2 independent).

In summary, C3d is able to induce a mild enhancement of the secondary humoral immune responses in the absence of CD4+ T-cells. These mild secondary immune responses, possibly in collaboration with other innate and acquired immune cells, stimulated by C3d are able to reduce morbidity and mortality in MHC $II^{-/-}$ mice.

VII. Chapter 7: Conclusions and Future Directions

VII.A. Conclusions

C3d is the final degradation product of the third component of complement (C3). This small molecule plays an important role during natural infections by coating invading microorganisms and thus enabling their interaction with immune cells that bear CR2 on their surface (**Figure 3**). In this way C3d links the innate with the adaptive immunity. The main cells that express CR2 are FDCs, B-cells and T-cells (106, 108, 115, 116, 378, 381). C3d interaction with CR2 on FDCs has been implicated in antigen presentation and maintenance of immune memory (Section I.C.4) (**Figure 2**) (115). On B-cells, cross-linking of the sIgM and CR2 by the antigen and C3d, triggers pathways that cross-talk and synergize in the activation of B-cells (**Figure 3**) (48, 124, 319, 381). The role of CR2 on T-cells remains elusive; however, cell activation and triggering of signaling pathways that influence cytokine secretion can be speculated. Therefore, C3d is a natural adjuvant with properties that have the potential to be beneficial for the development of vaccines. The adjuvant properties of C3d have been explored using several model antigens (*e.g.* hen egg losozyme (126)), microorganism-derived (*e.g.* HIV-1 envelope, measles and influenza hemagglutinins (85, 127, 132, 138), PS14 for *Streptococcus pneumoniae* (128)) and self-antigens (*e.g.* heterologus type II collagen(131)).

The enhancement of the immune response has been extensively studied in B-cells, which provided the first light on the classic mechanism(s) by which C3d functions. Initially, it was discovered that C3d interacts with CR2 (CD21) and that this molecule later binds

and stimulates CD19, which initiates a signaling pathway (Vav, Lyn, Fyn, IP3K, Btk) that activates the B-cell (46, 48). At the same time, it was known that the B-cell receptor (BCR or sIgM) was activated by antigens and triggered a signaling pathway that involved Src, which interacts with Lyn (CR2 pathway) amplifying the signaling provided by Src. Furthermore, both pathways stimulate Btk, downstream. Thus, these two pathways cross-talk and converge at an activation intermediate in the B-cell. Initial experiments co-ligated CR2 and sIgM with monoclonal antibodies and this provided evidence of a synergistic effect in B-cell activation (120, 307, 308, 321). Later, injection of chimera proteins of hen egg lysozyme (HEL) and two or three copies of C3d (HEL-$C3d_2$ and HEL-$C3d_3$) into HEL-transgenic mouse models reduced the amount of antigen needed to trigger optimal humoral immune responses (126). Furthermore, antigens conjugated to C3d bound inefficiently to B-cells derived from $CR2^{-/-}$ mice and cell activation was reduced *in vitro* (130). Hence, there was evidence to support that the main mechanism of enhancement of the immune response was dependent on CR2. However, there were reports indicating that other non-CR2 receptors were present in various immune cells, such as monocytes, neutrophils, and platelets (329-331). Furthermore, there was evidence that C3 products were important for T-cell migration and activation, suggesting that more than one mechanism could be implicated in the enhancement of the immune response by C3d (379).

Mechanisms of immune enhancement that are independent of CR2 are presented in this thesis using mice deficient for this receptor. Contrary to earlier reports, $CR2^{-/-}$ mice immunized with antigens fused to C3d mounted efficient humoral immune responses.

These responses were almost comparable to those induced in wild-type mice. Furthermore, this enhancement was proven to be independent of CR2 since the immune responses were comparable to that induced by chicken gamma globulin (CGG) in $CR2^{-/-}$ mice. CGG is a well known carrier protein that does not use CR2 for its mechanism of adjuvanticity. These results, however, suggested that C3d might work as a carrier protein (275). Nevertheless, Mitsuyoshi *et al* (382) later proved this theory as incorrect, using a TI type II antigen (PP14 from Pneumococcus) conjugated to OVA (known carrier protein) or C3d. These antigens were used to immunize CBA/N *xid* mice, which do not mount humoral immune responses to TI type II antigens. Following the third immunization PPS14-OVA mice developed IgM and IgG, while PPS14-C3d did not, demonstrating that PPS14 retained the TI type II characteristics when fused to C3d but acquired TD properties when conjugated to OVA. Thus, C3d does not function as a carrier protein and antigens fused to this molecule retain their characteristics. In summary, immunization of $CR2^{-/-}$ mice with antigens fused to C3d demonstrated that C3d can enhance the immune response by CR2-dependent and –independent mechanisms. This has opened the door to explore new pathways by which C3d enhances the immune response in B-cells and other immune cells.

Evidence that C3d enhances the immune responses in more than one cell type was provided from immunizations of mice deficient in CD4+ T-cells (MHC $II^{-/-}$). C3d was able to induce IgG class switch in the absence of CD4+ T-cells, a phenomenon attributed only to T-cell independent (TI) antigens. Despite that IgG induction was not efficient; mice that developed class switch demonstrated that it was the result of B-cell activation

by C3d, but also the result of activation of other immune cells. Evidence that B-cells were the main cells activated came from the IgG isotype induced, C3d induced class switch recombination mainly to IgG_{2b}, which is dependent on TGF-β. The main cell producers of TGF-β are B-cells, T-cells, monocytes and platelets. Among T-cells, CD4+ T-cells are responsible for 90% of the TGF-β produced, which are absent in $MHCII^{-/-}$ mice. Monocytes and platelets account for a small portion of the TGF-β produced; however, need to be considered because both have the potential to interact with C3d through non-CR2 surface receptors. It has been demonstrated that TGF-β is produced by B-cells upon activation and this cytokine has an autocrine role (367). Since C3d induces B-cells to IgG class switch in the absence of T-cells, it can be speculated that B-cells are activated by C3d inducing TGF-β production, which will ultimately be the responsible for the dominant IgG2b isotype. Evidence for activation of other immune cells came from the other IgG isotypes induced in MHC $II^{-/-}$ mice prior to virus challenge. These mice also class switched to IgG_1 and IgG_{2a}, which are induced mainly by IL-4 (along with IL-5 and IL-13) and IFN-γ. These cytokines can be produced by CD8+ T-cells, which can interact with C3d through CR2. Other cells that can potentially be involved in the production of one these cytokines are NK, NKT and γ/δ T-cell (267, 340, 383); however, there is not a direct link between C3d and these cells, as neither CR2 nor non-CR2 receptors have been identified. Finally, the last line of evidence that C3d induces activation of more than one cell comes from the results following virus challenge in MHC $II^{-/-}$. It is well known that live influenza virus is able to induce class switch to IgG in mice deficient in CD4+ T-cells. Consistent with this, MHC $II^{-/-}$ mice that received A/PR/8/34 class switched to IgG. The anti-sHA IgG level was slightly higher in the sHA-

C3d3 than in sHA group; however, not statistically significant. Despite of the small difference in antibody titer, sHA-C3d3 mice had reduced weight loss (morbidity) and prolonged survival. This suggested that antibody was not the only factor involved in the protection against live virus challenge, but other immune factors were also involved. The principal cells that might have a role include: CD8+ T-cells, monocytes/macrophages, neutrophiles and platelets. It is known that T-cells express CR2 (106-108, 378), thus CD8+ T-cells may be activated by C3d. Furthermore, C3 derived factors are implicated in lung migration and activation of T-cells to the lungs during influenza infection, implying another role of C3d in CD8+ T-cells (379, 380). C3d can also interact with monocytes, macrophages, neutrophils and plateles through non-CR2 receptors, which are involved in cell activation or cytokine secretion (375). A summary of the different cells that can interact with C3d through CR and non-CR2 receptor is described in **Figure 20**.

The outcome on the immune response by antigens conjugated to C3d depends on the characteristics of the antigen and the way by which it interacts with the B-cell or other cells. Thus, some antigens fused to C3d might benefit more than others because of their intrinsic properties and abilities to interact with the immune cells. Hence, more antigens need to be explored in order to have a complete understanding of how C3d benefits different antigens. Special focus should receive diseases known to require antibodies for their prevention, as C3d enhances mainly the humoral immune responses. It also remains to be explored the consequences of using other adjuvants in conjunction with C3d. For example, CpG ODNs could be co-inoculated with chimera C3d proteins or antigen-C3d proteins could be encapsulated in liposome particles.

Overall, C3d is a safe adjuvant that enhances mainly the humoral immune responses by interacting with CR2 and non-CR2 receptors. The final adjuvant outcome seems to be an additive effect of the interaction with several cells, of which B-cell seem to play a major but not unique role.

VII.B. Future Directions

Given that one of the most studied mechanisms by which C3d works depends on CR2, a deeper understanding of this controversial interaction will help in the improvement of C3d as an adjuvant.

The C3d-CR2 interaction is complex. Several models, based on the crystal structure, as well as mutational analysis, have been described; however, there was not a consensus and some data is contradictory (142, 143, 145-147, 149, 152, 384). Recently, a new model based on theoretical electrostatic potential and pK_a calculations tried to reconcile the controversial results and suggested that the association of C3d with CR2 is predominantly electrostatic in nature and involves the whole molecule and not only the limited association sites that were previously studied (151). Hence, this model clarifies why mutations in completely opposite regions impaired the C3d-CR2 interaction. Until now, several mutations that impair the C3d-CR2 mutation have been described. However, only one mutation that increases the affinity of C3d for CR2 has been identified. Considering that the C3d-CR2 interaction is probably the main mechanism responsible for the

enhancement of the immune response, increasing the affinity of C3d for CR2 will also further increase the enhancement effect. One of the future studies will address this question and several C3d mutants will be generated. Mutations that impair as well as the mutation that enhances the immune response will be introduced. These mutant molecules will be tested using surface plasmon resonance technology (Biacore) to determine the affinity of C3d for CR2. One important consideration in these experiments will be the use of the full length CR2 molecule (16 short consensus repeats), versus the two first consensus repeats that are normally used. It has been suspected that more than only the first two short consensus repeats are involved in the C3d-CR2 interaction, thus the use of the full length molecule would provide data more relevant for the subsequent experiments in animal models. Following the *in vitro* interaction experiments, three tandem copies of the C3d mutants will be cloned with antigenic molecules and used to immunize animals. It will be expected that C3d mutants that enhance the affinity for CR2, will also enhance the immune response in wild-type animals. Later, the enhancement effect on non-CR2 receptors will be initially investigated in mice deficient for CR2.

Given that the C3d- non-CR2 receptor interactions also contribute to the enhancement effect, it will also be important to characterize these effects. These effects, however, involve more cells and the non-CR2 receptor(s) has not been isolated making more difficult the characterization. Initial studies will require *in vitro* work, where highly purified B-cell, monocyte, macrophages, neutrophils and platelets would require being isolated from wild type as well as a various knock-out mice such as $CR2^{-/-}$, $CD4^{-/-}$, MHC $II^{-/-}$, etc. These cells would require stimulation with C3d conjugated to different

molecules including LPS and other antigens and determine 1) binding to the surface 2) activation of the cell 3) signaling inside the cell 4) possible outcome of the signaling, such as protein production (*e.g*. cytokines) or increase of mRNA. Some of this analysis could be benefited from the use of proteomics assays to detect specific protein production.

The role of C3d in NK, NKT and γ/δ T-cells is suspected; however, neither CR2 nor non-CR2 receptors have been described. Thus, initial experiment intending to identify possible interactions between C3d and these cells *in vitro* would be required. Depending on the results, further studies using specific knock-out mice and reconstitutions experiments to identify the *in vivo* role of these cells would be required.

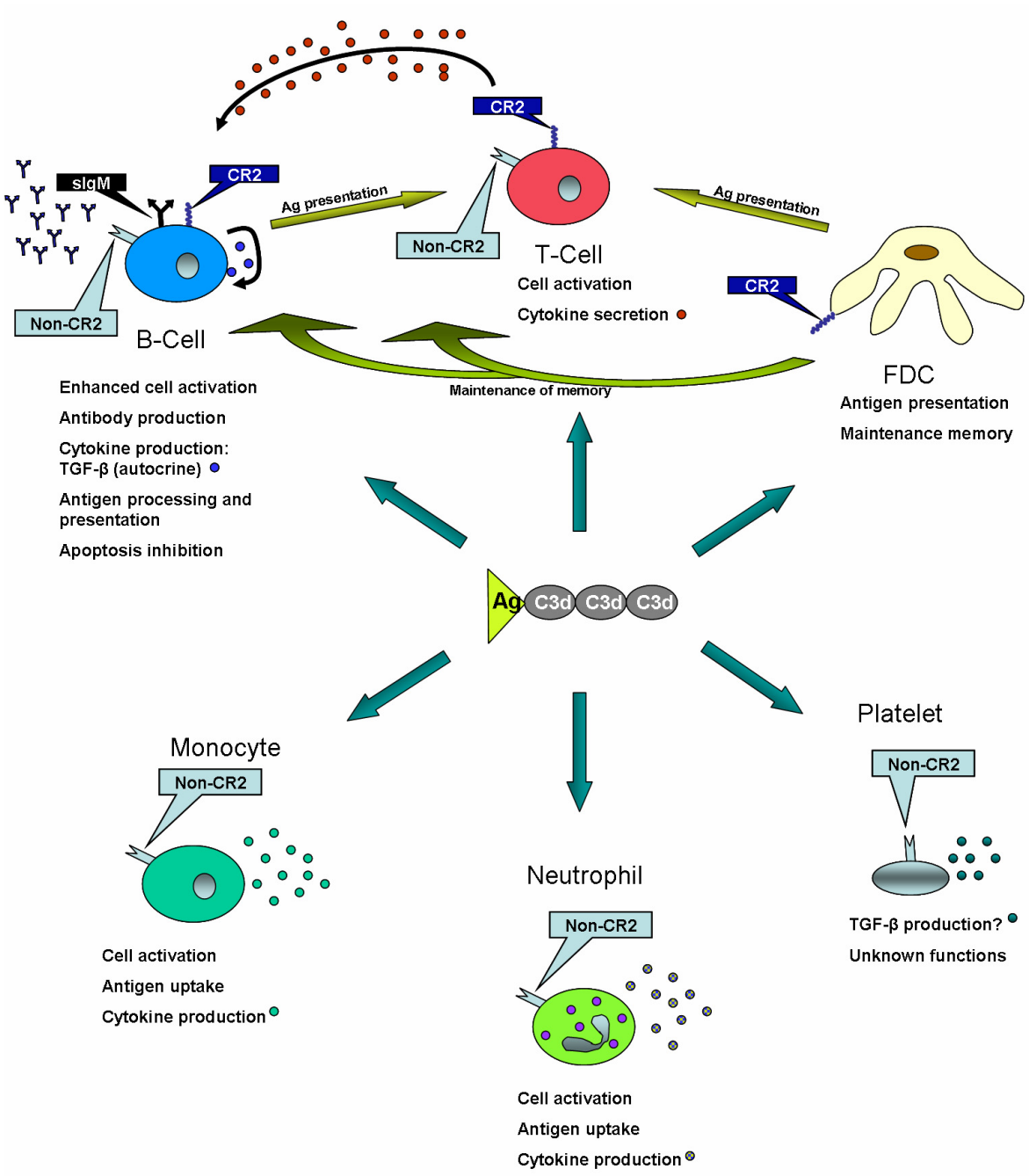

Figure 20. Cells that interact with C3d fused antigens and their possible outcome

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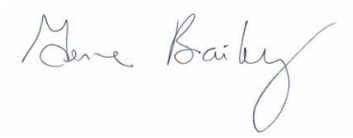

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