ENGINEERING IMMUNITY AGAINST HIV

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Kenneth K. Yu

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Some people say that science is an intensely personal enterprise, bringing to mind the image of a scientist working alone long into the night, in a solo quest to make Nature yield its secrets. There is some truth to that image, but it's far from the whole story. For me, I would have to say that science is an inherently social experience. It's hard to engage in it without becoming connected to people, and having one's self enriched by their presence. And thus it's a tremendous honor for me to write this acknowledgement, to express, perhaps inadequately, my deepest gratitude to the people without whom this work would not have been possible.

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for the lab, it was a little past 5 pm, the normal time they'd lock the doors to the buildings. As we neared Braun's east entrance, I started fumbling for my keys. Like a true experimentalist, you reached for the door handle and pulled the unlocked door open. With a smile on your face, you said to me, "Do the experiment!" To a theoretical physicist by training, that changed my world. Dear David, I have done the experiments, and the results are exciting. Thank you for opening the door.

ABSTRACT

An effective vaccine against the human immunodeficiency virus (HIV)-1 has so far been elusive. Anti-viral vaccines against other viruses work by stimulating the production of neutralizing antibodies that block infection. To be useful, an anti-HIV vaccine preparation needs to elicit potent neutralizing antibody response with sufficient breadth to cover the diversity of HIV variants. Despite sustained research efforts, such an immunogen has been difficult to develop. We could overcome this difficulty by using gene therapy to directly instruct the body to produce anti-HIV broadly neutralizing antibodies (bNAbs). In this thesis, I describe a technology I developed termed the "Molecular Rheostat" for directing the simultaneous expression of anti-HIV surface and secreted immunoglobulins using mutant 2A "self-cleaving" peptides. I describe the application of this system to the programming of hematopoeitic stem cells to generate anti-HIV B cells as a strategy to "vaccinate" against HIV infection. I then pivot to consider alternatives to B-cell programming to produce antibodies against HIV. I investigate the modification of non-lymphoid hematopoietic cells to produce antibodies using retroviral vectors and describe the use of lentiviral vectors to program muscle to produce anti-HIV broadly neutralizing antibodies. In addition to presenting a novel tool for controlling the simultaneous expression of full-length and truncated proteins, the work described here furnishes a foundation for future development into potential gene-therapeutic prophylaxis against HIV.

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CHAPTER 1: INTRODUCTION

AIDS at 30

The year 2011 marks the 30th anniversary of the first formal report of the disease that came to be known as AIDS (Acquired Immune-Deficiency Syndrome) caused by the human immunodeficiency virus (HIV). According to the most recent statistics available through UNAIDS, it is estimated that 34 million people globally were living with HIV/AIDS at the end of 2010, with 2.6 million new infections, and 1.8 million deaths directly attributed to AIDS (UNAIDS 2011). While the number of new infections has stabilized, the burden of the disease continues to grow globally.

In the summer of 2007, I went on a medical and humanitarian mission with the aid group, Project Africa Global, to Swaziland. Swaziland is a small sovereign kingdom inside South Africa with a population of a little over a million and the size of the State of New Jersey. We were the guests of the king and one of the princes, and we travelled in relative comfort and our lodging was pleasant. It was there that I witnessed with my own eyes the tragic consequences of the HIV/AIDS pandemic both on the individual level and on a society as a whole. That year, over 30 percent of Swazis were living with HIV/AIDS (and by some estimates, more than 40 percent). I remember the drives from our residence to the clinics in the countryside. Despite the seasonably warm weather and a clear blue sky, there was a palpable sense of doom and depression as we travelled on the rural roads. Fields lay fallow and unused, not because it wasn't the right season, but because

there was not enough man power to cultivate them. As we drove through the cities, at the height of the day, the streets seemed strangely quiet and abandoned. HIV/AIDS had cut down entire generations from Swaziland's population pyramid. The largest groups of people were those under the age of 18 and those older than 40. We arrived at a make-shift countryside clinic, and I beheld a sight that I would never be able to forget. As I worked with patients with various ailments (there were exactly 41 doctors in a country of one million, so a medical student was as close to a doctor as some of these people would ever see), a mother and her son came in to see us. Lovingly she carried her son in her arms. She came in for "severe ear infections", along with cough, blood tinged sputum, and fever at night that rattled the bones—the latter three being the classic signs of TB infection, and in the setting of this patient population, the ominous harbinger of full blown AIDS. But as I examined her, the thing that caught my eye, and the attention of everyone in the room, was a red, florid, alien-looking, fungal growth that had colonized much of her right ear and had also put down roots on the left side of her face. We tried our best to clean the wounds and gave her anti-fungal medications, but with extremely limited access to consistent anti-retroviral therapy and nutritional support, we realized that she did not have long. Her son suffered from diarrhea and a slew of other ailments that suggested to us that he too was infected. As the day in the clinic wound down, I began to reflect on the patients I saw. I thought of the people who were dying with HIV/AIDS and those who they would leave behind; then I thought of the young boy who

would lose his mother, and whose own life was threatened by the disease. I never wished more fervently for a vaccine.

Here in the United States, the CDC estimates that 1.2 million people are living with HIV infection and over half a million people have died since the epidemic began (National Center for HIV/AIDS 2011). The annualized medical costs per HIV infection in the U.S. was estimated to be approximately $24,000 per person per year (Farnham, Holtgrave et al. 2010). These numbers do not begin to capture the magnitude of the human suffering caused by HIV/AIDS; they say nothing of the fear and stigma associated with the disease.

The identification of the causative agent of AIDS, the human immunodeficiency virus or HIV, was announced by two separate research groups led respectively by Luc Montagnier and Robert Gallo in 1983 (Barre-Sinoussi, Chermann et al. 1983; Gallo, Sarin et al. 1983), for which Montagnier was awarded the Nobel Prize in Physiology or Medicine in 2008. Gallo and Montagnier's discovery brought great advances in the understanding of the molecular biology of the virus and the mechanisms of disease transmission and pathogenesis, and stimulated new developments in medicine at treatment, prevention, and control. The recognition that AIDS was transmissible by a virus led to early hopes for a quick vaccine. Those hopes were not entirely unreasonable, as our experiences with other viral diseases such as polio and smallpox suggested to us that it might be relatively easy to make a vaccine.

History turned out differently. Nearly 30 years after its initial identification, we still do not have a vaccine (yet). HIV is a virus so very different from those other viruses we had made effective vaccines for before that conventional vaccines did not work against it. For one thing, HIV belongs to the larger family of retroviruses that use the enzyme reverse transcriptase to make a copy of themselves that allows them to integrate into the host genome. Thus, once an infection is established, the virus effectively becomes a part of the host's genetic make-up. The reverse transcriptase, discovered independently by Dr. David Baltimore and Dr. Howard Temin in 1970, and for which discovery they shared the Nobel Prize in 1975, is the target of the first class of antiviral drugs. Several other classes of drugs have come online since then, through our improved understanding of the molecular biology of the virus. This knowledge has led to the development of HAART (highly active antiretroviral therapy), inspired by the work by Dr. David Ho, in which multiple drugs that target different molecular aspects of the HIV life cycle are given in combination. HAART was shown to be capable of suppressing the virus and controlling the progression of the disease for periods up to decades. However, these drugs do not provide a cure, are expensive, and cause significant side effects. The best way to stop or slow the epidemic is an effective vaccine that can prevent infection in the first place, and the need for it is as acute as ever (Baltimore 2002; Letvin, Barouch et al. 2002).

Toward an AIDS Vaccine: The Broadly Neutralizing Antibodies

Vaccines are antigen preparations that elicit immune responses against pathogens. The utility of vaccines is limited by the kinds of antibodies (Abs) that are made by the host immune system after vaccination. Most currently used anti-viral vaccines work by stimulating production of neutralizing antibodies (NAbs), which block viral infection (Zinkernagel, LaMarre et al. 2001; Burton 2002). HIV is an enveloped retrovirus that presents problems for antibody-based vaccine strategies. The virus rapidly mutates to change residues on its surface, sheds immunodominant decoy epitopes, masks immunogenic sites on its surface with host-derived carbohydrates, and/or hides conserved regions in the interfaces of oligomeric proteins (Burton, Stanfield et al. 2005; Berkley and Koff 2007). While neutralizing antibodies against HIV do emerge in the natural course of infection, they occur too late, after an infection has already been established, and are thwarted by rapid genetic mutation of the virus (Richman, Wrin et al. 2003). For all of these reasons, it has been exceedingly difficult to design an immunogen that would elicit an anti-HIV antibody response of sufficient quality and breadth to be protective (Burton, Desrosiers et al. 2004; Flynn, Forthal et al. 2005; Pitisuttithum, Gilbert et al. 2006; Johnston and Fauci 2007; Fauci, Johnston et al. 2008).

Broadly neutralizing antibodies (bNAbs) against HIV do exist; they are an unusual class of antibodies that neutralize a broad range of HIV variants (Burton, Stanfield et al. 2005). Produced by few individuals, bNAbs are rare, but some have been

immortalized as monoclonal Abs (Burton, Stanfield et al. 2005). Passive immunization with bNAbs has been shown to protect animals against simian-HIV (SHIV) challenge (Mascola 2002). Indeed, long-term-non-progressors (individuals who have remained free of disease without treatment more than 10 years after HIV infection) exhibit broadly cross-reactive bNAb responses (Pilgrim, Pantaleo et al. 1997). The existence of bNAbs suggests that it might be possible to prevent HIV infection and subsequent disease by producing bNAbs in individuals at risk for AIDS (Burton 2002; Ferrantelli, Rasmussen et al. 2002; Burton, Desrosiers et al. 2004). Broadly reactive human bNAbs shown to protect against HIV challenge in animal models include b12, 2G12, 2F5, 4E10, and more recently VRC01 (through work done in our lab), among some others. Some of these bNAbs exert their effects by preventing the trimeric HIV-1 envelope complex (GP120-GP41) from binding to the host receptor (CD4) or co-receptor (usually CCR5 or CXCR4); others inhibit fusion of the virus with a host target cell by binding to the envelope protein after virions have attached to a target cell (Xiao, Dong et al. 2002; Burton, Desrosiers et al. 2004). The bNAbs 2G12 and 2F5 have been verified to be safe for use in humans in phase I clinical trials (Wolfe, Cavacini et al. 1996; Cavacini, Samore et al. 1998; Armbruster, Stiegler et al. 2002), and evidence for anti-viral activity was seen in HIV-infected patients treated with these bNAbs (Stiegler, Armbruster et al. 2002).

Engineering Immunity Against HIV

It occurred to Dr. Baltimore that if we could directly instruct the immune system to produce broadly neutralizing anti-HIV antibodies, we might be able to use them as "vaccines" to prevent HIV infections. Gene therapy technology provides the means for us to genetically program immune cells. Using gene therapy to deliver broadly neutralizing antibodies, we would be able to provide them to people as a prophylaxis against HIV infections. We call this approach "Engineering Immunity".

The most natural way to provide broadly neutralizing antibodies to people is by engineering B cells, as they are the immune cells that are responsible for the production of antibodies. They begin their life in the bone marrow as descendants of the more primitive common hematopoietic stem and progenitor cells. As these cells develop into B cells, they undergo sequential RAG1/2-mediated DNA rearrangement of the heavy and light chain immunoglobulin gene loci in a process called V(D)J rearrangement. This is a pseudo-random process in which different V, (D), and J segments are combined together with the addition of certain non-templated nucleotides to produce a great diversity of antigen binding regions. Cells that successfully complete this process and assemble a functional IgM B cell receptor (BCR) on their surface are able to leave the bone marrow to continue further development in the peripheral lymphoid compartments (Burrows and Cooper 1993; Chen and Alt 1993). It has been shown in transgenic animals that provision of a pre-rearranged IgM heavy chain and light chain transgene shuts down the

rearrangement of endogenous heavy and light chain genes (allelic exclusion), and guides the ordered development of functional B cells with specificity defined by the transgene (Spanopoulou, Roman et al. 1994; Young, Ardman et al. 1994).

The mature B cells patrol the body in the general and lymphatic circulations, using their BCRs as antigen sensors. When a cognate antigen engages the BCR, the B cell becomes activated and enters into germinal center reactions in the lymph node or spleen in a dance of mutual activation with T cells; this process leads to further development into memory B cells or differentiation into antibody-producing plasma cells. The memory B cells will provide a more rapid and higher quality antibody response in the future when the same antigens are encountered again. The plasma cells produce antibodies against the inciting antigens, which leads to their eventual clearance from the body (McHeyzer-Williams and McHeyzer-Williams 2005).

As B cells differentiate into plasma cells, they switch from producing the membrane-bound BCR to making a soluble, secreted antibody. The switch is accomplished on the level of RNA processing by alternative splicing of the 3’ end of the heavy-chain primary RNA transcript (Peterson, Gimmi et al. 1991; Peterson 2007). This replaces the hydrophobic amino acids that form the membrane anchor with a hydrophilic tail that enables the secretion of the BCR as free antibody. The antibody retains the same specificity and isotype as the BCR.

Some of the cells in the germinal center reactions also go through a process called isotype switching, in which the heavy chain constant regions of the initial IgM BCR are replaced with that of another isotype, which encodes different effector functions. This involves a DNA rearrangement mediated by the enzyme activation-induced cytidine deaminase (AID) (Muramatsu, Kinoshita et al. 2000). While the IgM BCR is required for the normal development of B cells in the bone marrow, and the IgM antibody is generally the first antibody isotype produced against an antigen, alternate isotypes provide additional effector functions that enhance the ability of the antibody to clear certain types of pathogens or to function in different body compartments. For example, in addition to fixing complements on target cells, as IgM antibodies can, IgG antibodies also have the ability to direct the killing of antibody-bound infected cells by engaging Fcγ receptors on NK cells (termed ADCC, or antibody-dependent cell-mediated cytotoxicity). IgA antibodies are produced by plasma cells in mucosal areas and are transported across the epithelial barriers of the lung, gut, and genital tracts by binding the polymeric immunoglobulin receptors (pIgR) with their Fc portions. These antibodies are critical in the defense of mucosal surfaces from pathogens.

Our understanding of the humoral immune response as summarized above forms the framework for my efforts to engineer the immune system. This framework suggests to us that by delivering a cleverly designed, synthetic immunoglobulin gene to the hematopoietic stem and progenitor cells using gene therapy, we would be able to direct

the development of B cells that would produce broadly neutralizing antibodies against HIV with pre-programmed specificity and effector functions. Specifically, the synthetic immunoglobulin gene should 1) encode a mechanism that directs the production of both an IgM-like membrane-bound BCR and a secreted immunoglobulin isotype that has the desired effector properties, such as those of an IgG antibody, and 2) it should bind and neutralize HIV with the specificity and affinity of an anti-HIV broadly neutralizing antibody. Those were the two objectives that I set out to accomplish in my work when I joined Dr. Baltimore's team in 2006, and this work will be described in detail below. As the project proceeds, I also explored a few other alternatives to this original approach by looking at cell types other than B cells as targets for engineering to produce anti-HIV antibodies, and the results obtained are summarized in separate chapters of this thesis.

Overview of Thesis

Chapter 1 gives a short, personal introduction to the HIV/AIDS epidemic and briefly reviews some significant scientific advances that have been made in the fight against HIV/AIDS. It then gives a succinct review of the broadly neutralizing antibodies against HIV and the biology of B cells that form the background of the Engineering Immunity project.

Chapter 2 of this thesis describes a novel approach I have developed to genetically program hematopoietic cells to become anti-HIV B cells that we call a "Molecular Rheostat" for antibody genes. The focus will be on the *in vitro* development

and characterization of this technology. I will show that the Molecular Rheostats provides a useful tool for manipulating B cell specificity and gives us the ability to program them to produce a bNAb against HIV.

Chapter 3 describes my attempt to use the Molecular Rheostats to program B cells *in vivo*. It summarizes what we have learned from testing the system in both the human-immune-system (HIS) mouse model and the murine bone marrow adoptive transfer model. We describe certain limitations of the lentiviral vector system we developed and suggest what we might do to overcome the limitations

In Chapters 4 and 5 I pivot to look at alternative approaches to make broadly neutralizing antibodies *in vivo*. Chapter 4 describes the use of retroviral vectors to program non-lymphoid hematopoietic cells to produce antibody long-term. Chapter 5 describes my effort to study the feasibility of using lentiviral vectors to program muscle.

In Chapter 6 I give a short summary of this work, speculate on directions for future investigations, and offer some concluding remarks.

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CHAPTER 2: USE OF MUTATED "SELF-CLEAVING" 2A PEPTIDES AS "MOLECULAR RHEOSTATS" TO DIRECT SIMULTANEOUS FORMAION OF MEMBRANE AND SECRETED IMMUNOGLOBULINS

Abstract

In nature, B cells produce surface immunoglobulin and secreted antibody from the same immunoglobulin gene via alternative splicing of the pre-messenger RNA. Here we present a novel system for genetically programming B cells to direct the simultaneous formation of membrane-bound and secreted immunoglobulins that we term a "Molecular Rheostat" Immunoglobulin gene, based on the use of mutated "self-cleaving" 2A peptides. The Molecular Rheostats are designed so that the ratio of secreted to membrane-bound immunoglobulins can be controlled. Lentiviral transgenesis of the Molecular Rheostat constructs into B cell lines enables the expression of functional b12-based BCRs that signal to the cells and mediate the secretion of b12 IgG broadly neutralizing antibodies that can bind and neutralize HIV-1 pseudovirus. We show that these b12-based Molecular Rheostat constructs promote the maturation of EU12 B cells in an *in vitro* model of B lymphopoiesis. The Molecular Rheostat Immunoglobulins offer a novel tool for genetically manipulating B cell specificity with implications for B-cell based gene therapy.

Introduction

B cells are responsible for the production of antibodies in response to foreign antigens. The ability to manipulate the antigen specificity of B cells and that of the antibody produced by these cells could be useful for achieving immunization against deadly pathogens such as HIV. In this chapter, I describe a novel way of programming B cells by using mutated 2A peptides to direct the simultaneous formation of an IgM-like BCR and IgG antibody. The system is designed so that the ratio of surface-to-secreted immunoglobulins can be controlled by appropriate choice of mutations. We call this system a "Molecular Rheostat" for immunoglobulin gene expression.

B cells begin their life in the bone marrow as descendants of the more primitive common hematopoietic stem and progenitor cells. As these cells develop into B cells, they undergo sequential RAG1/2-mediated DNA rearrangement of the heavy and light chain immunoglobulin gene loci in a process called V(D)J rearrangment. Cells that successfully complete this process and assemble a functional B cell receptor (BCR) of the IgM isotype on their surface are able to leave the bone marrow to continue further development in the peripheral lymphoid compartments (Burrows and Cooper 1993; Chen and Alt 1993). The generation of the IgM BCR is central to B cell devevelopment and function. It is both necessary for the normal development of B cells (Kitamura, Roes et al. 1991; Kitamura and Rajewsky 1992; Wagner, Williams et al. 1994), and sufficient for directing B cell development. In transgenic animals. the provision of a pre-rearranged

IgM heavy chain and light chain transgene shuts down the rearrangement of endogenous heavy and light chain genes (allelic exclusion), and guides the ordered development of functional B cells with specificity defined by the transgene (Spanopoulou, Roman et al. 1994; Young, Ardman et al. 1994). See Chapter 1, pp. 7-9, for more details on the process of B cell development and developmentally regulated switch from membrane to secreted Ig production.

2A peptides are "self-cleaving" peptides that are derived from animal viruses and multicellular parasites of mammals (de Felipe 2004; Szymczak and Vignali 2005). They are involved in the processing and expression of polyproteins. Mechanistically, these peptides do not really undergo a "self-cleaving" event in the sense of breaking a *pre-existing* peptide bond; rather the presence of the 2A element in the mRNA causes the translating ribosome to undergo an intra-ribosomal, translational termination-and-restart event during the synthesis of nascent polypeptide chains. The peptide bond between the first and second polypeptide deriving from the same mRNA is in fact not formed during translation. As a result, when these two polypeptides are liberated from the ribosome, they appear as two separate proteins (de Felipe, Hughes et al. 2003; Doronina, de Felipe et al. 2008; Doronina, Wu et al. 2008). Because the apparent effect is as if a single polypeptide had been cleaved by an enzyme post-translationally into two separate polypeptides, for consistency with their historic description, I will still refer to 2A peptides as "self-cleaving" peptides, even though in reality they mediate a ribosomal

stop-and-restart event. Several 2A peptides appear to have near 100% cleavage efficiency in their native contexts, but they can be made to cleave at lower efficiencies when they are mutated at key amino acid residues or introduced into non-native sequences (Ryan and Drew 1994; Donnelly, Hughes et al. 2001; Donnelly, Luke et al. 2001). By engineering the peptides with reduced efficiency of cleavage, we show that we can co-express the BCR and antibody molecule simultaneously. We will call the system a "Molecular Rheostat" for immunoglobulin genes.

Materials and Methods

Constructs

The Molecular Rheostat constructs were created by cloning a transgene containing the EEK promoter, the b12 light and heavy chains, the 2A sequences, and and the 3' region of the human IgM BCR gene corresponding to the last 41 amino acids into either a pHAGE2 or pHAGE6 vector system. The Igα and Igβ genes were cloned into a FUW vector.

Transfections

293T cells were grown to 50–75% confluence on 30 cm dishes and were transfected in 15 ml D10 media (DMEM plus 10% heat-inactivated fetal bovine serum, supplemented with 20 mM L-glutamine, 1000 IU/ml penicillin, and 1000 µg/ml streptomycin, filtered through a 0.22 µm PES membrane bottle-top filter) for 24 h. The transfections used the

TransIT-293 reagent (Mirus Bio, Madison WI) or BioT (Bioland Scientific, Paramount CA) according to manufacturer's instructions using a total of 40 µg DNA.

Lentiviral Vector Production

293T cells were transfected with lentiviral vectors. After 24 h of incubation, the supernatant was pipetted off the cells and filtered through a 0.22 µm PES membrane bottle-top filter into a collection bottle. 15 ml of fresh D10 media was then filtered through the bottle-top filter into the collection bottle to reduce virus waste from supernatant that the filter absorbed. The collected supernatant was stored at 4°C, and 30 ml of fresh D10 media was added to the dish. This collection process into the same collection bottle was repeated 4 to 5 additional times at 12 h intervals. All of the collected supernatant was centrifuged at 10000 rpm for 12–24 h at 4°C to pellet the virus, and the supernatant was poured off the pellet. The pellet was re-suspended in 500–1000 µL DMEM media (for 293T transductions) or RPMI media 1640 (for OCI-Ly7 or EU12 transductions) and incubated on ice at 4°C for 12 h.

Lentiviral Transductions

0.5–1 × 10^6 293T, OCI-Ly7, or EU12 cells were suspended in 1 mL of D10 media for 293T transductions or C10 media (RPMI 1640 plus 10% heat-inactivated fetal bovine serum, supplemented with 25 µM β-mercaptoethanol, 1000 IU/ml penicillin, and 1000 µg/ml streptomycin, filtered through a 0.22 µm PES membrane bottle-top filter) for OCI-

Ly7 or EU12 transductions in 12 well plates, and 400–600µL of virus re-suspensions or dilutions thereof was added to each well. 10 mg/mL polybrene (Millipore, Billerica, MA) was added so that the final polybrene concentration was 10 µg/mL in each well. The transductions were incubated for 24 h before the cells were passaged.

Cell Line

The 293T-Igα/β cell line was created from a vector carrying the Igα and Igβ genes using the transfection, lentiviral production, and lentiviral transduction procedures above.

Tissue Culture

293T and 293T Ig-αβ cells were grown in D10 media. The cells were passaged 1:5 every other days. OCI-Ly7 and EU12 cells were grown in C10 media. The cells were passaged 1:5–1:10 every other day to maintain a density between 10^5–10^6 cells/ml.

Flow Cytometry

For flow cytometric analysis, cells were first washed in PBS with 2% FBS, and then stained with combinations of the following antibodies: anti-human-IgG-APC (BD Pharmingen, San Diego, CA), anti-human-IgG-PE (BD Pharmingen), anti-human-IgM-PE/Cy5 (BD Pharmingen), anti-CD10-PE (Biolegend, San Diego, CA). The cells were then analyzed on a BD FACSCalibur flow cytometer.

Cell Sorting

Cells were prepared as in flow cytometric analysis and were sorted with the assistance of Sylvia Chavira at the University of Southern California's Clinical Pathology Laboratory using a MoFlo FACS cell sorter.

Calcium Flux Assay

Calcium flux measurements were made essentially using the protocol described by Bondada, et al. [29], with the following modifications: cells were washed, pelleted, and resuspended in dye loading buffer (HBSS with Ca^{2+} and Mg^{2+} plus 4% 100mM probenecid, 2% 1 M HEPES buffer, and 1% heat-inactivated fetal bovine serum) and were incubated with 4 µg/mL Fluo-3 AM and 1 µg/mL FuraRed AM dyes in the presence of 0.02% (w/v) pluronic F-127 for 30 m. The cells were again washed, pelleted, and resuspended in dye loading buffer and were kept at room temperature until they were analyzed on a BD FACSCalibur flow cytometer equipped with a circulating 37°C water bath on the sample port. During analysis, cells were stimulated with goat $F(ab')_2$ anti-human IgG γ F_c-specific antibodies (Invitrogen, Carlsbad, CA) or with goat $F(ab')_2$ anti-human IgM µ F_c-specific antibodies (Southern Biotech, Birmingham, AL) and a ratiometric measurement between the Fluo-3 AM and FuraRed AM dye channels was made for 512 s. On some samples, ionomycin controls were performed to calibrate the dynamic signaling range.

ELISA

Supernatants from cultured cells were analyzed using Human IgG ELISA Quantitation Set (Bethyl Laboratories, Montgomery, TX) according to manufacturer's instructions.

Biacore Binding Assay

Biacore binding assays were performed as previously described by Klein et al. (2009), with the following modifications: All experiments were done in-house. b12 antibody supernatants were produced from transfection of 293T cells.

In Vitro *Neutralization Assay*

In vitro neutralization assays were performed as previously described by West et al. [30], with the following modifications: All experiments were done in-house. Pseudoviruses were produced by co-transfecting HEK293T cells with an Env SF162 expression plasmid and a replication-defective backbone plasmid, PSG3minusEnv. Each mutant F_c and unmodified fragment version of b12 samples was tested in duplicates.

Results

IgM Molecular Rheostat Immunoglobulin Genes Mediate Co-Expression of IgM-Like BCR and Secreted IgM Antibody

As a pilot experiment to test whether the mutated 2A peptides can mediate co-expression of surface and secreted immunoglobulins, we constructed the first-generation Molecular Rheostat Immunoglobulin genes by joining the secreted version of the b12 IgM heavy chain to the transmembrane domain of the IgM BCR via a mutated 2A peptide. The transmembrane domain is defined as the M1 and M2 exons from the human IgM locus and comprises the last 41 amino acids of the membrane bound IgM BCR (Figure 2.1A). We call these "IgM Molecular Rheostats". We chose the wild type F2A and two mutant peptides as well as another F2A-like element derived from a silk-worm virus, based on previous work by Donnelly et al. (Donnelly, Hughes et al. 2001), in which they observed reduced cleavage efficiencies when certain mutations are introduced. The four mutants we chose are designated F2A, F2A(3), F2A(14), and I2A(2). See Table 2.1 for the nomenclature and the amino acid sequence for each of the 2A elements.

We cloned these IgM Molecular Rheostat genes into a lentiviral vector plasmid (FMHW) that doubles as a mammalian expression vector under the control of a CMV promoter. We co-transfected this vector together with a separate vector carrying the b12

light chain (FEEK-b12L) and a mammalian expression vector carrying the human Igα and Igβ genes (phIgαβ) into 293T cells (Figure 2.1A). We analyzed the cells and their supernatants by FACS and human IgM ELISA 48 hours later. All transfected cells showed surface expression of the IgM Molecular Rheostat BCR and secreted IgM into their supernatants (Figure 2.1B and 2.1C).

IgG Molecular Rheostat Mediates Expression of an IgG/M Chimeric BCR and Secreted IgG Antibody

We next attempted to adapt the Molecular Rheostat format to the production of an IgG antibody in an effort to mimic an isotype-switched secretory IgG while preserving the signaling properties of an IgM, which is required for normal B cell development. Furthermore, we wished to explore whether we could manipulate the ratio of surface-bound to secreted immunoglobulins by making appropriate mutations in the 2A elements. To test these ideas, we constructed a library of chimeric IgG Molecular Rheostat immunoglobulins, in which a complete secretory b12 IgG is joined to the transmembrane anchor of the IgM BCR via different 2A peptides (Figure 2.2). The library includes all 2A peptides listed in Table 2.1.

To reduce the number of vectors that need to be transfected and anticipating the need to use the vectors in the context of lentiviral transduction, where it would be advantageous to work with a single vector, we fused the b12 light chain with the Molecular Rheostat transgene by joining the b12 light chain to the b12 heavy chain via a

different F2A element, F2Aopt. F2Aopt is codon-optimized for human expression and contains a furin cleavage site before the 2A element.

Additionally, to ensure consistency of Igα and Igβ expression across the cells used to test the Molecular Rheostat constructs and reduce the number of vectors that need to be transfected, we engineered 293T cells that express human Igα and Igβ by repeatedly co-infecting 293T cells with two lentiviral vectors, FUW-Igα and FUW- Igβ, which carry the Igα and Igβ transgenes, repectively, under the control of a ubiquitin C promoter. The resulting cells are denoted 293T-Igαβ cells.

We transfected the library of IgG Molecular Rheostat constructs into the 293T-Igαβ cells, and 48 hours later analyzed the cells and their supernatants for surface IgG by FACS and secreted IgG by ELISA, respectively. All transfected cells showed surface expression of the IgG Molecular Rheostat BCR and secreted IgG into the culture supernatant (Figure 2.2B and 2.2C). Significantly, while the surface expression of the Molecular Rheostat BCR appears comparable across all constructs, there is a range of levels of secreted IgG. This suggests that the different Molecular Rheostats could be used to produce a range of ratios of surface to secreted immunoglobulins.

IgG Molecular Rheostat Mediates Expression of a Range of Ratios of Surface BCR to Secretory IgG in the Human B-Cell Line OCI-Ly7

To validate the results that the IgG/M Molecular Rheostat constructs can mediatea range of expression ratios of surface BCR to secreted antibodies in human B cells, we used lentiviral vectors to deliver the constructs into the OCI-Ly7 B cell line, which expresses an endogenous IgM BCR on its surface and therefore should possess the necessary machinery (such as Igα and Igβ co-receptors) for BCR surface expression. To provide an independent marker of lentiviral transduction than the expression of the Molecular Rheostat immunoglobulins, we constructed a lentiviral vector, pHAGE2-EEK-IRES-ZsGreen, which contains an Internal Ribosomal Entry Site (IRES) driving a ZsGreen fluorescent protein gene. Based on the results in Figure 2.2B, we selected six of the IgG/M Molecular Rheostat genes and cloned them into the first position (before the IRES-ZsGreen) of the pHAGE2-EEK-IRES-ZsGreen vector. We then infected OCI-Ly7 cells with the IgG Molecular Rheostat vectors at low MOI (~ 0.1) to ensure that every cell that was infected had at most one copy of the transgene (Figure 2.3A). 48 hours after infection, we FACS-sorted out the ZsGreen positive cells and allowed these cells to expand for another 48 hours.

The cells and supernatants were analyzed by FACS and ELISA (Figure 2.3B, left and right panels, repectively). The different mutants produced a range of ratios of surface-to-secreted immunoglobulins. Significantly, there is an inverse relationship

between the amount of IgG Molecular Rheostat BCR expressed on the surface of the cells vs. the amount of IgG antibody that was detected in the supernatants, indicating that the mutant 2A elements were behaving like a "rheostat", tuning the ratios of surface-to-secreted immunoglobulins. Also notably, the rank order of the ratios of surface-to-secreted immunoglobulin expression recapitulates what was observed from the transfection into 293T-Igαβ cells (see Figure 2.2B and C). For example, from Figure 2.2B and C, F2A(-2) would be expected to make more secreted IgG than F2A(-4), and this was indeed the case when the constructs were expressed in the OCI-Ly7 B cell line. Furthermore, F2A(-2) made less surface Molecular Rheostat BCR than F2A(-4), as would be expected if the F2A(-2) peptide mediated more efficient cleavage than the F2A(-4) peptide. The library of mutants thus gives us a Molecular Rheostat system that we can use to direct tunable ratios of expression of surface vs. secreted immunoglobulins.

IgG Molecular Rheostat Constructs Produce Functional b12 IgG/M Chimeric BCRs are Signaling Competent and Bind to HIV GP120

To test whether the IgG/M chimeric BCR produced by the IgG Molecular Rheostat genes is functional, we developed a ratiometric Fluo-3/FuraRed calcium flux assay in which anti-BCR crosslinking antibodies are used to examine whether the BCRs are able to signal in the OCI-Ly7 B cells. We chose two of the 2A peptides from the library, F2A, which cleaves with high efficiency, and I2A(2), which does not cleave well.

As the ZsGreen protein interferes with the Fluo-3 calcium-sensitive dye used in the assay, we cloned those two IgG Molecular Rheostat immunoglobulins into lentiviral vectors that do not have the IRES-ZsGreen marker gene. Lentiviral infections of OCI-Ly7 B cells with these vectors resulted in a variegated pattern of expression of the BCRs. The vector containing the I2A(2) element showed generally higher levels of surface BCR expression than F2A, as expected. While both populations responded to BCR stimulation using a control anti-IgM antibody (Southern Biotech, Birmingham, AB) and an anti-IgG antibody (Sigma, St Louis, MO), the responses were detectable but modest (data not shown). We believe the modest response was due to the effect of averaging the calcium signals over the large range of surface expressions. To ensure we have more homogenous populations for use in BCR stimulations, we FACS sorted out the top 10% of IgG positive cells from each of the populations (Figure 2.4B), and performed the calcium flux assays on the sorted cells. The cells responded robustly to anti-BCR stimulation (Figure 2.4A), with a dose-response correlating with the levels of surface IgG Molecular Rheostat BCR expression and the concentrations of anti-Ig used. The higher anti-IgG dose (100 ug/ml) gives a stronger calcium signal than the lower dose (20 ug/ml); the higher amount of surface Molecular Rheostat BCR also generates a stronger and more lasting response.

Additionally, to see whether the IgG/M chimeric BCR would bind to HIV antigens, we co-stained the sorted OCI-Ly7 cells with fluorescently labeled HIV $gp120_{MN}$ and anti-IgG interacting with the anti-GP120 epitope of b12 and the γ heavy

chain constant region of b12 IgG, respectively (Figure 2.4C). We found that the Molecular Rheostat BCRs on the cells bound to HIV GP120.

IgG Molecular Rheostat Constructs Produce b12 IgG Antibody that Neutralizes HIV Pseudovirus with Same Potency as Unmodified b12 IgG

To determine whether secreted b12 IgG from the Molecular Rheostat system can neutralize infectious virus, we performed an *in vitro* pseudovirus neutralization assay using an Env SF162 pseudotyped HIV-1 pseudovirus on the TMZ-b1 reporter cell line with supernatants from 293T cells transfected with several different IgG Molecular Rheostat constructs according to a protocol previously described by Klein et al. (Klein, Gnanapragasam et al. 2009). The neutralization curves demonstrated that secreted Molecular Rheostat b12 IgG antibodies neutralized the Env SF162 pseudovirus as potently as the control b12 IgG antibody (L+H), with IC_{50} values nearly identical to that of the control b12 IgG (Figure 2.5A). We also performed a surface-plasmon resonance GP120-binding assay. The antibodies tested bound GP120 as well as the control b12 IgG antibody, consistent with the neutralization assay results (Figure 2.5B).

Expression of IgG Molecular Rheostat Immunoglobulins Promote Maturation of EU12 Cells in an In Vitro *Model of B Cell Development*

The promotion of B cell development is one of the major functions performed by the IgM BCR. It thus also offers a stringent test of BCR function. To test whether the IgG

Molecular Rheostat Immunoglobulin BCR can direct B cell development, we adopted a model of human B cell development using the EU12 system (Zhang, Wang et al. 2003; Zhang 2007). The EU12 cells are derived from a B cell leukemia patient, and the cells are $CD19^{+}$ and exist in a spectrum of primitive ($CD34^{+}$ and $CD10^{-}$, or $CD34^{+}$ and $CD10^{+}$) to more mature ($CD34^{-}$ and $CD10^{+}$, or $CD34^{-}$ and $CD10^{-}$) states. These cells lack a functional BCR, but rarely an IgM BCR is generated spontaneously and the cells proceed to acquire a more mature phenotype.

We isolated early-stage, CD 34^{+} EU12 cells by FACS sorting. These cells were then transduced with lentiviral vectors carrying IgG Molecular Rheostats that give rise to respectively low, intermediate, and high surface BCR expression. A luciferase-carrying vector was used as a control. The cells were allowed to expand, and 4 weeks after transduction the surface expression of IgG Molecular Rheostat BCR and maturation markers were analyzed by FACS (Figure 2.6). The EU12 cells transduced with Molecular Rheostat constructs tuned for different levels of surface BCR *vs.* secreted antibody expression showed the expected levels of surface BCR expression (F2A was used for maximum secretion; F2A(11) for intermediate; F2A(19) for maximal surface). Using ZsGreen as a measure of the amount of gene expression from the entire cassette in each cell, the level of surface IgG Molecular Rheostat BCR expression correlates with the ZsGreen expression level for each of the three Molecular Rheostat constructs (Figure 2.6A).

Gating on the high-expressing cells, we analyzed CD34 and CD10 expression by FACS. We found that the cells that had been transduced with Molecular Rheostats tuned to higher BCR expression and less secreted antibody have larger populations of cells that down-regulated CD10 (Figure 2.6B). This provides further evidence that the IgG/M chimeric BCRs produced by the IgG Molecular Rheostat Immunoglobulins are functional BCRs and can promote maturation of B lineage cells.

Discussion

To provide a compact system for genetically manipulating the BCR and antibody specificity of B cells with a lentiviral vector, we created the Molecular Rheostat Immunoglobulins to direct tunable simultaneous formation of the membrane-bound and secreted immunoglobulins by using mutant 2A "self-cleaving" peptides (Figure 2.7).

This system provides a synthetic approximation to the natural process of the mRNA alternative splicing-mediated switch to make membrane and secreted immunoglobulins. By fusing an IgG to the membrane anchor of IgM through a mutant 2A peptide that functions as a Molecular Rheostat, we constructed both IgM and IgG/M chimeric versions of Molecular Rheostat immunoglobulins. We showed that such a design could produce both membrane bound and secreted immunoglobulins and demonstrated that we could generate a library of mutant 2A elements to provide a range of tunable ratios of membrane-bound to secreted immunoglobulins by appropriate choice of mutations. We also showed that the surface chimeric IgG Molecular Rheostat BCRs signal to B cells and that these BCRs bind to HIV gp120 antigens. We showed that the secreted version of b12 IgG produced by the Molecular Rheostat constructs also bound GP120 and neutralized HIV-1 pseudovirus equally as well as unmodified b12 IgG. While the responsiveness of the BCRs was seen upon stimulation with anti-IgG antibodies that can cross-link the BCRs, it is possible that this responsiveness would be observed upon stimulation with any molecule(s) that can cross-link the BCRs, including multimeric

forms of gp120 and possibly HIV spike complexes. Finally, we provided evidence suggesting that the chimeric BCR produced by the Molecular Rheostat system can direct maturation of B cells using a cell line model of B cell maturation. In EU12 cells transduced with vectors carrying the Molecular Rheostat Immunoglobulins, we observed increasing $CD10^-/CD\ 34^-$ populations in the cells that received increasingly more surface-biased Molecular Rheostat constructs, suggesting that the chimeric IgG Molecular Rheostat BCRs are capable of directing the normal B cell maturation progression from $CD10^-/CD34^+$ to $CD10^+/CD34^+$ to $CD10^+/CD34^-$ to $CD10^-/CD34^-$. We note, however, that the $CD10^-/CD34^+$ populations were also greater in cells that were treated with Molecular Rheostat immunoglobulins biased toward higher surface BCR expression. At first glance, this might be explained by the downregulation of CD10 alone as a result of the expression of chimeric BCR. However, that the ratio of the most mature $CD10^-$, $CD34^-$ double negative population to the most primitive $CD10^-, CD34^+$ population also increases with the use of surface-baised Molecular Rheostat immunoglobulins suggests that the chimeric Molecular Rheostat BCR gives the more mature cells a proliferative advantage over the more primitive cells. This is consistent with the hypothesis that the Molecular Rheostat immunoglobulin genes promoted maturation of the cells.

While one might imagine encoding the entire heavy chain and light chain locus into a vector to program B cells, the heavy chain locus alone is ~ 1 Mb, too big to incorporate into a lentiviral vector with a coding capacity of ~ 10 kb. We had attempted

earlier to remove the introns of the heavy chain locus, except for the one required for the alternative splicing of the secreted and transmembrane exons. Our efforts to get those constructs to splice were not successful. We thus created the Molecular Rheostat system to mimic the natural system, incorporating the additional feature of expressing isotype-switched IgG antibodies while maintaining the signaling properties of an IgM transmembrane domain. Our results suggest that the Molecular Rheostat system, which is small enough to be introduced into cells with a lentiviral vector, could be used to direct the *in vivo* maturation of anti-HIV B cells. A detailed *in vivo* characterization of the Molecular Rheostat system in animal models would be necessary to test this idea. We propose to use this system to transduce hematopoietic stem cells or B cells in transplant models as a prophylactic "vaccine" against HIV infections. Work is currently under way to study the use of these Molecular Rheostat Immunoglobulins *in vivo* as a vaccination strategy against HIV, but the system may be used to manipulate B cells to target other antigens.

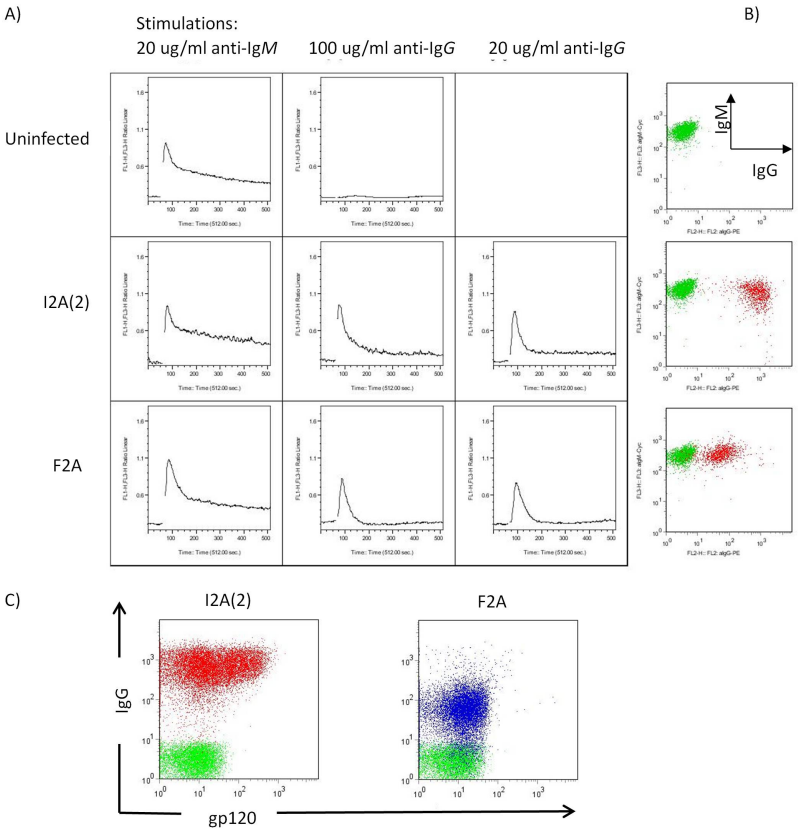

Figure 2.4 Molecular Rheostat BCRs generate calcium signals in response to anti-BCR stimulations and bind to HIV gp120. A) Calcium response of cells to anti-BCR stimulation. First column: response of endogenous IgM BCR to anti-IgM stimulation. Second column: high dose (100 ug/ml) anti-IgG stimulation. Third column: low dose (20 ug/ml) anti-IgG stimulation. B) BCR expression post-sorting. Endogenous IgM expression (vertical) *vs.* surface IgG staining from IgG Molecular Rheostat BCR (horizontal). Red: sorted cells expressing the Molecular Rheostat Immunoglobulins. Green: uninfected control cells. C) Anti-IgG and $gp120_{MN}$ labeling of sorted cells. Red

and Blue: I2A(2) and F2A Molecular Rheostat Immunoglobulin vector transduced cells, repectively. Green: untransduced control cells.

2A Mutant	**Mutation Type**	**Amino Acid Sequence**
F2A	Wild-type	QLLNFDLLKLAGDVESNPGP
F2A(-7)	7aa N-terminal deletion	LKLAGDVESNPGP
F2A(-6)	6aa N-terminal deletion	LLKLAGDVESNPGP
F2A(-5)	5aa N-terminal deletion	DLLKLAGDVESNPGP
F2A(-4)	4aa N-terminal deletion	FDLLKLAGDVESNPGP
F2A(-3)	3aa N-terminal deletion	NFDLLKLAGDVESNPGP
F2A(-2)	2aa N-terminal deletion	LNFDLLKLAGDVESNPGP
F2A(-1)	1aa N-terminal deletion	LLNFDLLKLAGDVESNPGP
F2A(3)	Point mutation	QLLNFDLLKLAGDV**Q**SNPGP
F2A(11)	Point mutation	QLLNFDLLKLAGDVE**I**NPGP
F2A(14)	Point mutation	QLLNFDLLKLAGDVES**E**PGP
F2A(19)	Point mutation	QLLNFDLLKLAGDVESNP**A**P
I2A(0)	Wild-type	TRAEIEDELIRRGIESNPGP

I2A(1)	Point mutation	TRAEIEDELIR**A**GIESNPGP
I2A(2)	Alternative codon	TRAEIEDELIRRGIESNP**G**P
I2A(3)	Point mutation	TRAEIEDELIRRGIESNP**A**P

Table 2.1 Nomenclature and amino acid sequences of different 2A peptides.

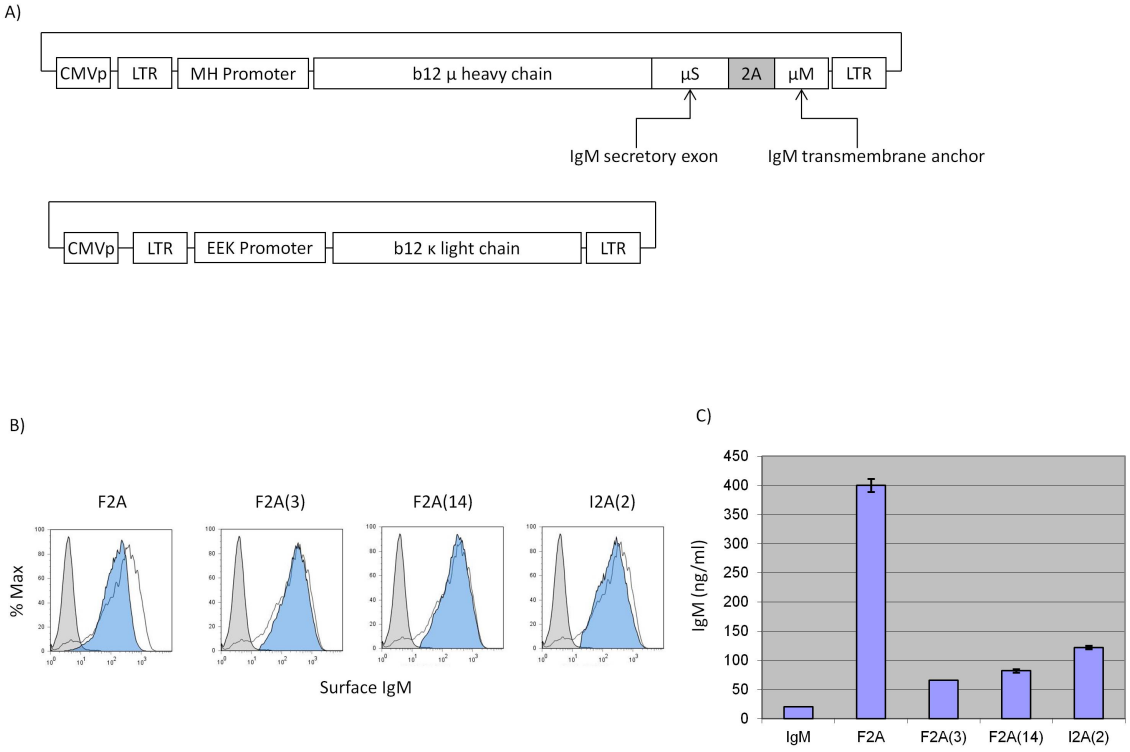

Figure 2.1 A) Schematic representation of the IgM Molecular Rheostat Immunoglobulin contructs. 2A: location of self-cleaving 2A elements. CMVp: CMV promoter. LTR: long terminal repeat. MH and EEK promoters: internal B cell specific promoters. b12 µ heavy chain: IgM heavy chain with variable region corresponding to that of the b12 broadly neutralizing antibody. B) Surface staining for human IgM. All 293T cells transfected with first-generation Molecular Rheostat contructs show surface staining for IgM. White: Membrane-bound IgM control. Blue: Molecular Rheostat

contructs. Gray: GFP control. C) IgM ELISA of supernatants of transfected cells. IgM: membrane-bound IgM control.

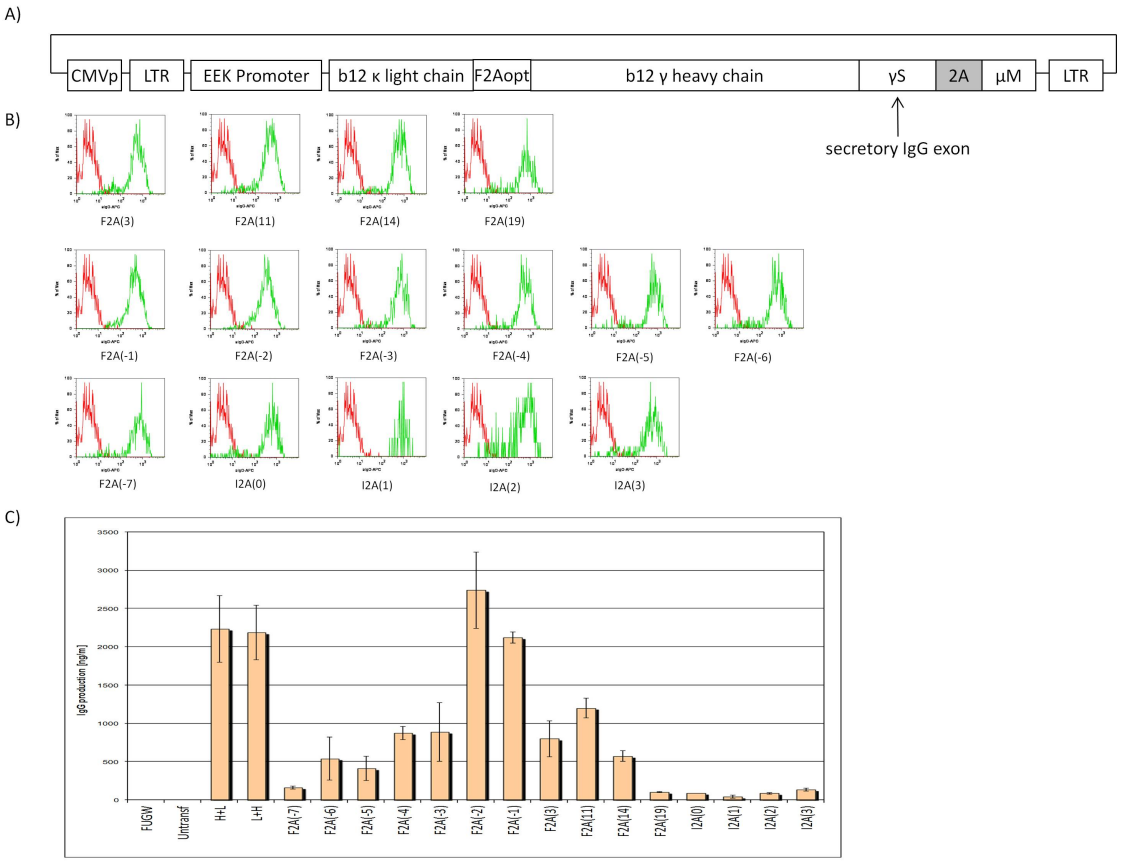

Figure 2.2 A) Schematic representation of the IgG/M Molecular Rheostat constructs. 2A: location of mutant self-cleaving 2A elements. 2Aopt: optimized 2A element with a furin cleavage site at 5' end. CMVp: CMV promoter. LTR: long terminal repeat. EEK: internal B cell specific promoter. b12 γ heavy chain: IgG heavy chain with the variable region corresponding to that of the b12 broadly neutralizing antibody. B) Surface staining for human IgG. Green: Molecular Rheostat Contructs. Red: Secretory IgG (L+H) control. C) IgG ELISA of supernatants of transfected cells. FUGW: GFP containing

vector control. L+H and H+L: secretory b12 IgG controls. H+L has the light chain in the first position and heavy chain in the second position; L+H is in the opposite order.

Figure 2.3 A) Experimental design for measuring the ratioed expression of surface-to-secreted immunoglobulins by IgG Molecular Rheostat constructs. B) Inverse relationship between surface expression of IgG Molecular Rheostat BCRs and secreted IgGs in the supernatants of sorted cells. NV: untransduced control. L+H: secretion only b12 control. The Molecular Rheostat contructs are denoted by the mutant 2A elements they contain.

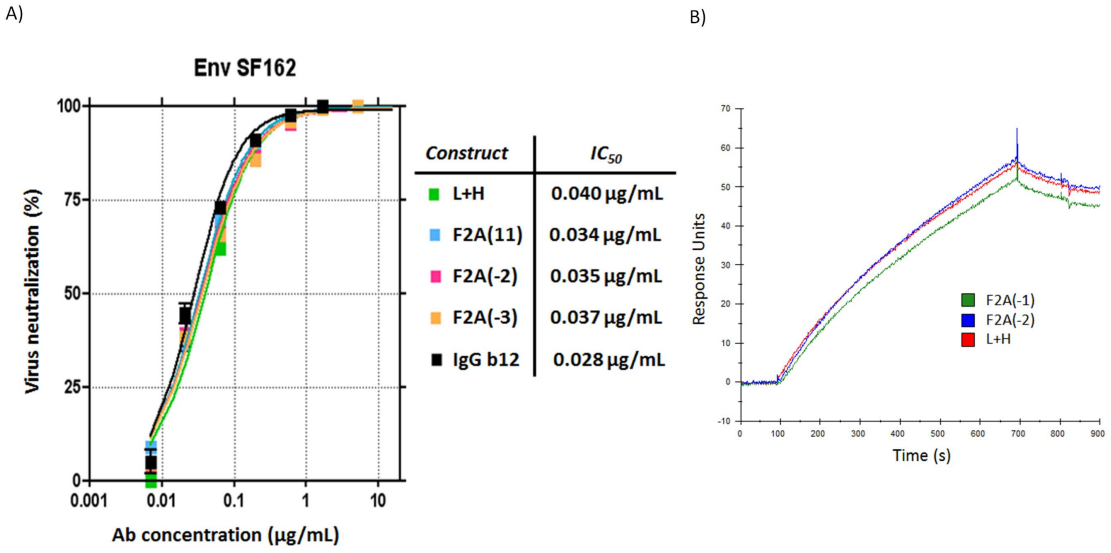

Figure 2.5 Secreted b12 IgG from the Molecular Rheostat constructs neutralized Env SF162 pseudovirus and bound to GP120 as well as control b12 IgG. A) *In vitro* neutralization assay against Env SF162 pseudovirus. Green: L+H, the control b12 IgG. Red, Magenta, and Orange: different b12 IgG Molecular Rheostat antibodies. Black: a different batch of b12 IgG purified included as positive control for the assay. B) Surface Plasmon Resonance GP120 binding assay.

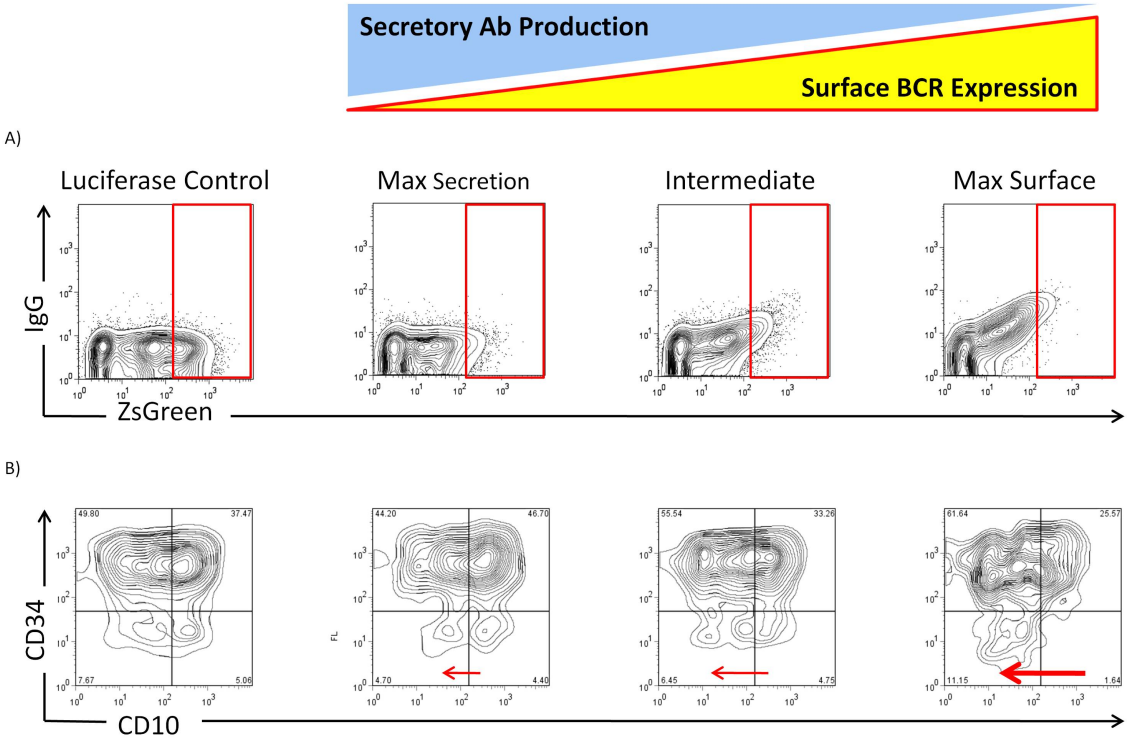

Figure 2.6 Molecular Rheostat BCRs promote maturation of EU12 cells. A) CD 34^{+} EU12 cells (early B cells) transduced with IRES-driven ZsGreen expressing Molecular Rhestat constructs were analyzed by flow cytometry. Surface BCR levels correlate with ZsGreen intensity. Cells transduced with Molecular Rheostats tuned for higher surface expression showed more surface BCR expression with the same ZsGreen expression. B) Red arrows indicate the normal B cell development progression. Cells transduced with constructs that express higher surface IgG/M BCR levels show greater mature B cell populations.

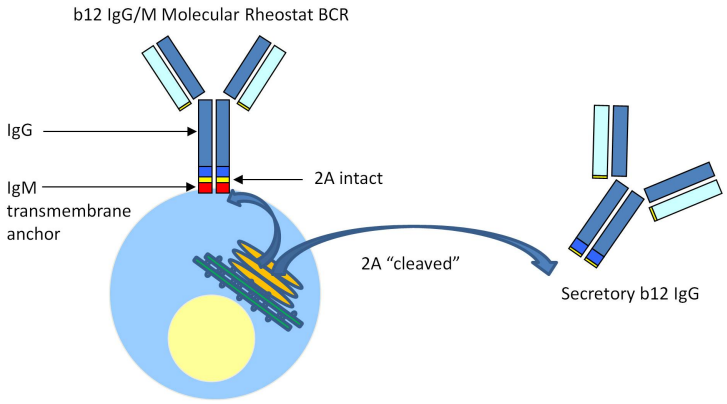

Figure 2.7. A model of how the b12 IgG Molecular Rheostat immunoglobulin system directs tunable simultaneous formation of surface BCR and secreted IgG.

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CHAPTER 3: *IN VIVO* CHARACTERIZATION OF THE MOLECULAR RHEOSTAT IMMUNOGLOBULINS

Introduction

In this chapter I describe the use of the humanized immune system (HIS) mouse model and a murine bone marrow transplant model to study the Molecular Rheostat immunoglobulin genes in the context of human B cell development *in vivo.* HIS mice are immunodeficient mice that have been transplanted with human cord blood $CD34^+$ cells (Traggiai, Chicha et al. 2004). The human cells engraft in the mice after transplant and reconstitute predominantly lymphoid lineage cells, primarily B and T cells, and some dendritic cells. The human T cells in these animals are capable of being infected by HIV (Zhang, Kovalev et al. 2007). In a more advanced model, the liver and thymus from the same cord blood donor are also grafted under the kidney capsule. These animals are referred to as bone marrow-liver-thymus mice (BLT). In the BLT mice, a limited, though inconsistent, immune response to HIV antigens is possible (Brainard, Seung et al. 2009). In collaboration with Dr. Ryan O'Connell, I undertook a study to look at whether the Molecular Rheostat immunoglobulin genes could promote the development of B cells that express b12-based anti-HIV IgG/M chimeric BCR. To complement the HIS model, I have also begun a study to characterize the Molecular Rheostat immunoglobulin genes using a murine bone marrow adoptive transfer model. The results from these studies are described below.

Materials and Methods

Lentiviral Vector Constructs and Viral Vector Production

The constructs we used were described in detail in Chapter 2. 293T cells were transfected with lentiviral vectors. After 24 h of incubation, the supernatant was pipetted off the cells and filtered through a 0.22 µm PES membrane bottle-top filter into a collection bottle. 15 ml of fresh D10 media was then filtered through the bottle-top filter into the collection bottle to reduce virus waste from supernatant that the filter absorbed. The collected supernatant was stored at 4°C, and 30 ml of fresh D10 media was added to the dish. This collection process into the same collection bottle was repeated 4 to 5 additional times at 12 h intervals. All of the collected supernatant was centrifuged at 25,000g for 90 mins at 4°C to pellet the virus, and the supernatant was aspirated. The pellet was re-suspended in 500–1000µL DMEM media (for 293T transductions) or RPMI media 1640 (for OCI-Ly7 or EU12 transductions) and incubated on ice at 4°C for 12 h. The resuspended vectors were aliquoted and frozen at -80°C until use.

Tranduction of $CD34^+$ Cells

Transduction of $CD34^+$ cells was performed using a modified protocol described by Luo et al. (Luo, Maarschalk et al. 2009). Briefly, The $CD34^+$ cells were cultured for 24 hours before transduction in StemSpan media (Stemcell Technologies, Vancouver, BC, Canada) with 10% FBS and recombinant human cytokine supplementation: 20 ng/ml

SCF and 20 ng/ml TPO (from R&D Systems, Mineapolis, MN), and 25 ng/ml Flt3 ligand (from eBioscience, San Diego, CA). Spin-infection was performed in Retronectin-coated 24-well plates (~4000 cells per well) for each construct with 1 ml/well of StemSpan media with the human cytokines at an MOI of between 50–100 for the three constructs, at 2500 rpm at 30°C for 90 mins. The cells were washed once with StemSpan media and resuspended in 250 ul of PBS and kept on ice until injection.

$CD34^+$ Cell Transplantation

Newborn pups were given 300 rads of radiation from a Cs^{137} irradiator. The human cells were resuspended in sterile PBS and injected intrahepatically by using a 27 gauge needle in 50 ul of PBS per mouse.

Transduction of Murine Bone Marrow Cells and Transplantation of Transduced Cells

Transduction of murine bone marrow cells and transplantation of the transduced cells were carried as described by Yang et al. (Yang and Baltimore 2005). Briefly, we harvested bone marrow from donor C57/BL6 mice and cultured the cells in DMEM plus 10% heat-inactivated fetal bovine serum supplemented with recombinant murin IL-3 (20 ng/ml), IL-6 (50 ng/ml), and SCF (50 ng/ml). The bone marrow cells were transduced with the pMIG-aHEL retroviral vector by spin-infection once per day for three days beginning 24 hours after the harvest. The cells were injected into irradiated Rag1

recepient mice one day after the last spin-infection. The Rag1 mice were pre-conditioned with 1100 rads of radiation from a Cs^{137} source.

Mouse Serum Collection and Human IgG ELISA

75 ul of blood was collected from each mouse using a heparin-coated microcapillary tube by retro-orbital bleeding and transferred into a microcentrifuge tube and kept on ice. The blood was then incubated at 37°C for 30 mins, and then spun down at 1150g at 4°C. The serum was collected from the top of the tube. Sera were analyzed using Human IgG ELISA Quantitation Kit (Bethyl Laboratories Inc., Montgomery, TX) according to manufacturer's instructions.

Results and Discussion

Using Human-Immune System Mice to Study Human B Cell Development under the Influence of the Molecular Rheostat Immunoglobulin Genes

Concentrated VSVg pseudotyped lentiviral vectors encoding one membrane-biased and one intermediate b12 IgG Molecular Rheostat immunoglobulin controlled by F2A(-4) and F2A(11), respectively, and a control luciferase vector were produced by transient transfection in 293T cells. We note that the F2A(-4) construct would have given rise to a higher level of surface BCR expression than F2A(11), based on previous work. The vectors were of the pHAGE2-series with an IRES-ZsGreen marker gene in the second position. The supernatants were concentrated by a three-stage low-speed

centrifugation process I developed in which the viral pellet obtained from a previous stage of centrifugation was combined with the supernatant from the next stage and re-spun.

The supernatants were spun at 10,000 rpm for 8 hours at 4°C. The total volume of supernatant processed for each lentiviral vector was approximately 1.5 L. The viral pellet from the final spin was resuspended in approximately 500 uL of the residual media in the centrifuge bottle after pouring off the supernatant. The vectors were aliquoted in 50 ul volumes and flash-frozen in an ethanol/dry ice mixture and stored at -80°C. The resulting vectors were titered on EU12 cells. The titers were on the order of 10^8 TU/ml (1.32, 4.59, and 3.22×10^8 TU/ml for F2A(-4), F2A(11), and luciferase control, respectively).

Frozen $CD34^+$ cord blood cells were purchased from Lonza (Lonza Walkersville Inc., Walkersville, MD). We transduced 100,000 $CD34^+$ cells for each experimental group (no vector control, F2A(-4), F2A(11), and luciferase), which will be injected into four Rag2/γC knockout pups per group. The newborn pups were given 300 rads of radiation from a Cs^{137} irradiator and the human cells were injected intrahepatically by Dr. Ryan O'Connell using a 27 gauge needle. We lost one group of pups (F2A(11)) as the box containing them fell off a cart used to transport them and the pups died. The remaining groups were placed with their mothers.

The mice were bled 10 weeks later to assess reconstitution by staining for human CD45 in the lymphocyte gate. We found that the mice had between 1%–5% human

reconstitution based on the human CD45 marker. The mice that had the best reconstitution were sacrificed (F2A(-4) mice, luciferase, and untransduced control) and their spleen and bone marrow were analyzed. Despite the low proportion of human cells, the mice showed reconstitution of the human B and T cell compartment. Gating on CD19, we found that the number of ZsGreen^{+} cells in the mice that carried the Molecular Rheostat immunoglobulin genes was much lower than those of the luciferase control, and close to the level of the untransduced cells (Figure 3.1A). The bone marrow of these mice was analyzed (Figure 3.2B). We found that there were many fewer ZsGreen^{+} than ZsGreen^{-} cells, which might partially explain why there were also few ZsGreen^{+} cells in the periphery. However, it does not explain why there were many fewer ZsGreen^{+} cells in the mice that received the Molecular Rheostat vectors. We therefore gated on the ZsGreen^{+} and ZsGreen^{-} cells. We found that in the ZsGreen^{-} compartment, both Molecular Rheostat and luciferase mice showed the normal pattern of B cell maturation, comparable to that of the untransduced control. However, in the ZsGreen^{+} compartment, there appears to be a lack of the more mature CD20^{+} CD10$^{lo/-}$ cells. However, due to the expense, technical difficulty, and the low levels of consistent reconstitution we achieved with the HIS mice, we decided that we would eschew the HIS mouse model and further analzye this phenomenon in a full murine model of bone marrow transplantation.

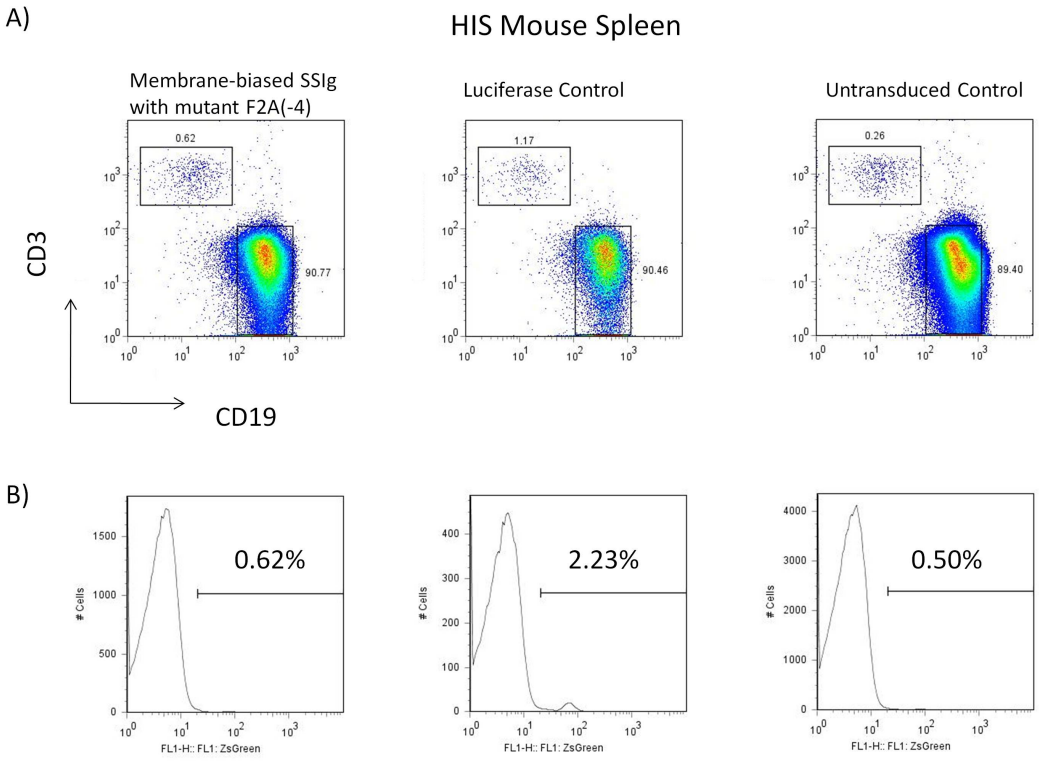

Figure 3.1 FACS analysis of the bone marrow of HIS mice reconstituted from human $CD34^+$ cells transduced with Molecular Rheostat and luciferase control vectors. A) The mice had reconstituted the B and T cell compartments. B) ZsGreen expression gating on the $CD19^+$ cells.

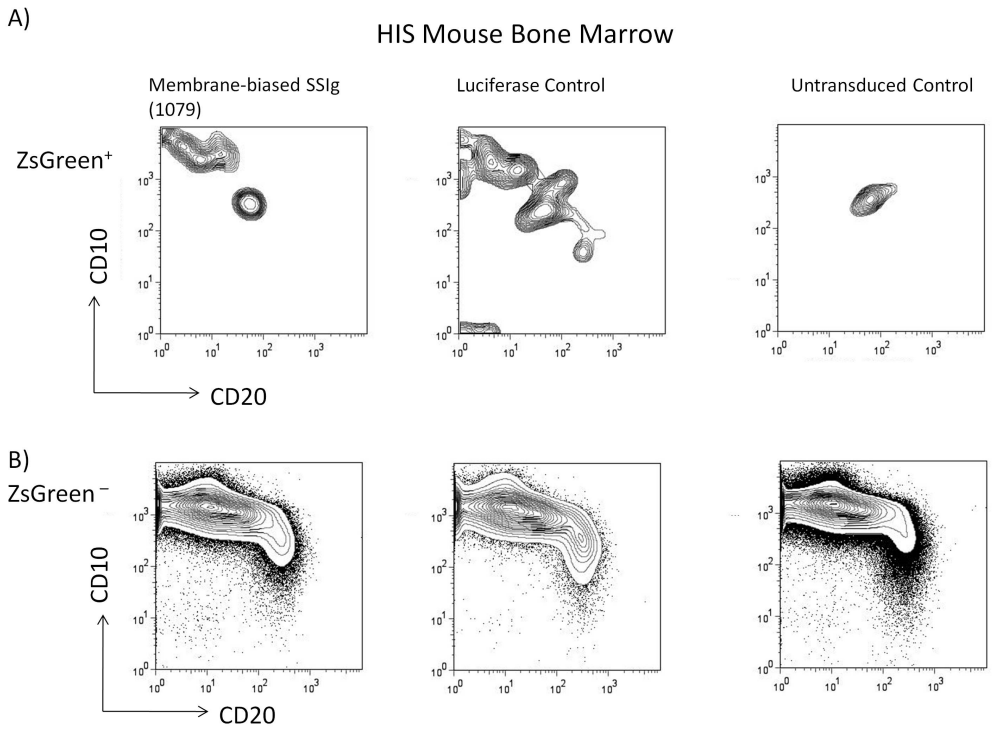

Figure 3.2 FACS analysis of the bone marrow of HIS mice reconstituted from human $CD34^+$ cells transduced with Molecular Rheostat and luciferase control vectors.

Using the Murine Bone Marrow Transplant Model to Study B Lymphopoiesis under the Control of Molecular Rheostat Immunoglobulin Genes

To study the Molecular Rheostat immunoglobulins in an *in vivo* system that provided more consistent engraftment and is more amenable to detailed analysis than the HIS mouse model, we elected to use the murine bone marrow radiation chimera model. We harvested HSC-enriched bone marrow from wild-type C57/BL6 mice that had been injected with 5-fluorouracil five days prior, and transduced the bone marrow cells in culture by spin-infection with VSVg-pseudotyped concentrated pHAGE2-EEK-IRES-ZsGreen lentiviral vectors that carry four different Molecular Rheostats genes and a luciferase control gene, respectively (Figure 3.3A). Gating on the c-Kit^{+} CD19^{-} cells, it could be seen that we achieved high levels of transduction in the enriched marrow (Figure 3.3B), as measured by the shift of the ZsGreen^{+} populations as compared to the control, untransduced bone marrow. The amount of vectors we gave was normalized such that the bone marrow cells all received equivalent, ~ 50 MOI of the vectors. We observed that the expression of ZsGreen is approximately eightfold higher in the luciferase vector infected cells than the vectors that carried the Molecular Rheostats. This was consistent with what we observed when the same vectors were used to transduce cell lines. We also note that over the course of the culture prior to injection into mice, we observed by fluorescence microscopy increasing numbers of doublet cells (cells undergoing active cell division) in which there was a uniform distribution of fluorescent material, indicative to real transduction events where gene expression occurs from

integrated vectors, as opposed to a speckled pattern characteristic of pseudo-infected cells, where pre-formed fluorescent protein was carried into the cells by the vectors. Taken together, these suggested to us that the high levels of transduction we achieved were not pseudo-infection.

We injected the cells into five groups of congeneic $Rag1^{-/-}$ mice, which do not have any endogenous B and T cells. The mice had been given a lethal, 1100-rad dose of radiation from a Cs^{137} source. We bled the mice and analyzed the peripheral blood by FACS eight weeks post-transplant (Figure 3.4). We found we had reconstitution of both the B and T cell compartments. However, none of the cells expressed the ZsGreen marker gene (Figure 3.5). These were surprising findings given that we achieved high levels of transduction of the pre-transplant bone marrow cells. We performed PCR on the peripheral blood to detect integrated lentiviral vectors using specific primers against the WPRE elements of the lentiviral vector backbone. The lentiviral vectors were detected in all the recipient animals (Figure 3.6). In a parallel experiment, we also performed PCR against a Ho-1 (heme-oxgygenase 1) gene as an internal control, and we found that the levels of WPRE detected were higher than Ho-1, suggesting to us that on average, more than one copy of the vector was integrated into the cells. We selected the animals with the highest amount of detected vectors and analyzed their spleen and bone marrow by FACS (Figure 3.7). We found that there was no detectable expression of the ZsGreen marker gene in the $CD19^{+}$ compartment (or other compartments). We were unable to detect any human IgG expression, whether on the surface of B cells or intracellularly.

Taken together, the immunophenotypic analysis coupled with the molecular data suggested to us that the lentiviral vector driven by the EEK promoter is either silenced or that the EEK promoter is inactive. However, our earlier results with the bone marrow culture suggested to us that the promoter was active *in vitro*. Thus, the lentiviral vector backbone and/or the EEK promoter might be silenced *in vivo* through a yet unknown mechanism.

We performed luciferase imaging on the mice that received the luciferase vector, and IgG ELISA on the serum collected from the animals. While we found that two of the four mice that received the luciferase-vector transduced cells had luciferase activity by imaging, there was no detectable human IgG in the serum in the animals that received the lentiviral vectors carrying the Molecular Rheostat immunoglobulin genes. From the PCR data, more copies of the vectors were present in at least some of the animals that received the Molecular Rheostat genes than in those that received the luciferase vector (contrast Lane 11 and Lane 8 in Figure 3.6). We suspect that the detection of enzymatic reaction of luciferase bioluminescence is much more senstive than the detection of protein using anti-IgG antibodies by FACS. Thus the EEK promoter could still be active but is greatly attenuated compared to the activity in freshly transduced cells. Taken together, these data suggest that it is likely that yet-unidentified mechanisms are acting either on the lentiviral vector backbone or some elements within the promoter/transgene cassette to progressively attenuate expression from the integrated vectors *in vivo*. Further work is required to elucidate the precise mechanism(s).

We decided to focus our studies on evaluating the impact and function of the IgG Molecular Rheostat immunoglobulin genes on the development and function of B cells. To work around the issues related to lentiviral vector silencing, we decided to proceed with the use of an orthogonal retroviral vector system derived from the murine stem cell virus (MSCV) designated pMIG, which is a MSCV retroviral vector that has an IRES-GFP marker gene. This vector has been used extensively by Dr. Lili Yang, Dr. Dinesh Rao, and others in the lab, and found to be reliably active *in vivo*. I re-cloned the gene cassettes into this vector system and was able to perform successful transduction and transplantation of the HSCs following the same protocol as that described above, except for the vectors used in transduction. Four weeks is the minimum time generally required for reconstitution after adoptive bone marrow transfer before B lineage cells can be analyzed, and at the time of writing, we are on the verge of analysis.

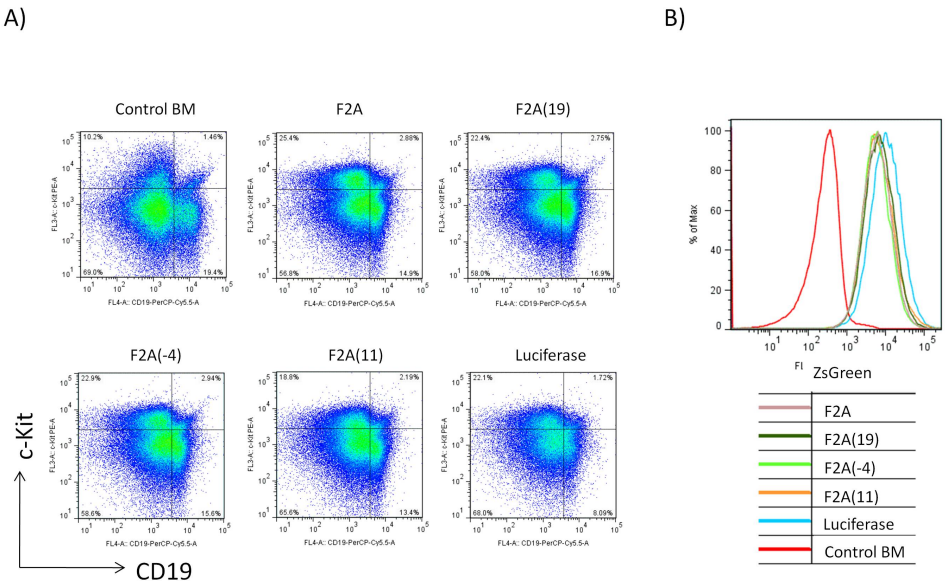

Figure 3.3 Pre-transplant FACS analysis of HSC-enriched bone marrow. A) FACS analysis. B) ZsGreen expression in the progenitor/stem cell compartment (c-Kit^{+} CD19^{-}).

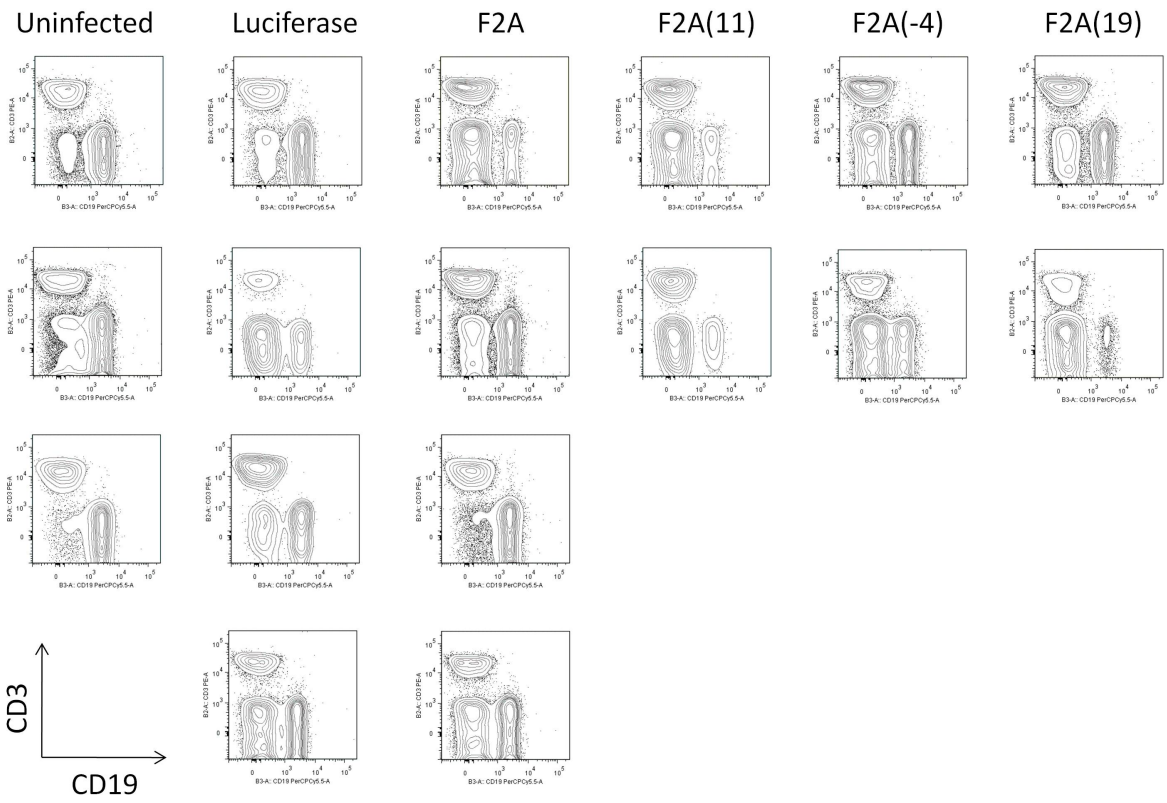

Figure 3.4 FACS analysis peripheral blood of Rag1/WT radiation chimera. Each column is one group of animals that received the lentiviral vector indicated at the top of the column.

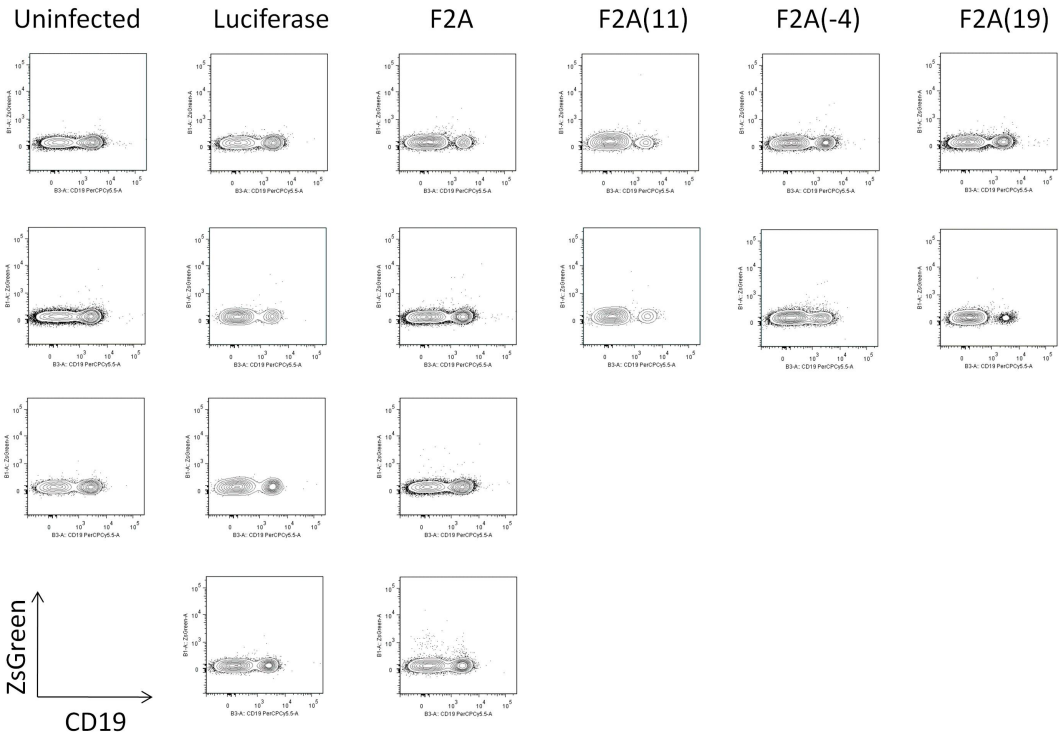

Figure 3.5 ZsGreen expression in peripheral blood of Rag1/WT radiation chimera. Each column is one group of animals that received the lentiviral vector indicated at the top of the column.

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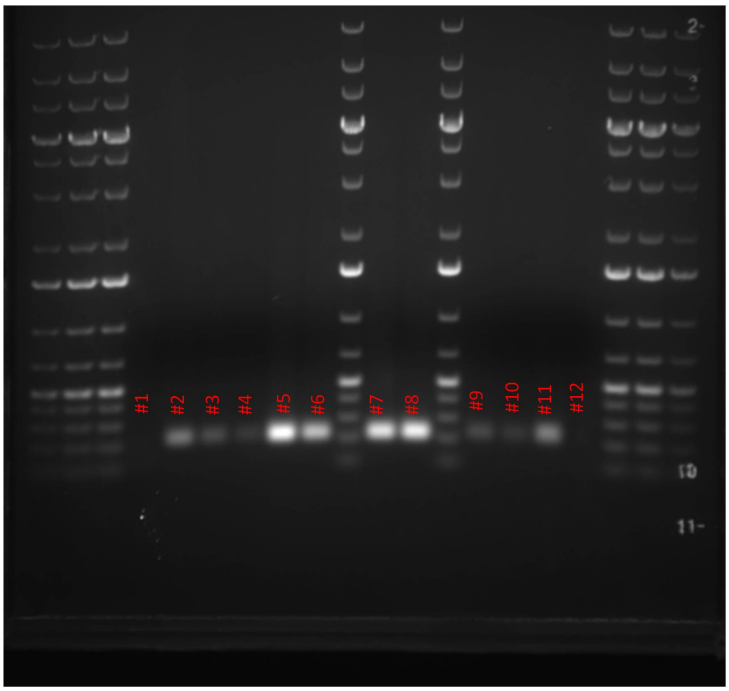

Figure 3.6 Detection of lentiviral vector elements in the peripheral blood of the bone marrow transplant recipient mice. The bolded numbers in the lengend indicate the animals that were sacrificed for analysis of the spleen and bone marrow. For each of the Molecular Rheostat constructs, the animals with the highest detected amount of integrated lentiviral vectors were selected for analysis.

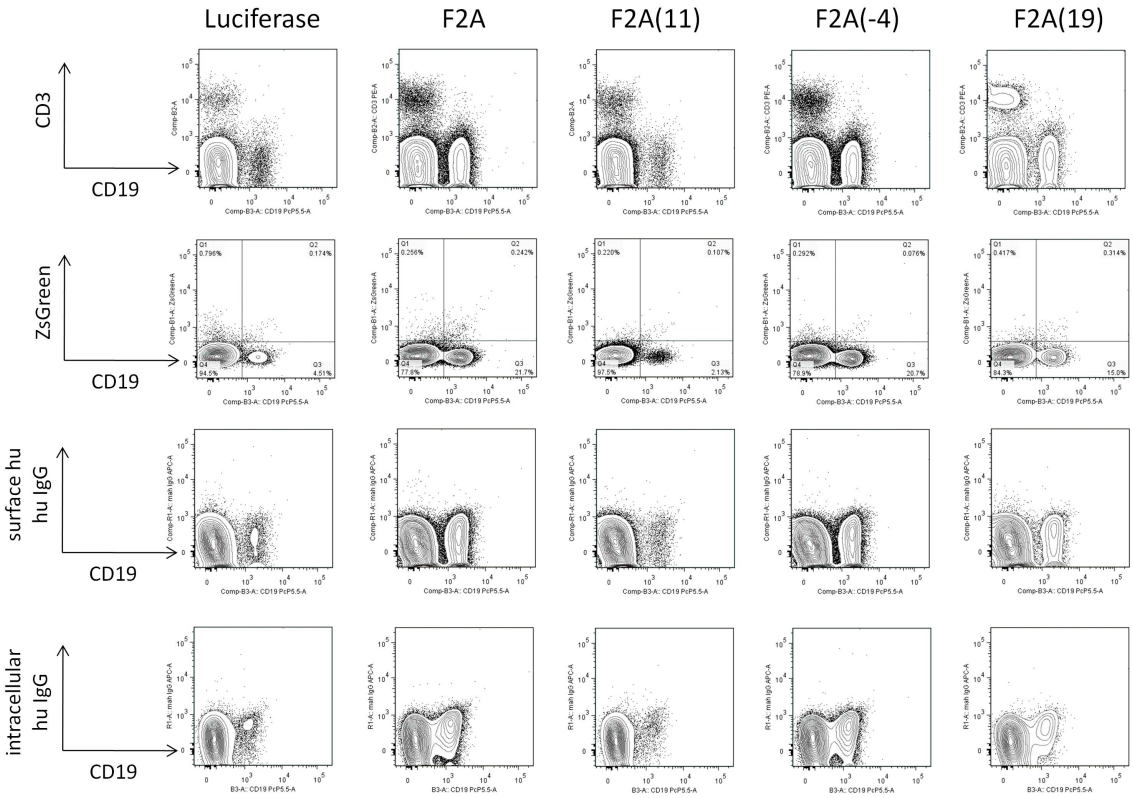

Figure 3.7 FACS analysis of the bone marrow of mice with the highest level of integrated lentiviral vectors.

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CHAPTER 4: THE USE OF NON-LYMPHOID HEMATOPOIETIC CELLS FOR ANTIBODY PRODUCTION

Introduction

Given the challenges we faced in programming B cells via lentiviral modification of the HSCs to produce HIV-responsive B cells that can produce anti-HIV broadly neutralizing antibodies, we asked whether it might be possible to make use of non-B cells to accomplish this task. The advantage of a direct gene therapy approach to the production of antibodies is its relative simplicity, compared to designing a system that could "slot in" to the normal B cell developmental process. Furthermore, use of non-B lineage cells avoids altogether the "mispairing problem", which arises when a transgenic antibody is co-expressed with an endogenous antibody in the absence of allelic exclusion, resulting in a combinatorial pairing of the light chains and heavy chains from two different antibodies. This could result in non-functional or potentially deleterious self-reactive antibodies. However, some disadvantages of using non-B cells involve the loss of all the natural regulatory controls that a "natural" antibody response to antigens have, and importantly, they lack the amplification mechanism of clonal expansion that natural B cells employ when responding to foreign antigens. The advantages were sufficiently compelling that we decided to explore the possibilities of expressing antibodies from non-lymphoid hematopoietic cells and muscle cells. We elected to study the former approach due to the relative ease of experimental manipulation of the murine bone

marrow transplant models and the established use of bone marrow transplantation in human therapies against cancer and other hematopoeitic disease; and we decided to examine muscle cells as a target for antibody production due to their easy accessibility and high protein synthetic capacity. This chapter will focus results on the work done on the hematopoietic cells that are not of the B or T lineage. The next chapter will describe our work on muscle cells.

Materials and Methods

pMIG-aHEL Vector

The aHEL IgG1 antibody is contructed by PCR cloning the entire κ light chain and the heavy-chain-variable region of the anti-HEL antibody from the MD4 mouse genomic DNA. The light chain is fused to the heavy-chain-variable-region DNA via a F2Aopt element (described in Chapter 2). The light chain-F2Aopt-heavy-chain-variable-region DNA is then grafted onto a murine anti-human CD34 IgG1 antibody by SOE (splicing-by-overlapping-extension) PCR. The cassette is then cloned into the pMIG vector between the Not1 and BamH1 sites.

Tranduction of Murine Bone Marrow Cells and Transplantation of the Transduced Cells

Transduction of murine bone marrow cells and transplantation of the transduced cells were carried out as described by Yang et al. (Yang and Baltimore 2005). Briefly, we harvested bone marrow from donor Rag1 mice and cultured the cells in DMEM plus 10%

heat-inactivated fetal bovine serum supplemented with recombinant murin IL-3 (20 ng/ml), IL-6 (50 ng/ml), and SCF (50 ng/ml). The bone marrow cells were transduced with the pMIG-aHEL retroviral vector by spin-infection once per day for three days beginning 24 hours after the harvest. The cells were injected into irradiated Rag1 recepient mice one day after the last spin-infection. The Rag1 mice were pre-conditioned with 300 rads of radiation from a Cs^{137} source.

Serum Collection and Mouse IgG ELISA

75 ul of blood was collected from each mouse each time using a heparin-coated microcapillary tube by retro-orbital bleeding and transferred into a microcentrifuge tube and kept on ice. The blood was then incubated at 37°C for 30 mins, and then spun down at 1150g at 4°C. The serum was collected from the top of the tube. Murine IgG ELISA was performed using a Mouse IgG ELISA Kit (Bethyl Laboratories Inc., Montgomery, TX) per manufacturer's instructions.

Results and Discussion

The Use of Non-Lymphoid Hematopoietic Cells to Produce Antibodies

We asked whether it was possible to produce antibodies in non-lymphoid hematopoietic cells. We employed an *in vitro* and *in vivo* models in parallel. Specificially, we were interested to see whether myeloid cells could be made to produce

antibodies. For the *ex vivo* studies, we chose to use bone-marrow-derived-macrophages (BMMs) as a representative myeloid lineage cell because the culture conditions for them are well established (Zhang, Goncalves et al. 2008). They were chosen also because they up-regulate the Xbp-1 gene on activation by LPS (Martinon, Chen et al. 2010; Zeng, Liu et al. 2010; Dickhout, Lhotak et al. 2011), a gene that is required for and similarly induced in the B-cell-to-plasma-cell transition, and required for the secretion of immunoglobulins (Reimold, Iwakoshi et al. 2001; Calfon, Zeng et al. 2002; Iwakoshi, Lee et al. 2003).

Human antibodies interact differently than mouse antibodies with murine Fc receptors. To more faithfully monitor the effects that antibody expression might have on mouse immune cell function, we chose to use a mouse antibody instead of a human one in this study. We cloned a mouse anti-HEL IgG1 gene into the retroviral vector, pMIG (Figure 4.1A). We harvested mouse bone marrow cells and cultured them in the presence of 10 ng/ml of M-CSF (macrophage-colony stimulating factor), a culture condition conducive to the differentiation and survival of macrophages from bone marrow stem and progenitor cells. During the culture period, the cells were transduced by spin-infection once per day, for four days (Figure 4.2B). At the end of the ten-day culture, we changed the media by carefully removing most of the media from each well. At this time, only adherent cells were kept. Cells were challenged with 10 ng/ml lipopolysaccharide (LPS) for two days, in the absence or presnce of M-CSF for one or two days. We observed a marked transition of macrophage morphology 24 hours after the LPS challenge, with the

cells taking on an activated, more spread out morphology and many fewer cells staying in the spherical morphology. We quantitated the amounts of antibody in a 24-hour period for two days by aspirating the media, washing the cells, and adding fresh media to the cultures. We found that the macrophages were able to produce a substantial amount of antibody (Figure 4.2). To give a sense of scale, OCI-Ly7 cells, a DLBCL (diffuse large B cell lymphoma) B cell line, when transduced with a human antibody gene by lentiviral vectors, are capable of producing on the order of 50 ng/ml of IgG in a culture volume of 10 ml containing a total of roughly 5 million cells over a period of 48 hours, giving an output of 50 ng/ml × 10 ml / 5 million cells / 2 days = 5×10^{-14} ng/cell/day. In the case of the BMMs, the number of cells at the end of the 10-day culture approximately quardruples the initial number of input bone marrow cells at the start of the culture, giving us approximately 2 million cells in each 1 ml culture in the 24-well plate. The number of cells reaches a plateau at this time due to near confluent growth. Thus, the per-cell antibody output from the macrophages can be estimated to be 50 ng/ml × 1ml / 2 million cells / 1 day = 2.5×10^{-14} ng/cell/day. This estimate of the antibody production capacity of an activated macrophage is on the same order of magnitude as that of a B-lineage cell that constitutively secretes antibodies. This is rather impressive.

This level of antibody output bodes well for the use of engineered non-lymphoid hematopoietic cells to produce antibodies, but here it also raises a legitimate concern that the high levels of antibody output from the cells that do not normally make antibodies might disrupt the normal functioning of these cells, by, e.g., impacting the synthesis and

secretion of other crucial proteins, such as secreted cytokines. Extensive *in vivo* characterization would be required to fully address this concern, but to get an initial handle on the issue, we quantitated the amount of the inflammatory cytokines IL-6 and TNFα secreted by the transduced BMMs on LPS-challenge as a measure of the overall function of these cells. We found that the amounts of cytokines secreted by the antibody-vector transduced cells were not significantly different from those produced by cells transduced by control-vectors carrying either IRES-GFP alone or a Luciferase-IRES-GFP cassette (Figure 4.3). Taken together, these data suggest that we might be able to use non-lymphoid cells to produce a substantial amount of antibody *in vivo*. To test this hypothesis, we turned now to a bone marrow adoptive transfer model.

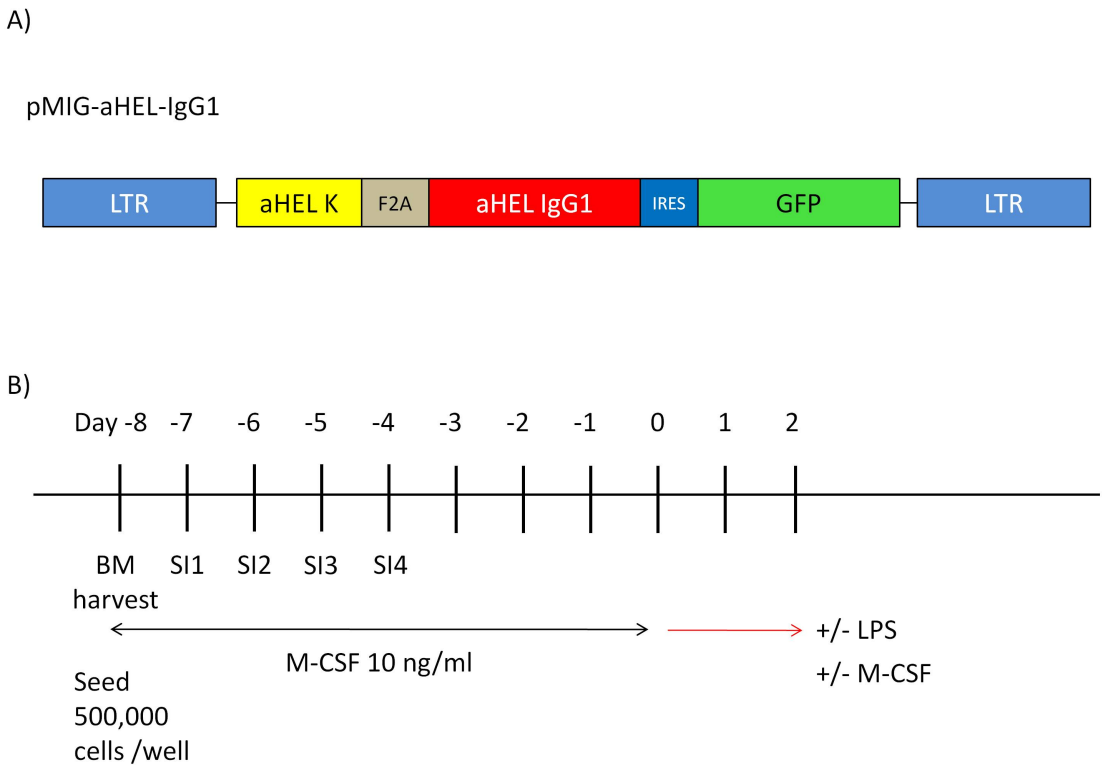


Figure 4.1 A) Schematic representation of the pMIG-aHEL-mIgG1 retroviral vector. B) Experimental design for the BMDC model of ectopic expression of antibody genes in the non-lymphoid hematopoietic cells. After transduction and culture for ten days, the cells were challenged with LPS in the absence or presence of continued M-CSF stimulation.

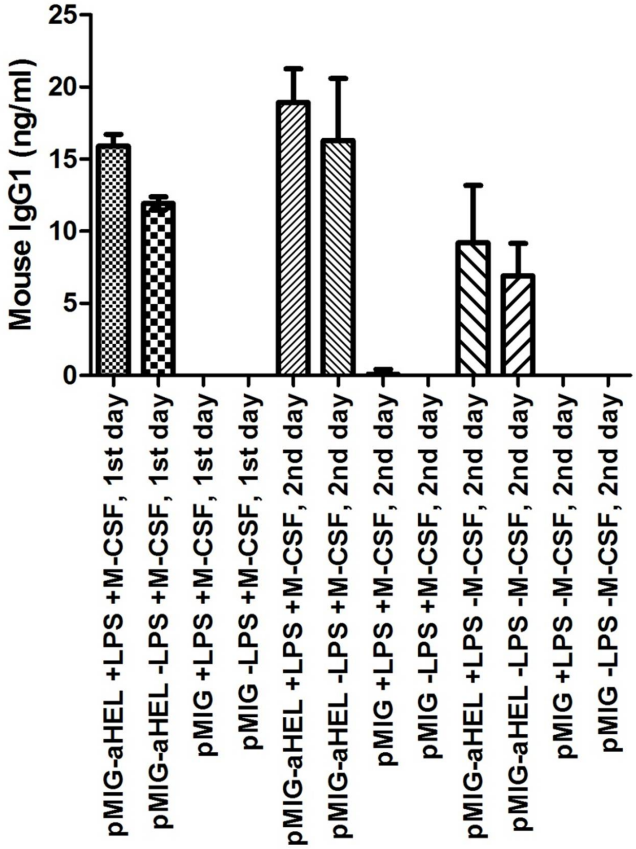


Figure 4.2 Antibody production by bone marrow-derived-macrophages transduced with pMIG-aHEL antibody vector or pMIG control vector.

A) B)

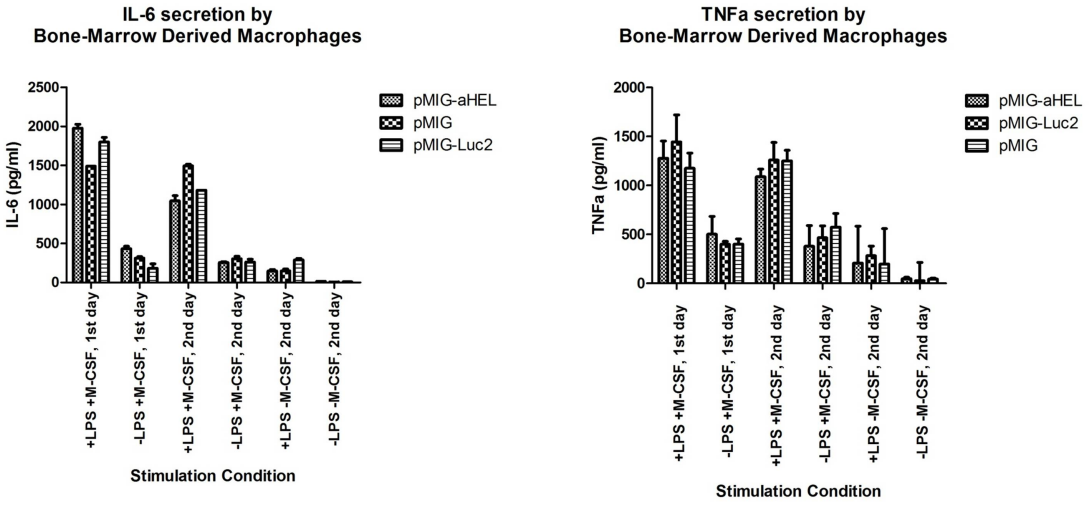


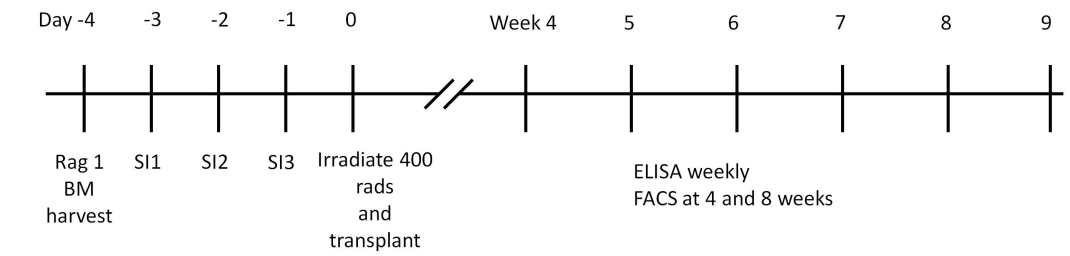
Figure 4.3 Cytokine production from vector-transduced bone-marrow-derived-macrophages.

To study whether non-lymphoid hematopoietic cells could be made to produce antibody *in vivo*, we chose to use the Rag-1 knockout (Rag1) mouse radiation chimera model. The Rag1 mouse does not produce any functional T or B cells due to homozygous deletion of the RAG1 gene. Therefore, antibody measured in blood would have to have been derived from the antibody vector. The strain of Rag1 mice that we propagated in the lab was more sensitive to ionizing radiation than a Rag1 strain based on C57/BL6 mice, commercially available from Jackson Laboratory (Bar Harbor, ME). The lethal dose for the strain we used was achieved at 450 rads, due to modes of radiation death from organ damage not related to bone-marrow failure; this is in contrast to wild-type C57/BL6 mice and C57/BL6-based Rag1 mice, which can tolerate a lethal dose of up to 900 rads, and die due to bone-marrow failure. We therefore chose to use a dose of 400 rads to maximize clearance of endogneous marrow while preserving the viability of the transplant recepients. This “sublethal” dose of radiation is not myeloablative in the Rag1 mice.

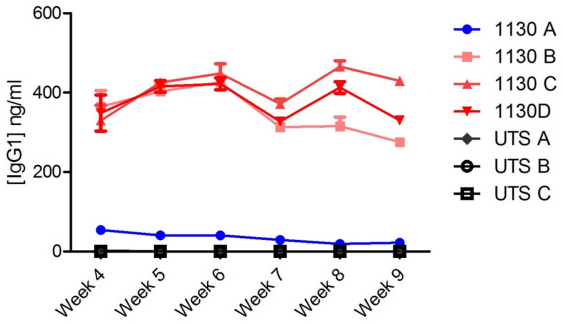
The experimental design is outlined in Figure 4.4A. 5-FU-enriched bone marrow cells were cultured in cytokine-enriched media and transduced with the pMIG-aHEL vector by spin-infection. The cells were injected into irradiated Rag1 hosts. The animals were bled weekly, starting four weeks post-transplant, to monitor serum IgG levels, and on week 4 and week 8, for both IgG ELISA and FACS analysis to assess the degree of engraftment (Figure 4.4B and C). We found that three animals in the experimental group that received the pMIG-aHEL treated cells had relatively high levels of reconstitution

(1130B, C, and D) as assessed by GFP positivity to be around 10–20% of the peripheral blood leukocytes. These animals maintained high levels of antibody concentrations (mean: 350 ng/ml). One animal, 1130A, had a low level of reconstitution (1.68% at week 4, and 1.49% at week 8), and had a low level of serum antibody (mean: 25 ng/ml). We observed that the levels of antibody produced appeared to be correlated with the degree of reconstitution as assessed by the percentage of cells that expressed GFP. The levels in the serum rose and fell with the levels of reconstitution.

A)



B)



C)

%GFP+ cells		
Animal	**Week 4**	**Week 8**
1130 A	1.68%	1.49%
1130 B	9.20%	2.41%
1130 C	12.51%	12.11%
1130 D	24.81%	8.37%

Figure 4.4 A) An outline of experimental design for studying antibody production from non-lymphoid hematoipoietic cells in pMIG-aHEL transduced Rag1 mice. B) Serum antibody levels post-transplant. The time shown is the number of weeks after bone marrow transplant. UTS: Untransduced control mice. 1130 A–D: pMIG-aHEL transduced-bone-marrow recipient mice. C) Percent reconstitution by vector-transduced cells.

The normal concentrations of IgG antibody present in mouse serum is approximately between 1–10 mg/ml, depending on a variety of factors, including strain, gender, and age. Thus the levels of antibody we achieved represented less than 0.035% of the total serum antibody concentration in the mouse. To give additional perspective, a previous attempt by Dr. Lili Yang in our laboratory to express antibodies in murine bone marrow transplant models that also employed the pMIG vector resulted in a serum concentration of approximately 1 ug/ml in *wild-type* mice, which had normal B and T cells. We were thus able to achieve approximately 40% of that level using non-lymphoid cells alone. We note that this value was achieved with only 10% of chimerism. We believe that the antibody concentrations could be higher if the levels of engraftment could be improved in wild-type animals that can tolerate myeloablative preconditioning prior to transplantation.

In summary, we showed that non-lymphoid hematopoietic cells are viable alternative cell types to lymphocytes for engineering antibody production. The engineered myeloid cells (BMMs) are functionally normal. The Rag1 BM transplanted animals produced sustained and high levels of antibodies from non-lymphoid cells, and the levels of antibody production are correlated with the degree of transduced cell engraftment. The expression of antibody from engineered non-lymphoid hematopoietic cells thus represents a potentially useful alternative to engineering B cells.

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CHAPTER 5: LENTIVIRAL VECTOR-MEDIATED BROADLY-NEUTRALIZING ANTIBODY PRODUCTION FROM MUSCLE

Introduction

Muscle is an easily accessible organ that has a high capacity for protein synthesis. We thus explored the feasibility of producing broadly-neutralizing antibodies from muscle using lentiviral-vector-mediated delivery of broadly-neutralizing-antibody genes. Muscle has been the target for adenovirus- (Ad) and adenovirus-associated-virus- (AAV) based gene therapy (Vincent-Lacaze, Snyder et al. 1999; Schnepp, Clark et al. 2003). While Ad vectors are able to provide high levels of transgene expression, their usefulness in gene therapy is limited by short duration of sustained gene expression, which is possibly related to its high immunogenicity, itself also a shortcoming for Ad-vector-mediated gene delivery (Yang, Haecker et al. 1996; St George 2003). AAV-based vectors, on the other hand, have limited useful coding capacity (~ 3–4 kb) . Despite their limitations, the use of optimized AAV-based vectors is curently under active investigation by Dr. Alex Balasz in our laboratory and the results are promising. As an alternative to Ad- and AAV-based vectors, lentiviral vectors provide a large useful coding capacity compared to AAV (10 kb) and have been used successfully to stably transduce myogenic progenitors long-term *in vitro*, though their *in vivo* performance in transducing adult muscle is inconsistent (Li, Kimura et al. 2005). In this chapter, I

describe the results from my effort to investigate lentiviral vectors as a candidate to deliver broadly-neutralizing antibodies to muscle *in vivo*.

Materials and Methods

Lentiviral Vector Production Using High-Speed Ultracentrifugation

293T cells were transfected with lentiviral vectors. After 24 h of incubation, the supernatant was pipetted off the cells and filtered through a 0.22 µm PES membrane bottle-top filter into a collection bottle. 15 ml of fresh D10 media was then filtered through the bottle-top filter into the collection bottle to reduce virus waste from supernatant that the filter absorbed. The collected supernatant was stored at 4°C, and 30 ml of fresh D10 media was added to the dish. This collection process into the same collection bottle was repeated 4 to 5 additional times at 12 h intervals. All of the collected supernatant was centrifuged at 25,000g for 90 mins at 4°C to pellet the virus, and the supernatant was aspirated. The pellet was re-suspended in 500–1000µL DMEM media (for 293T transductions) or RPMI media 1640 (for OCI-Ly7 or EU12 transductions) and incubated on ice at 4°C for 12 h. The resuspended vectors were aliquoted and frozen at -80°C until use.

Lentiviral Vector Production Using HYPERFlask Cell Factories

293T cells from one 60% confluent 15-cm tissue culture dish are seeded in 500 ml of D10 media in a Corning HYPERFlask. The cells were well mixed with the media before seeding. 3 days later, transfection was carried out using the BioT reagent (Bioland Scientific, Paramount, CA). 600 ug of vector and helper plasmids was added to 30 ml of DMEM, and mixed thoroughly by swirling with 900 ul of BioT, incubated at room temperature for 5 minutes, and added to 470 ml of fresh, pre-warmed D10 media. The mixture was used to replace the spent media in the HYPERFlask. The media was replaced 24 hours later with fresh pre-warmed media. Thereafter, 500 ml of supernatant was harvested every 12–24 hours and replaced with fresh media. The supernatant was filtered through a .45 um PES membrane bottle-top filter and kept at 4°C until concentration. Vector concentration was performed using a 500 ml bottle in a JA-20 rotor at 10,000 rpm for 6–8 hours. The supernantant was decanted and the remaining pellet resuspended in 500–1000 ul of DMEM. (If higher concentration is desired, the pellet could be resuspended with the next batch of viral supernatant. This could be repeated up to two times without significant loss of titers.) The final pellet was resuspended in 500–1000 ul of DMEM, aliquoted, flash-frozen by immersion in an ethanol/dry-ice mixture, and kept at -80°C until use.

Results and Discussion

The choice of promoters is crucial to the expression power of vectors. To help find the optimal vector design for use *in vivo,* we cloned a series of lentiviral vectors based on Alex Balasz's pHAGE2 lentiviral vectors with different promoters (Figure 5.1A). The CAGJ promoter contains the CMV early enhancer/promoter, a chicken β-actin intron, and a rabbit β-globin splice acceptor. The UbC promoter is derived from the UbC promoter that also contains an intron. The CASI promoter is designed by Dr. Alex Balasz and contains the CMV early enhancer/promoter, and a synthetic intron based on consensus splice sequences that are used by the Berkeley Drosophila Genome Project Neural Network Splice Site Prediction program NNSPLICE 0.9 (Reese 1996). To have a simple way of non-invasively tracking the efficiency of transduction *in vivo* by bioluminescence imaging, the vectors carry a firefly luciferase transgene. We also constructed vectors that carry the IgG_1 broadly neutralizing antibodies b12 and VRC01 (Figure 5.1B). As there was concern that the EV71 IRES element might cause reduction in viral titer, we also cloned versions of the antibody vectors that do not include an IRES-ZsGreen marker gene.

To determine the effect of IRES-ZsGreen on vector titer, we transduced 293T cells with the vectors, and 48 hours later titered them by ZsGreen expression and intracellular staining for IgG antibody (Figure 5.2, right panel). We used the vectors that had both an antibody gene and the IRES-ZsGreen marker gene to establish a standard curve betweeen titer measured by ZsGreen expression and IgG intracellular staining (ICS). We derived a conversion factor of the transduction units (TU) measured by FACS

analysis of ZsGreen expression to ICS of IgG: 1.965 ZsGreen TU = 1 IgG ICS TU. Using this scale as a basis, we calculated the titers of all the vectors (Figure 5.2, left panel). We found that the IRES-ZsGreen containing vectors had a similar, if not slightly better, titer than those that did not carry the IRES-ZsGreen marker gene. For this reason, all subsequent work was carried out with vectors that carry the IRES-ZsGreen marker gene for ease in titering the vectors by FACS analysis of ZsGreen expression.

Figure 5.1 Design of the lentiviral vectors for *in vivo* tranduction of muscle.

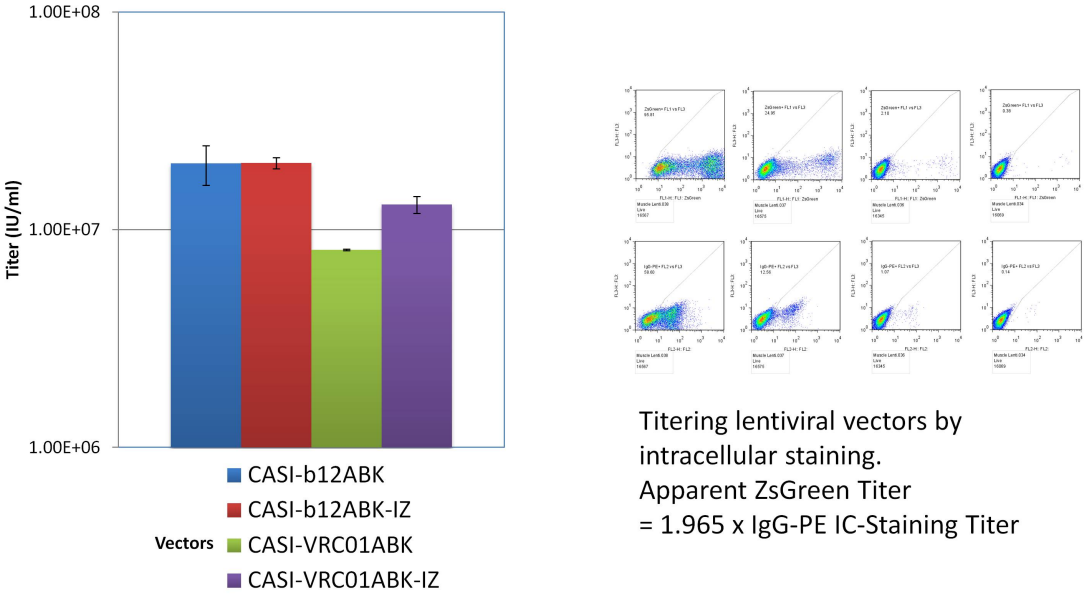

Figure 5.2 Determination of the effects of the IRES-ZsGreen marker gene on viral vector titer.

To study the dynamics of muscle transduction *in vivo*, we produced concentrated vectors for injection using standard ultracentriguation. The titers we achieved were on the order of 10^7 TU/ml. We injected these vectors into the gastrocnemius muscle of NSG mice, and performed serial luciferase imaging (Figure 5.3). We observed luciferase expression in the mice as early as three days post injection (Figure 5.4, left panels). The levels rose over time, and the peak expression occurred around 1 month after injection. The expression persisted for as long as 106 days after transduction (Figure 5.4, right panels), the length of our experiment. The detailed dynamics of the time course of luciferase expression is shown in Figure 5.5. From these traces, we found that the UbC promoter provided the highest levels of total transgene expression *in vivo* as measured by luciferase bioluminescence output (Figure 5.5, top panel). However, the CAGJ promoter is able to drive the highest level of transgene expression per TU of vectors injected (Figure 5.5, bottom panel). Based on these results, we chose the UbC vector for our study of antibody expression from muscle cells *in vivo*, since it provided the highest overall level of transgene expression.

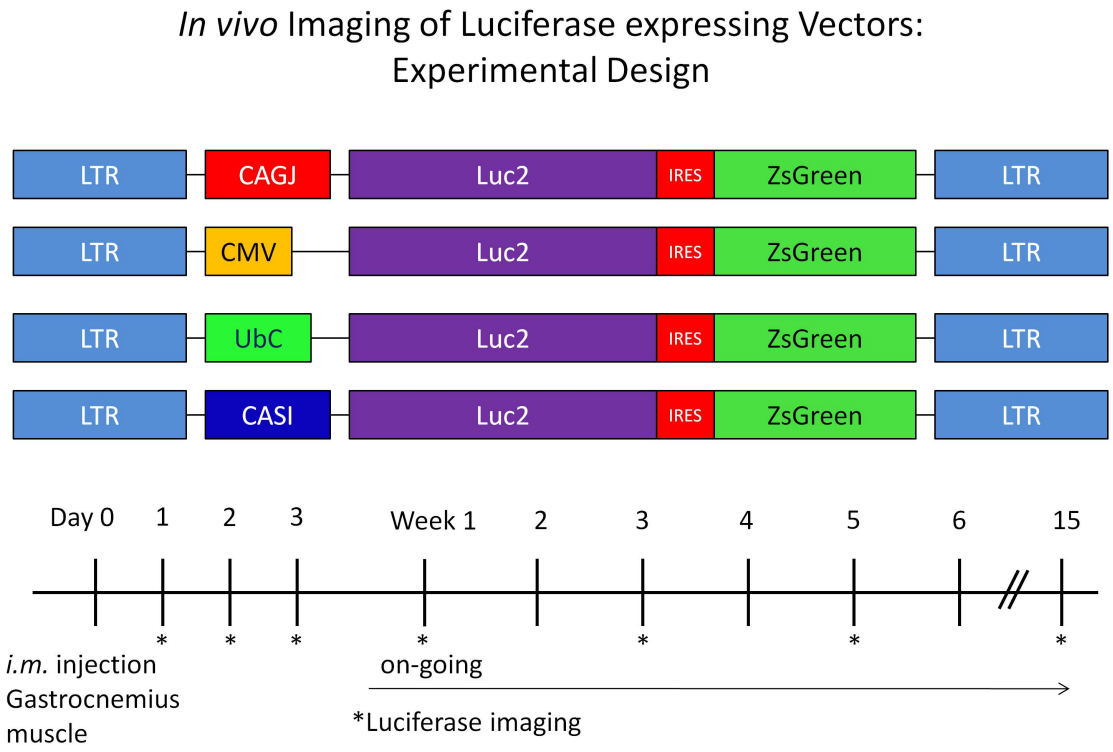

Figure 5.3 Design of luciferase imaging experiment to track the dynamics of muscle cell transduction *in vivo*.

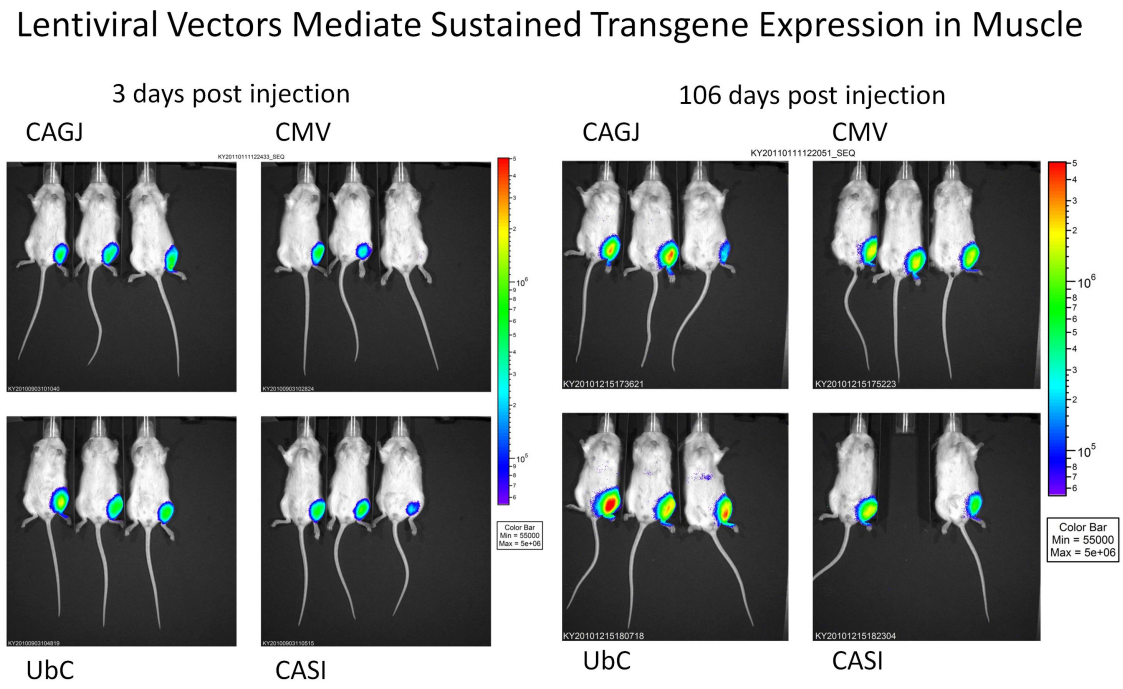

Figure 5.4 Luciferase bioluminescence imaging of transgene expression *in vivo*.

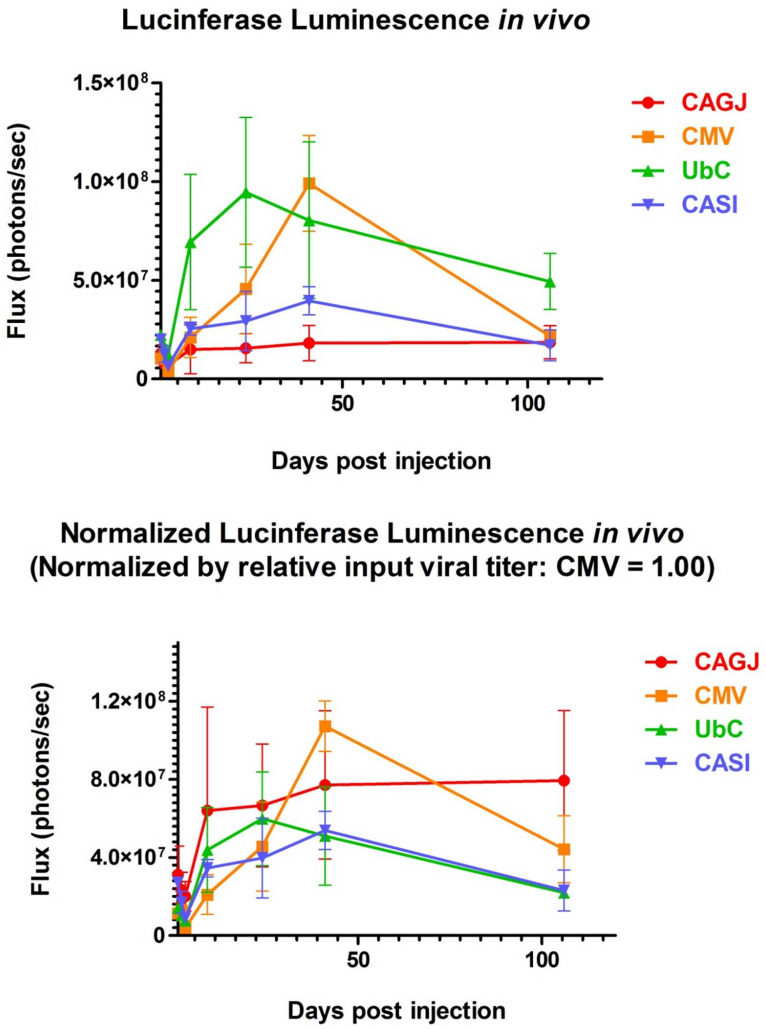

Figure 5.5 Time series of luciferase bioluminescence *in vivo*. Top panel: total luciferase expression as measured by bioluminescence output. Bottom panel: per-TU expression power driven by different promoters, calculated by dividing the bioluminescence output by that of the relative titers of the different vectors. The relative titer of the CMV-driven vector is taken to be 1.0.

Our first attempted injection of lentiviral vector into muscle using vectors concentrated by the standard method of ultracentrifugation (titer: ~ 5×10^7 TU/ml, 200 ul injected per mouse) yielded no detectable amount of antibody after 30 days. This was in contrast to what we saw with the lentiviral vectors carrying luciferase, which had readily detectable transgene expression. We suspected this was due to the very high sensitivity of the luciferase imaging technique. We felt that if we could improve our vector production system and raise the concentration of the vector, we might be able to achieve detectable levels. We therefore proceeded to develop a protocol for large-scale virus production using the HYPERFlask (Corning Inc., Corning, NY) high-density culture system also commonly called "cell factories", and employing centrifugation at 10,000g for an extended period (8 hours) We also switched from pHAGE2 to a pHAGE6 vector backbone to reduce the number of helper plasmids needed for transfection from four to two. The vectors and protocol are schematically illustrated in Figure 5.7. At the end of protocol development, we were able to consistently achieve a viral vector titer of 5.0×10^9 TU/ml, a 100-fold improvement from the standard method using high-speed ultracentrifugation. We proceeded to test these "ultraconcentrated" vectors on mice.

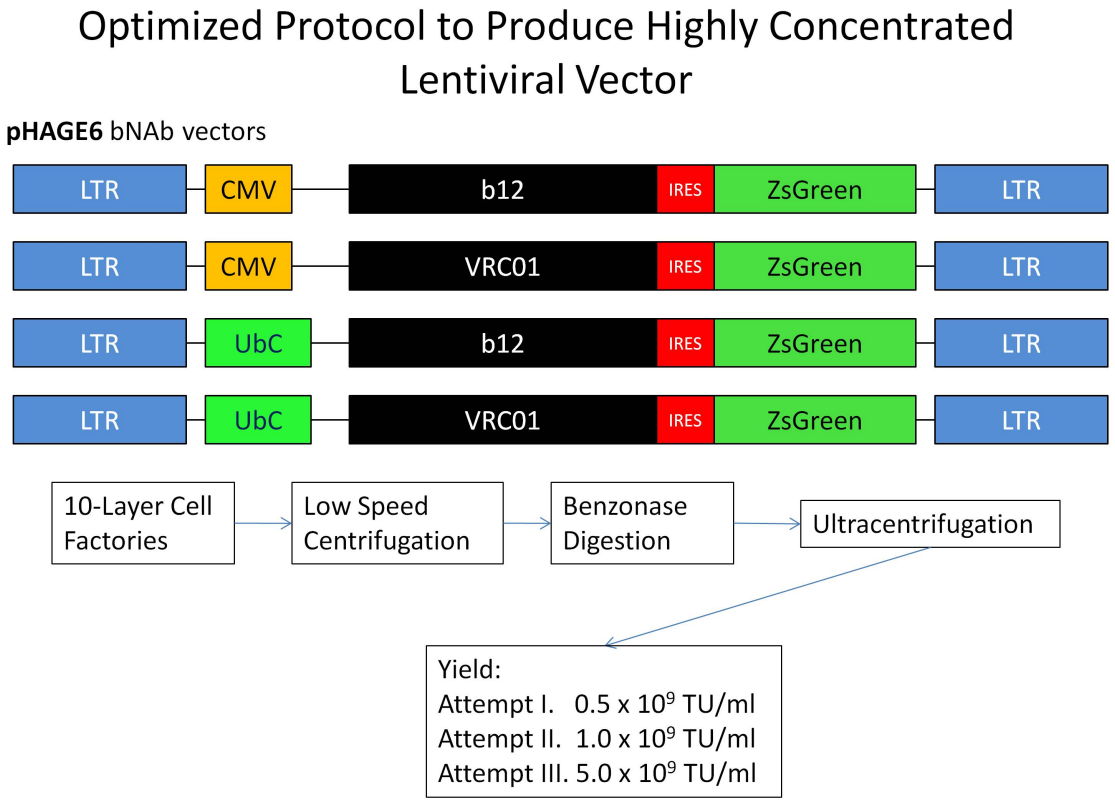

Figure 5.7 Schematic representation of the pHAGE6 vectors and optimized protocol to produce highly concentrated lentiviral vectors using HYPERFlask cell factories.

We injected 200 ul of the ultraconcentrated UbC vectors carrying the IgG human broadly neutralizing b12 antibody gene into the gastrocnemius muscle in each leg of the mice. To increase the amount of vectors we could inject into the mice, we separated the animals into three groups. One group received two injections of the vector, one in each leg; a second group received three injections, two in one leg and one in the other; the last group received five doses of the vector, two injections in one leg and three injections in the other. Injections into the same leg were performed 24 hours apart. We monitored the serum concentration of human IgG antibody by ELISA.

We were able to achieve detectable levels of human IgG with the ultraconcentrated vectors, and peak serum concentration occurred between two to four weeks after injection (Figure 5.8). However, even in the animals that received the highest amount of vectors, the levels hovered around 3 ng/ml. We also explored the levels of antibody produced in intravenously injected animals, and the levels were slightly higher (5 ng/ml).

These levels of antibody achieved by intramuscular injection of our best lentiviral vector preparation at present fell far short of the levels achieved by adoptive transfer of vector-modified hematopoietic cells. These levels are unlikely to be protective as the *in vitro* IC_{50} of the b12 IgG against primary isolates of HIV is on the order of 1 ug/ml (Binley, Wrin et al. 2004). Based on these data, we thus ruled out the use of intramuscular injections of lentiviral vectors as prophylaxis against HIV at this time.

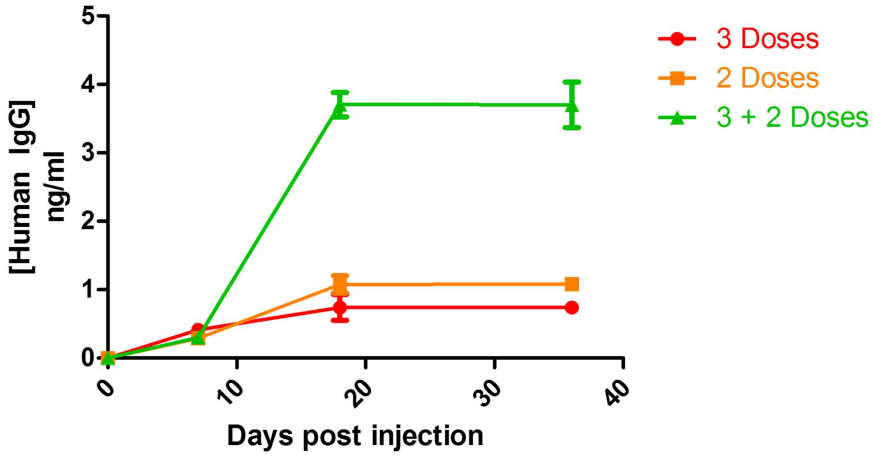

Figure 5.8 Antibody levels in NSG mice injected intramusclarly with ultraconcentrated lentiviral vectors.

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CHAPTER 6: LOOKING AHEAD

Summary and Future Directions

I have described in the preceding chapters my work toward engineering immunity against HIV. I showed how the specificity of B cells and the antibodies they produce might be manipulated to make anti-HIV broadly-neutralizing antibodies by using a Molecular Rheostat system I developed for the simultaneous and tunable expression of surface BCR and secreted antibody. I described my experience of using it to program hematopoietic cells *in vivo* and some of our surprising findings, and the need for the development of a vector-promoter combination that would provide sustained expression *in vivo*. I then investigated the use of two parallel approaches to engineer non-B cells to produce antibodies. I showed how non-lymphoid hematopoietic tissues could be made to produce antibodies and hold promise for further development as a potential gene therapy candidate, and ruled out the use of lentiviral vectors in engineering muscle tissues to produce antibodies. I will now suggest some potential paths for future investigations.

First, while we were not able to demonstrate at this time the directed development of B cells from hematopoietic cells *in vivo* under the control of the Molecular Rheostat immunoglobulin system, we believe this was due to a limitation of the lentiviral vector system we currently have. An important underlying issue in our choice of the HIS mouse model over a purely murine model is the concern for differences between murine and

human immune responses and their demonstrated differential susceptibility to HIV. However, difficulties encountered with the HIS mouse model caused us to redirect our effort of characterizing the Molecular Rheostat from using human to mouse cells. Further work is required to delineate the specific reasons why the lentiviral vector system performed poorly in the mouse. One possibility is the human EEK promoter is not as active in a murine model. Further studies using alternative promoters might be informative. We are currently studying the Molecular Rheostat immunoglobulin system using an alternative retroviral vector system adapted for murine cells (pMIG).

Second, an alternate path to anti-HIV B-cell generation might be the use of pre-existing B cells rather than relying on development from HSCs *de novo*. This poses the challenge of antibody mispairing. But the issue might not be as serious an issue as previously thought, as Luo et al. showed that in the absence of a transgenic membrane BCR to exert allelic exclusion, engineered B cells were still capable of producing bNAb with programmed specificity (Luo, Maarschalk et al. 2009). However, these cells were activated in a polyclonal, non-specific manner. Delivering the Molecular Rheostat immunoglobulin system to developed B cells *ex vivo* and transplanting them back could potentially provide anti-HIV B cells that would be activatable on stimulation by HIV antigens. Lentiviral vector systems now exist for efficient transduction of resting human peripheral blood B cells using measles virus pseudotyped vectors (Frecha, Costa et al. 2009). However, an *in vivo humanized* model suitable for antigen specific activation of B

cell immune response is still required. One possibility would be to use the BLT HIS mouse model. One could purify out the human B cells from the BLT mouse, modify them with a measles pseudotyped vector carrying the Molecular Rheostat antibody genes, and re-transfuse them back into the mouse.

Third, in addition to functioning as the cells that produce antibodies, B cells are also involved in immunoregulatory roles (Lund and Randall 2010). They are an important class of antigen presenting cells, especially under conditions where antigens are limiting (Malynn, Romeo et al. 1985; Lanzavecchia 1990), and this is accomplished in an antigen-specific manner by an immuno-phenotypically identifiable subset of B cells (B reg or B10 cells) (Yanaba, Bouaziz et al. 2008). Engineered B cells that express IL-10 have also been used in this capacity (Scott 2011). Certain well-known immune disorders such as celiac diseases have well defined inciting antigens. A Molecular Rheostat system could be used to engineer B cells specific for the gluten peptides and make it possible to specifically downregulate that part of the immune system that is involved in the disease.

Fourth, the use of non-lymphoid hematopoietic cells to produce antibodies holds promise for use as a prophylactic strategy against deadly infectious pathogens like HIV. However, the risks associated with bone marrow transplantation are real and significant. Thus, it might be difficult to apply this approach on a large scale. Nevertheless, in a setting where bone marrow transplantation is indicated for other serious conditions such

as cancer or to correct for other genetic diseases, this might be an appropriate adjunct therapy option.

Concluding Remarks

The search for an effective vaccine against HIV continues. I have outlined above some potential pathways for future investigation that would build upon the work described in this thesis. As a member of the Baltimore lab and the Engineering Immunity team, it is appropriate for me to acknowledge the work that is being done in parallel in the lab in pursuit of an HIV vaccine under Dr. Baltimore's leadership. The alternative approach using optimized AAV vectors spearheaded by Dr. Alex Balazs was published online today in the journal *Nature* and offers one of the most promising prophylactic strategies against HIV (Balazs, Chen et al. 2011). As I reflect upon my experience here, I am encouraged that we are closer than ever before to an effective vaccine against HIV. I am grateful for the opportunity to work with and learn from my many talented colleagues and friends in this lab and at Caltech, and the chance to make a personal contribution towards solving an important problem facing science, medicine, and humanity, that has also left an indelible impression on me. It's an exciting time to be a scientist and a physician, and I look forward to my continued adventure in this enterprise of science and discovery for the benefit of mankind. I want to thank Dr. Baltimore for his guidance, support, and mentorship, and for giving me the opportunity to start my scientific journey here in this most stimulating environment: au départ d'une grande aventure!

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