DEVELOPMENT OF MHC CLASS II TETRAMER FOR DETECTION OF INFLUENZA SPECIFIC TCR

By

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A Thesis Submitted to The Honors College

In Partial Fulfillment of the Bachelors Degree
With Honors In

Physiology

THE UNIVERSITY OF ARIZONA

MAY 2015

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Abstract:

T cells play a critical role in adaptive immunity by utilizing genetic recombination to produce diverse TCRs that strongly recognize specific antigens. The TCR recognizes peptides presented by APCs via the MHC class II molecules on their surface. The recognition of a specific peptide stimulates the CD4+ T cell to signal downstream processes, which result in the mounting of an antigen specific immune response. With age the available naïve CD8+ T cell pool declines, making the aged population more vulnerable to infections and less responsive to immunotherapies such as vaccines. We hypothesize there is a similar decline in CD4+ T cells, further reducing response to pathogens, such as influenza, with age. To identify CD4+ T cells specific for influenza we engineered an HLA-DR class II tetramer presenting influenza peptide. DR4 was chosen due to the frequency in the Caucasian population. The tetramer reagent will not only allow us to study the changes in the diversity of influenza specific CD4+ T cells, but will also be a valuable tool for the analysis of effector functions for a variety of adaptive immune cell populations in response to influenza virus.

Statement of Purpose:

Our goal was to build a useful and highly desirable tool to measure Influenza specific CD4+ T cells. Conducting antigen specific studies on T cells requires special techniques, one of which is using an MHC tetramer in order to identify the T cells specific to the antigen of interest among a large pool of cells. The T cells that are identified by the tetramer can then be sorted and individually evaluated for a variety of functions. Our lab is specifically interested in evaluating how well the receptor is able to recognize influenza peptide and the quantity of the T cell's receptors that recognize the specific influenza antigen in the aged population compared to the younger population. The tetramer can be used to answer many other questions posed of influenza antigen specific T cells.

A final purpose of the study was to adapt a newly developed technique for making tetramers. A laboratory we collaborated with had made MHC class I tetramers using a specific technique developed by Dr. Michael Kuhns in the Immunobiology Department at the University of Arizona. The technique involves piecing together a plasmid containing the genes of interest which is incorporated into a baculovirus. The baculovirus is used to transduce the plasmid into insect cells that can synthesize the proteins of interest, and secrete these proteins into the media to be collected. It was with the help of his former graduate student, Neha Deshpande, that we were able to use this technique to make the MHC class II tetramer that we were interested in utilizing. With the successful building of our tetramer, it is additional data for Dr. Kuhns that his technique works for both mouse and human MHC class II molecules.

Statement of Relevance:

According to the Centers for Disease Control (CDC), the aged population does poorly after influenza infection, and experiences a higher frequency of influenza related deaths compared to younger adults. The influenza vaccine does not result in as robust of a protective response in the aged population as it does in the younger adult population (1). To investigate potential mechanisms of this immune dysfunction, there is an NIH contracted group of laboratories spanning multiple universities that is tasked to evaluate different components of the aging immune response to influenza, which includes my laboratory. My lab is responsible for evaluating the CD4+ T cell potential contribution to the decreased ability of the aged population to mount a protective immune response to influenza after vaccination.

Although this study is specific to influenza, the ability of the aged population to mount a protective immune response to many pathogens is compromised (6,7,8). Findings in the aged population's CD4+ T cells response to influenza compared to the younger adult population's CD4+ T cells could be relevant to other infections. The CDC describes influenza as highly virulent, capable of rapid mutation, and it circulates in a large number of immunologically distinct strains, making it frequently recognized by the immune system as a new antigen. The virus is presented to the host as a novel antigen after mutation which contributes largely to the susceptibility of the aged population as well as children and immunocompromised people.

An MHC class II tetramer would allow our laboratory to isolate individual CD4+ T cells that recognize influenza and evaluate their functionality as well as enumerate how many CD4+ T cells are present that recognize influenza in both the young and aged population both pre and post vaccination. With this tool we could determine whether there is a defect in the CD4+ T cell component of the immune system in the aged population and how this defect is contributing to the aged population's lack of protection from the influenza vaccine.

Introduction:

The available number of naïve T cells in the periphery decreases with age due to thymic involution reducing the thymic output (2)(**Figure 1**). T cells are essential to mount a productive immune response to newly encountered pathogens. Thymic involution, along with other dysfunctional aspects of the immune system that occur with age, contribute to the aged population's decrease in protection from Influenza by the Influenza vaccine when compared to the younger adult population. It has been shown previously that the CD8+ T cell TCR diversity is reduced during aging conveying less protection from infection (3).

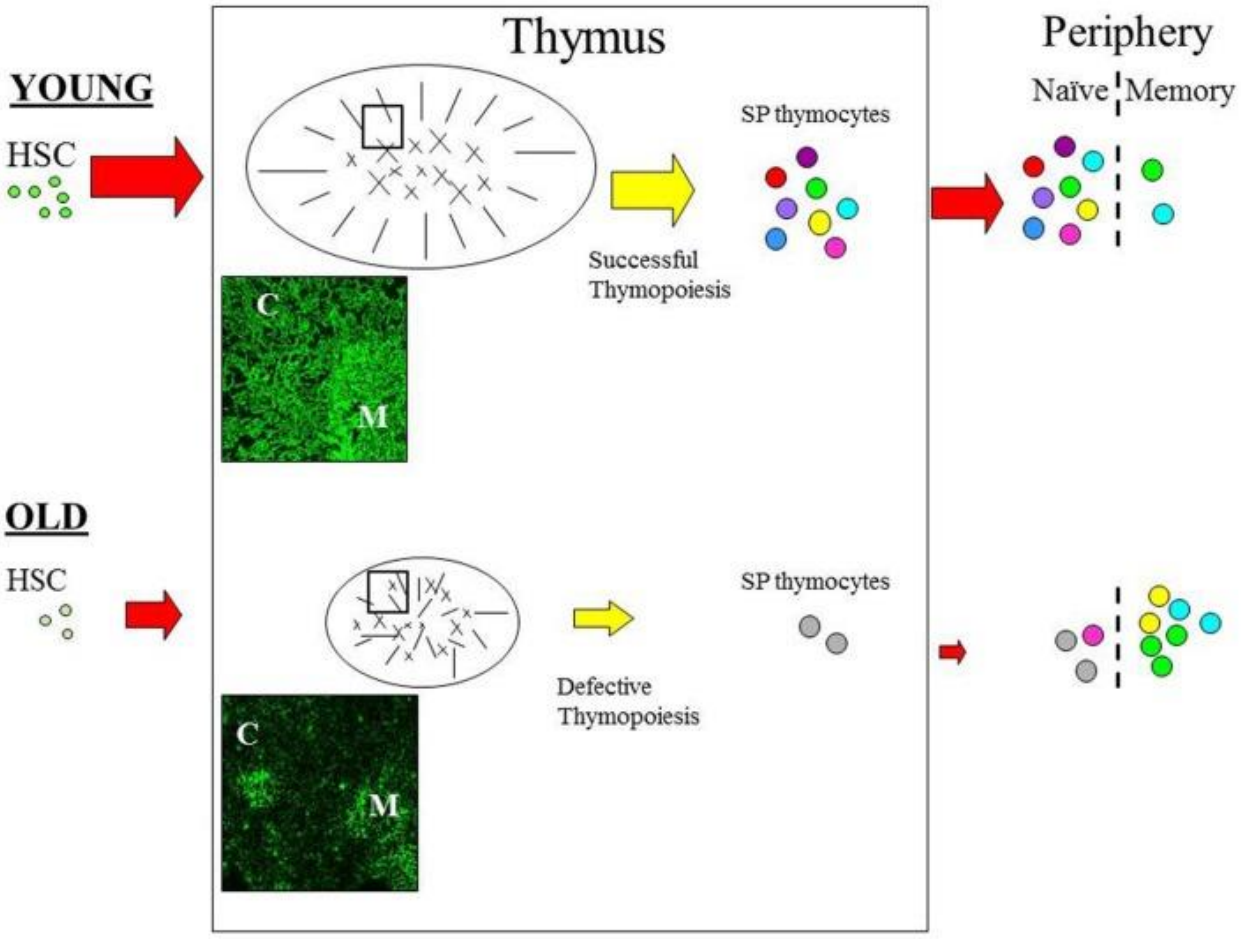

Figure 1. Memory/Naïve availability in Aged vs Young individual (2)

The aim of this study was to create a tool to evaluate the effect of aging on the influenza specific CD4+ T cell population. Post infection, antigen presenting cells (APC) encounter the virus, which is phagocytosed and broken down into protein fragments (peptides) inside the cell. The major histocompatibility complex (MHC) class II molecules within the APC are then loaded with a virus derived peptide. The MHC class II molecule then merges with the cell membrane, presenting the MHC class II molecule containing a peptide fragment of the encountered antigen to the environment. CD4+ T cells interact via their T cell receptor (TCR) with the MHC class II molecule on the APC and an immune response is mounted against the specific antigen (**Figure 2**). In order to evaluate the influenza specific TCR diversity and the quality of the response to Influenza, we built an MHC class II tetramer protein that will present Influenza peptide to CD4+ T cells in order to identify Influenza specific TCRs. One of the most frequent DR MHC class II molecules possessed by 29.8% of the population is the DR0101 α chain and the DR0401 β chain (together called DR4) MHC class II type (9, 10)). An immunodominant influenza peptide is conjugated to the MHC class II molecule in order to identify antigen specific CD4+ T cells.

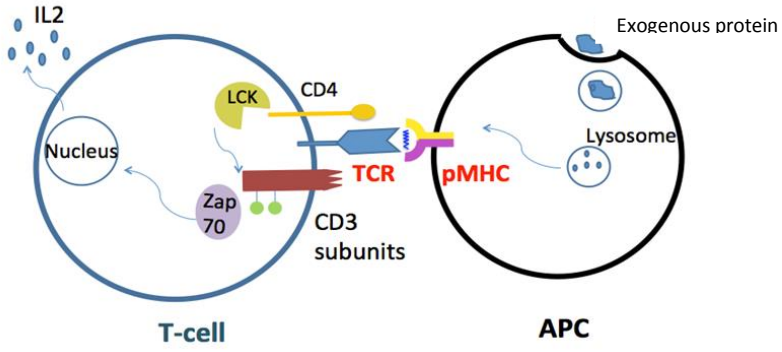

Figure 2. CD4+ T cell interaction with APC

The specific peptides of the influenza virus that were selected for this study were previously tested by Dr. William Kwok's laboratory at Virginia Mason Research Center for stability within MHC molecules. Our laboratory chose 5 tested peptides (amino acid regions 265-284, 329-348, 393-412, 433-

452, and 441-460) from the H1N1 2009 influenza virus strain that were shown to be stable when conjugated to MHC class II molecules.

Table 1.

Sequences of the 2009 H1N1 HA protein that contain $CD4^+$ T-cell epitopes

Amino acid region	Peptide sequences[a,b,c]	HLA restriction	Response frequency
1–20	*MKAILVVLLYTFATANADTL*	DR0404	1 of 4
33–52	**VDTVLEKNVTVTHSVNLLED**	**DR0701**	**7 of 7**
57–76	*KLCKLRGVAPLHLGKCNIAG*	DR0701	1 of 7
113–132	**IDYEELREQLSSVSSFERFE**	**DRB5**	**4 of 4**
121–140	**QLSSVSSFERFEIFPKTSSW**	**DR1101**	**2 of 3**
		DRB5	4 of 4
129–148	ERFEIFPKTSSWPNHDSNKG	DR1101	2 of 3
161–180	***FYKNLIWLVKKGNSYPKLSK***	**DR1101**	**3 of 3**
		DRB5	**3 of 4**
209–228	YQNADAYVFVGSSRYSKKFK	DR1101	2 of 3
217–236	*FVGSSRYSKKFKPEIAIRPK*	DRB5	1 of 4
225–244	*KKFKPEIAIRPKVRDQEGRM*	DRB5	1 of 4
241–260	*EGRMNYYWTLVEPGDKITFE*	DRB5	1 of 4
249–268	*TLVEPGDKITFEATGNLVVP*	DR0401	2 of 7
		DR0701	6 of 7
257–276	***ITFEATGNLVVPRYAFAMER***	DR0401	1 of 7
		DR0701	**7 of 7**
265–284	***LVVPRYAFAMERNAGSGIII***	**DR0401**	**7 of 7**
		DR0404	4 of 4
305–324	**TSLPFQNIHPITIGKCPKYV**	**DR0701**	**6 of 7**
313–332	*HPITIGKCPKYVKSTKLRLA*	DR1101	2 of 3
321–340	PKYVKSTKLRLATGLRNIPS	DR1101	2 of 3
329–348	LRLATGLRNIPSIQSRGLFG	DR0401	4 of 7
		DR0404	2 of 4
393–412	**TNKVNSVIEKMNTQFTAVGK**	DR0101	2 of 4
		DR0401	**4 of 7**
		DR0404	2 of 4
401–420	EKMNTQFTAVGKEFNHLEKR	DR0401	1 of 7
		DR1101	2 of 3
409–428	AVGKEFNHLEKRIENLNKKV	DR1101	2 of 3
433–452	LDIWTYNAELLVLLENERTL	DR0101	2 of 4
		DR1501	2 of 2
441–460	ELLVLLENERTLDYHDSNVK	DR0101	2 of 4
		DR0401	5 of 7
		DR0404	2 of 4
457–476	SNVKNLYEKVRSQLKNNAKE	DRB5	3 of 4
465–484	KVRSQLKNNAKEIGNGCFEF	DR1101	2 of 3
513–532	*NREEIDGVKLESTRIYQILA*	DR0701	1 of 7
521–540	KLESTRIYQILAIYSTVASS	DR0101	2 of 4
		DR0404	2 of 4

Figure 3. Table of tested peptides from Dr. Kwok's lab (4)

The H1N1 2009 influenza virus strain was chosen because it has been included in the vaccine given to the general population for 5 consecutive years according to the CDC. The approach to building the DR4 tetramer was to PCR amplify the DR4 genes from an HLA DR4/CD4 mouse and use bacterial cloning to yield a plasmid containing the DR4α/β genes as well as the selected peptide sequences. The plasmid chosen for cloning was pUC19 (**Figure 4**) and the bacteria used for cloning were DH5α *E. coli*.

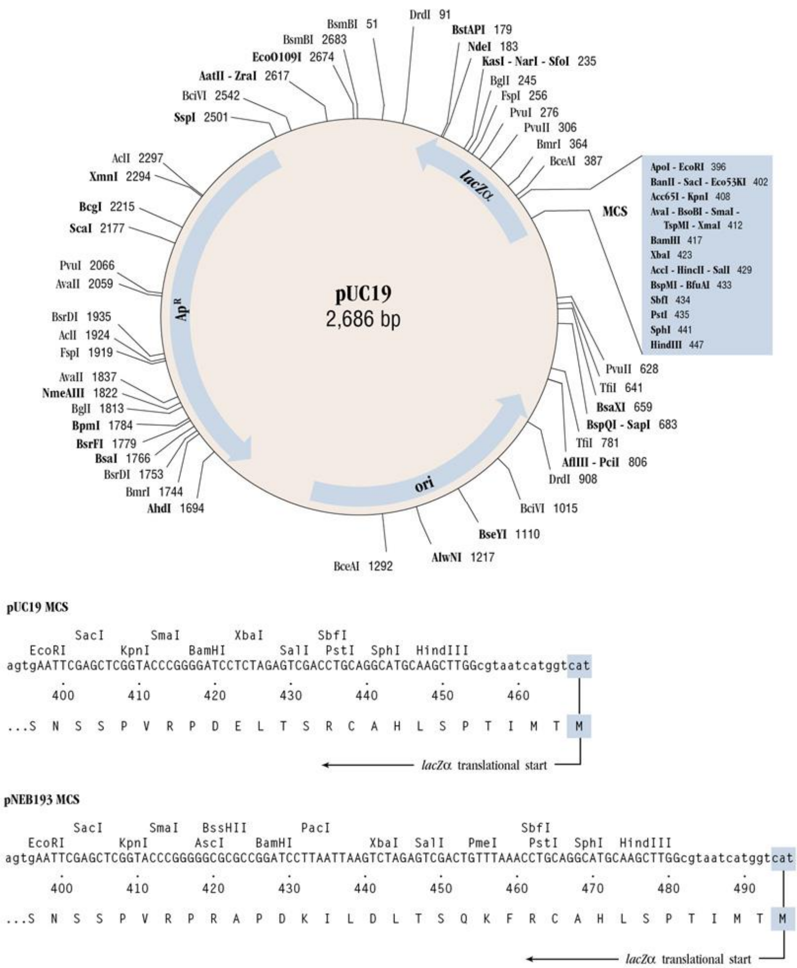

Figure 4. pUC19 map (5)

After cloning in DH5α cells, a surface expression test of the MHC class II molecules was tested in 58α-β- mammalian cell lines to assay for folding efficacy and stability before the monomers were

secreted into media free floating. After doing this surface expression stability test, the more stable construct sequences were used in Hi5 insect cell lines that secreted the protein monomers into the media, from which they were collected. The α and β chains were conjugated together by the acidic and basic leucine zippers that are encoded on the respective plasmids the protein sequences are on. The monomers can then biotinylated by a BirA enzyme, which allows the monomers to associate with a streptavidin bead. Four biotinylated monomers associate with a single streptavidin molecule forming a completed tetramer (**Figure 5)**.

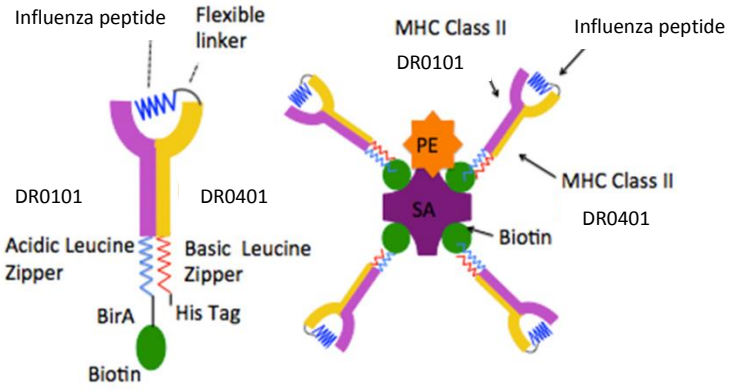

Figure 5. Diagram of monomer and tetramer components

Methods:

Isolation of DR4 genes

The hDR4 gene was present in HLA DR4/CD4 double transgenic mice obtained from Dr. Hugh McDevitt's at Stanford University School of Medicine (11, 12, 13). Splenocytes were harvested from an hDR4+ mouse and processed by grinding the tissue through a 70um cell strainer to produce a single cell suspension. Red blood cells were lysed using ACK Lysis buffer. The remaining leukocytes were then lysed using Trizol, allowing isolation of the RNA from the leukocytes. RNA isolation was conducted using the Trizol manufacturer protocol (Ambion cat. 15596-026, Man0001271). The RNA was then synthesized into cDNA using the SuperScript III protocol (Invitrogen cat. 18080-051), which was then used for PCR amplification of the hDR4 gene. In order to amplify these genes, engineered primers that hybridize near the hDR4 cDNA were used and the genes were amplified separately (**Table 1**). The DR4α forward primer was designed with a HindIII cut site, DR4α reverse was designed with a BglII cut site, DR4β forward was designed with an SpeI cut site, and DR4β reverse was designed with a BglII cut site in order to facilitate cloning.

DR4α Full Length Gene Primers	**DR4β Full Length Gene Primers**
Forward - AATAAGCTTCTCGACGCCACCATGGCCATAAGTGGAGTC	Forward - ATTACTAGTGCCCCGAGGAAGTGGAGGTGGAGGGTCTGGGGACACCCGACCACGTTTC
Reverse - AATGAATTCAGATCTTCAGGCGGCCGCCAGAGGCCCCCTGCGTTC	Reverse - AATAGATCTTCAGGCGGCCGCGCTCAGGAATCCTGTTG

Table 1. Primers used for DR4 gene amplification

Reagent	**Amount**
cDNA	1µL
Forward/Reverse Primers	1µL each of 10µM stock
dNTP	1µL
Phusion Buffer	10µL
Phusion (Taq polymerase)	.5µL
PCR Grade H2O	35.5µL

Table 2. PCR recipe used

Temperatures	Times
98°C	2m
98°C	30s x40
58°C	30s x40
72°C	30s x40
72°C	10m
4°C	Hold

Table 3. PCR times and temperatures

Cloning using Full Length DR4 genes, PUC19 and DH5α cells

The full length DR4α cDNA and pUC19 plasmid were then digested with HindIII and EcoRI restriction enzymes and ligated together using T4 DNA ligase. The plasmid containing full length DR4α was transformed into DH5α *E. coli* for bacterial cloning of the plasmid. Simultaneously, full length DR4β and a pUC19 plasmid provided by our collaborator, Dr. Kuhns, containing a peptide sequence (E641 West Nile virus peptide) as well as a full length MHC class II protein sequence ($A^b\beta$ mouse MHC) were digested with BglII and SpeI restriction enzymes. The full length DR4β cDNA sequence was ligated into the plasmid in place of the MHC class I sequence and then transformed into DH5α *E. coli* for bacterial cloning of the plasmid.

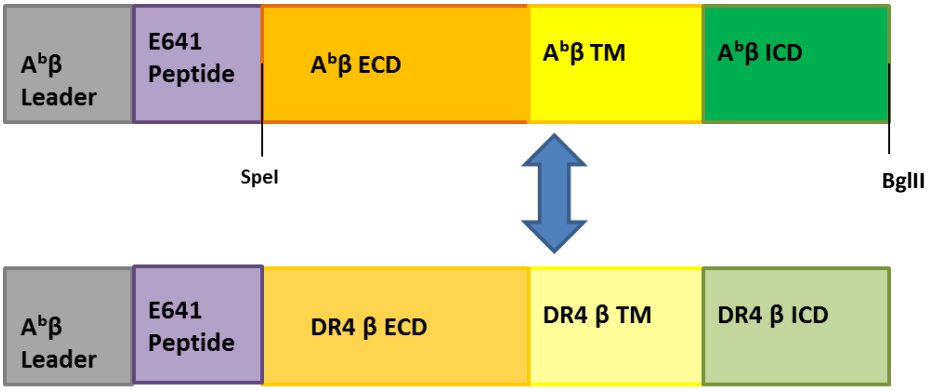

Figure 6. Schematic of switching $A^b\beta$ MHC sequence with DR4β MHC sequence

5 different influenza peptide sequences were designed with one blunt end and a SalI restriction overhang on the other side. During digestion the blunt end is phosphorylated, increasing the ligation

efficiency as seen in our experiments. BamHI restriction enzyme was used to digest the blunt end of the peptide sequence to leave an overhang. The plasmid containing full length DR4β and the West Nile virus peptide sequence were digested with BamHI and SalI restriction enzymes. The West Nile virus peptide sequence was purified from the plasmid by gel electrophoresis. The desired influenza peptide sequences were then ligated into the pUC19 plasmid. The plasmids, with the influenza peptide of interest and full length DR4β cDNA, were transformed into DH5α cells for bacterial cloning.

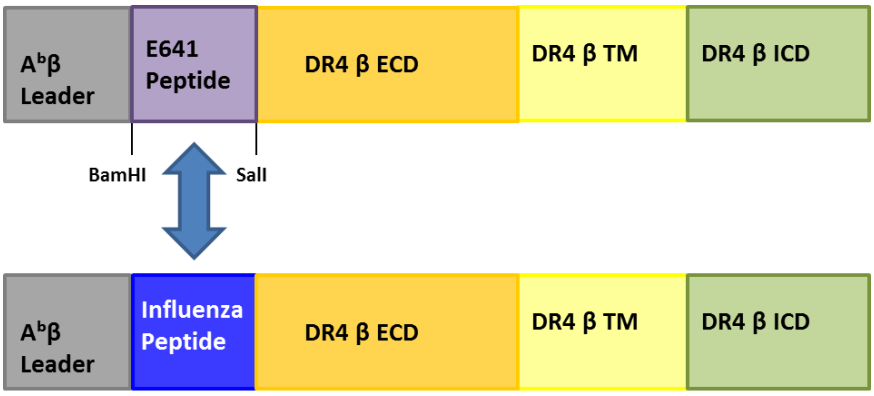

Figure 7. Schematic of peptide swapping

Cloning using Full Length DR4 genes, mammalian retroviral plasmids, and mammalian cell lines

Full length DR4α cDNA and a retroviral plasmid containing zeocin resistance (called Z4) were digested with XhoI and BglII restriction enzymes and ligated together. The plasmid was transformed into DH5α cells. Full length DR4β plus the influenza peptide and a retroviral plasmid containing puromycin resistance (called P2) were digested with XhoI and BglII restriction enzymes and ligated together. The plasmid was then transformed into DH5α cells.

ΦE mammalian cells were transfected with the Z4 plasmid containing DR4α in order to obtain retrovirus that contains the Z4 plasmid. A similar protocol was performed separately for each of the P2

plasmids containing the 5 different influenza peptide sequences, in order to obtain retroviruses containing the P2 plasmids.

ΦE DR4α Cell Lines	**ΦE DR4β Cell Lines**
1. DR4α	1. DR4β 265-284
	2. DR4β 329-348
	3. DR4β 393-412
	4. DR4β 433-452
	5. DR4β 441-460

Table 4. ΦE Cell Lines used to make retrovirus containing respective plasmids. 1. DR4α – cell line used to create retrovirus containing pUC19 with DR4α cDNA. 1. DR4β 265-284 – cell line used to create retrovirus containing pUC19 with DR4β and 265-284 Influenza peptide sequence. 2. DR4β 329-348 – cell line used to create retrovirus containing pUC19 with DR4β and 329-348 Influenza peptide sequence. 3. DR4β 393-412 – cell line used to create retrovirus containing pUC19 with DR4β and 393-412 Influenza peptide sequence. 4. DR4β 433-452 – cell line used to create retrovirus containing pUC19 with DR4β and 433-452 Influenza peptide sequence. 5. DR4β 441-460 – cell line used to create retrovirus containing pUC19 with DR4β and 441-460 Influenza peptide sequence

58α-β- T cells are mouse cells that do not express human class II genes, making them ideal to check for DR4 surface expression. These cells were co-transduced with the retrovirus contain Z4 and 1 of the retroviruses containing the respective P2 plasmid. This was done 5 times so that each 58α-β- cell line was co-transduced with the retrovirus containing Z4 and a different retrovirus containing the respective P2 plasmid.

58α-β- Cell Lines
1. Z4 DR4α + P2 DR4β 265-284
2. Z4 DR4α + P2 DR4β 329-348
3. Z4 DR4α + P2 DR4β 393-412
4. Z4 DR4α + P2 DR4β 433-452
5. Z4 DR4α + P2 DR4β 441-460

Table 5. 58α-β- cell lines with retroviral co-transduction schematic

After 1 week passed for proliferation to occur after infection, the 58α-β- cells were stained with an antibody to DR molecule and evaluated by flow cytometry for surface expression of the MHC class II molecules of interest.

Sub-Cloning of Extracellular Domain of DR4 genes, PUC19, and DH5α cells

Using the pUC19 plasmids containing full length DR4α and DR4β, new primers were used to amplify just the extracellular domain (ECD) of both DR4α and DR4β. DR4α forward was designed with a BamHI cut site, DR4α reverse was designed with an XbaI cut site, DR4β was designed with an SpeI cut site, and DR4β reverse was designed with an XbaI cut site new primers to make cloning more efficient.

DR4α ECD Primers	**DR4β ECD Primers**
Forward - ATTGGATCCATCAAAGAAGAACATGTGATCATCCAGGCC	Forward - ATTACTAGTGCCCCGAGGAAGTGGAGGTGGAGGGTCTGGGGACACCCGACCACGTTTC
Reverse - AATTCTAGAGTTCTCTGTAGTCTCTGG	Reverse - AATTCTAGACTTGCTCTGTGCAGATTC

Table 6. Primers used for PCR isolation of DR4 ECDs

The ECD of DR4α and pUC19 were digested with BamHI and XbaI and ligated together. The plasmid was transformed into DH5α cells. The ECD of DR4β and pUC19 containing both the influenza peptides of interest and full length DR4β were digested with SpeI and XbaI restriction enzymes in order to remove the full length DR4β cDNA and replace it with the DR4β ECD by ligation. The plasmid was transformed into DH5α cells.

Cloning of ECD of DR4 genes, baculoviral vectors, and insect cell lines

The pUC19 containing DR4α ECD and a baculoviral vector with the acidic leucine zipper sequence (pBV acidic) were digested with BamHI and XbaI restriction enzymes and ligated together. The pUC19 plasmids encoding DR4β ECD and the respective influenza peptide sequences and a baculoviral vector with the basic leucine zipper sequence (pBV basic) were digested with BamHI and XbaI enzymes and ligated together. The plasmids were transformed into separate DH5α cells.

SF9 cells were transfected with the pBV acidic plasmid containing DR4α ECD in order to produce p0 baculovirus containing the pBV acidic plasmid. The p0 viruses were the first generation of progeny and were expected to be at low concentration within the media. A similar protocol was performed separately for each of the pBV basic plasmids containing the 5 different influenza peptide sequences, in order to obtain p0 baculovirus containing the pBV basic plasmids. New SF9 insect cells were then transduced separate with each different p0 virus in order to produce the respective p1 baculovirus progeny at a higher concentration within the media.

SF9 pBV acidic Cell Lines	**SF9 pBV basic Cell Lines**
2. DR4α	6. DR4β 265-284
	7. DR4β 329-348
	8. DR4β 393-412
	9. DR4β 433-452
	10. DR4β 441-460

Table 7. SF9 cell line schematic to make baculovirus containing respective plasmids

Hi5 insect cells were co-transduced with the p1 baculovirus containing pBV acidic plasmid and 1 of the p1 baculovirusus containing the respective pBV basic plasmid. This was done 5 times so that each Hi5 cell line was co-transduced with the p1 baculovirus containing pBV acidic plasmid and a different p1 baculovirus containing the respective pBV basic plasmid. After time passed for proliferation to occur after infection, the media from the Hi5 cells was collected and kept separately at 4°C so as to keep the secreted proteins separate and distinguishable.

Hi5 Cell Lines
1. DR4α + DR4β 265-284
2. DR4α + DR4β 329-348
3. DR4α + DR4β 393-412
4. DR4α + DR4β 433-452
5. DR4α + DR4β 441-460

Table 8. Hi5 Cell Line Schematic

FFLISA Test for Secreted Monomers in Media

A fluorescent linked immune-sorbent assay (FFLISA) was performed, which means the proteins were stained with an antibody that recognizes DR4α chain, which was conjugated to a fluorescent tag, and an antibody that recognizes DR4β chain, which was biotinylated and associated with a streptavidin bead. The bead allows for multiple biotin molecules to associate with it, making the entire larger complex easier to detect by flow cytometry. The complexes were then evaluated on the flow cytometer to identify presence of protein in the media.

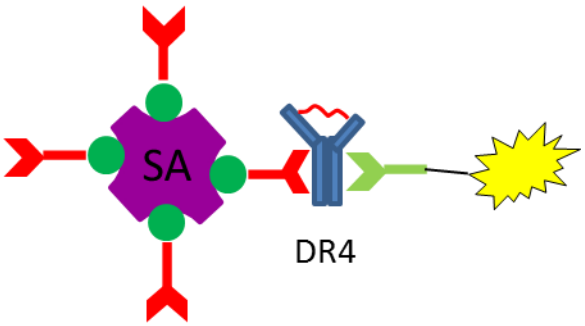

Figure 8. FFLISA test. Detection of monomers within the media.

Results:

Isolation of DR4 genes

After PCR amplification of DR4α and DR4β genes from the hDR4 mouse the PCR product was run on a .8% agarose gel in order to verify amplification of the desired genes. These PCR products were then ligated into pUC19 vectors to be transformed into DH5α cells for cloning.

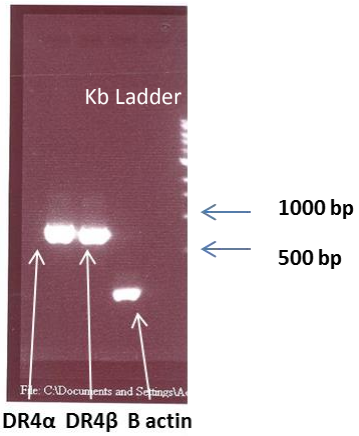

Figure 9. Gel image of PCR product

Cloning using Full Length DR4 genes, pUC19 and DH5α cells

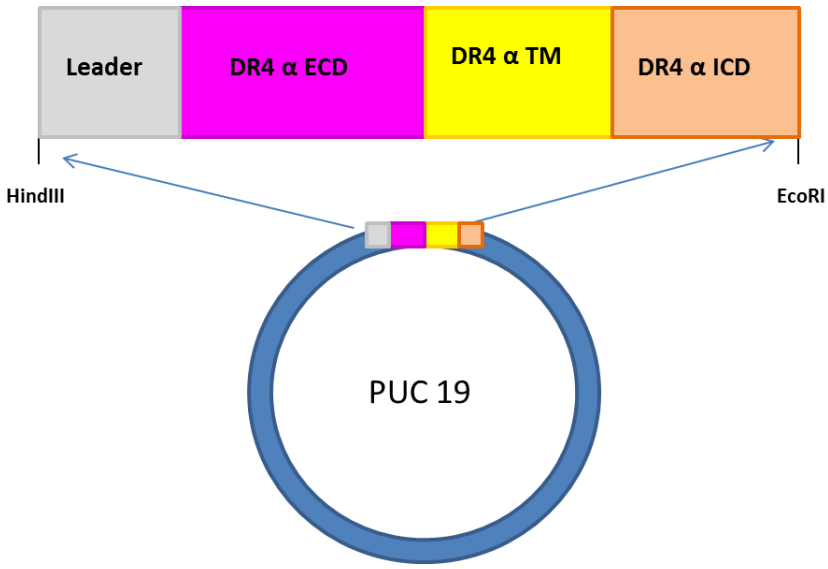

Figure 10. Full length DR4α plus mouse leader sequence ligation schematic into pUC19

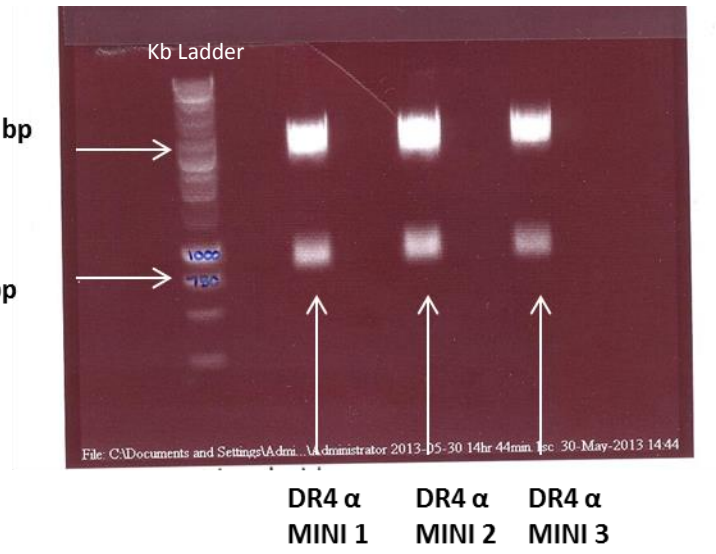

Figure 11. Gel picture after digestion of miniprep

In order to verify cloning of the transformed plasmid, the plasmids were isolated using a QIAprep Spin Miniprep kit (Qiagen cat. 27106). The plasmids were then digested using restriction enzymes that were used prior to ligation of these plasmids (HindIII and EcoRI). After digestion, the samples were run on a .8% agarose gel and visualized using a UV light box. The pUC19 plasmid backbone as well as the DR4α cDNA insert migrated at the sizes expected. The undigested plasmid was then submitted to the DNA sequencing facility on the University of Arizona campus in order to evaluate the ligated DNA for any mutations or other changes that may have occurred during cloning, no point mutations were found.

Cloning of DR4β was conducted differently due to the availability of a plasmid already containing a West Nile virus peptide sequence with enzyme cut sites on either side of it as well as a mouse Class II protein sequence with cut sites on either side of it. These cut sites allow us to "cut and paste" the chosen influenza peptide sequences and the DR4β gene sequence. First, the MHC Class II protein sequence and the DR4 MHC Class II full length gene sequence were cut with SpeI and BglII, gel purified, and the DR4 cDNA was lighted into the pUC19 vector containing the West Nile virus peptide.

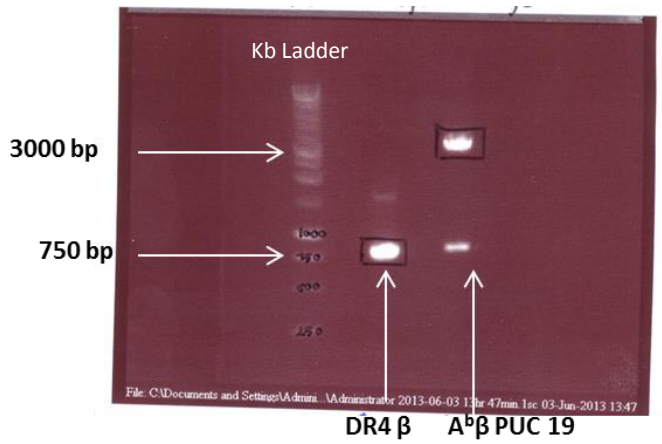

Figure 12. Gel Picture after digestion of plasmid with $A^b\beta$ and DR4β Full Length PCR product

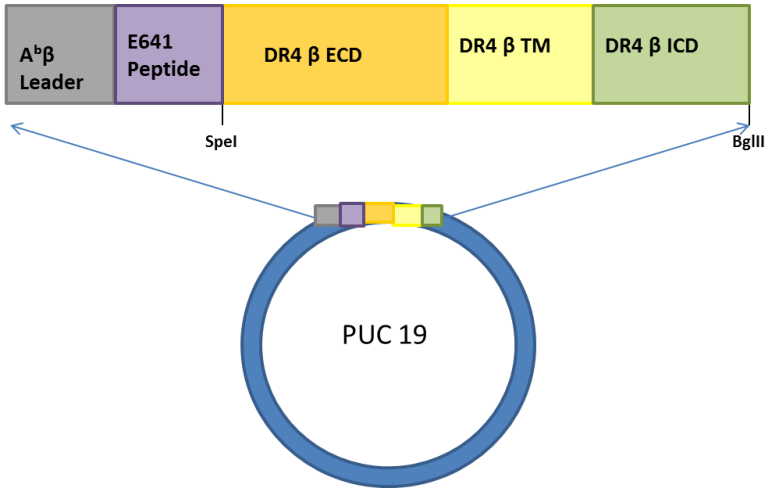

Figure 13. Ligation of DR4β into pUC19 in place of $A^b\beta$ but with $A^b\beta$ leader sequence

After ligation of the DR4β full length cDNA into the pUC19 vector containing West Nile virus peptide sequence, a test for successful ligation and cloning was conducted. Plasmid was propagated into DH5α cells and isolated using a Miniprep kit (Qiagen cat. 27106). The isolated plasmids were then digested using the same enzymes used previously, BglII and SpeI. After the digestion reaction the samples are loaded onto a .8% agarose gel for migration of DNA band. Both the pUC19 plasmid

backbone containing the E641 peptide and the DR4β full length cDNA insert migrated through the gel as expected due to their sequence length.

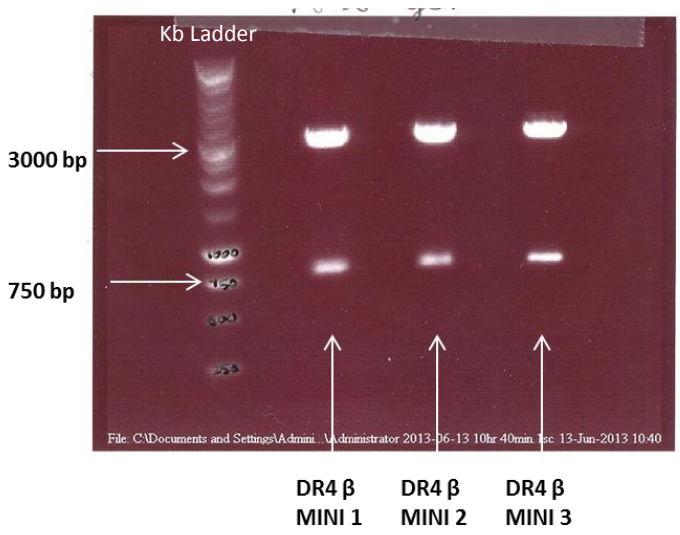

Figure 14. After cloning, miniprep and digestion

The plasmids were then submitted to the sequencing lab, no point mutations were found. After verification of the DNA sequence, the pUC19 plasmid now containing the E641 peptide sequence and the DR4β full length cDNA sequence was digested in order to remove the E641 peptide sequence.

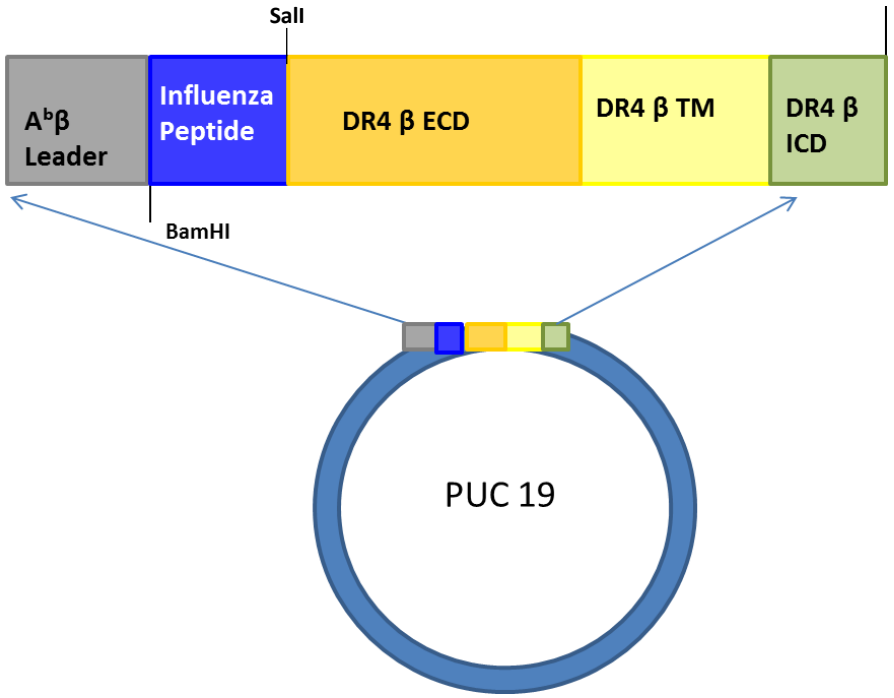

Figure 15. Plasmid schematic after ligation of influenza peptide coding sequence in place of the E641 peptide

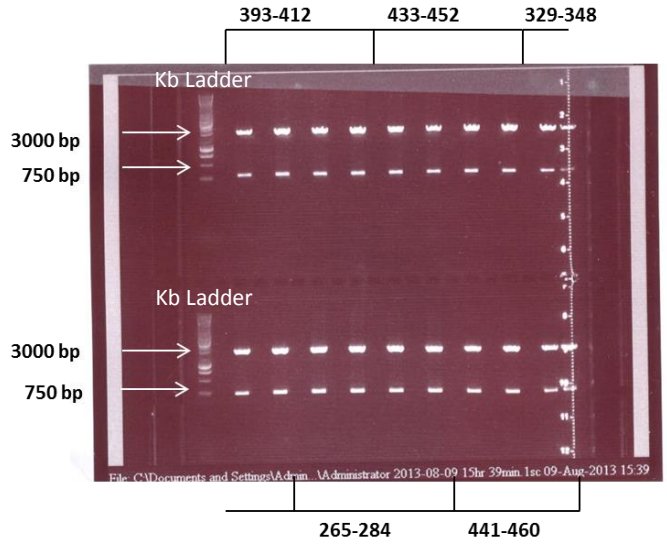

Figure 16. Restriction digestion to verify presence of the DR4β cDNA and influenza peptide sequence, chosen influenza peptide amino acid sequences are labelled

After bacterial cloning has occurred the plasmids were isolated using a Miniprep kit and then digested using one restriction enzyme used to digest the peptide sequence (BamHI) and one restriction enzyme used to digest the DR4β sequence (BglII) in order to capture the entire insert. The digested DNA separated on a .8% agarose gel and evaluated on a UV light box for DNA migration. Each of the samples had the expected pUC19 plasmid backbone length as well as insert length (**Figure 16**). The plasmids were also submitted to the sequencing lab, no point mutations were found.

Cloning using Full Length DR4 genes, mammalian retroviral plasmids, and mammalian cell lines

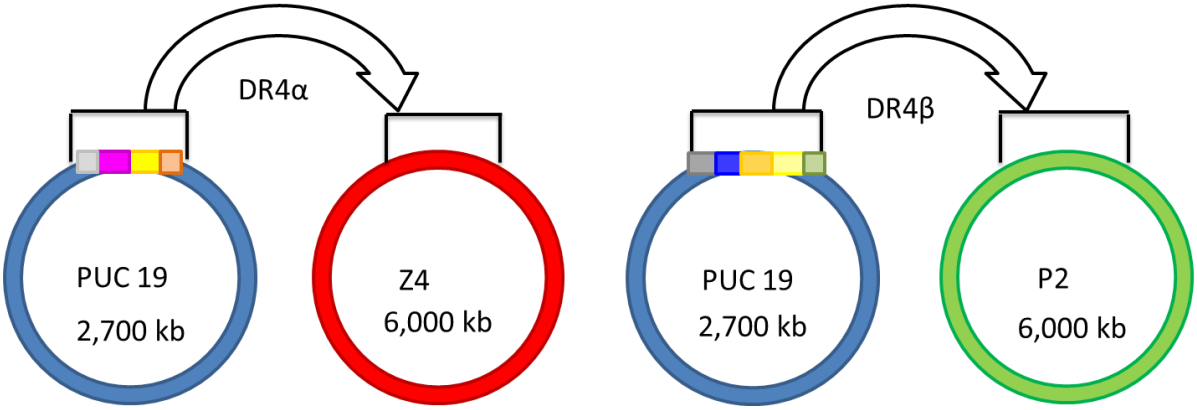

Figure 17. Shuttling genes of interest into retroviral vectors

The full length genes of DR4α and DR4β were then sub-cloned into mammalian retroviral expression vectors. The vectors are incorporated into a retrovirus that will infect the 58α-β- cells in order to test the protein's folding ability. The full length genes are shuttled into the respective retroviral vectors separately. After cloning in DH5α cells, the Z4 and P2 plasmids are isolated using a Miniprep kit. The plasmids were then digested and run on a .8% agarose gel in order to evaluate migration patterns of the DNA. Some of the selected DH5α colonies did not have the expected number of bands, indicating the insert was not ligated into the plasmid successfully (**Figure 18)**. The samples chosen to proceed with were lane 1 of the DR4α ECD containing plasmids, lane 1 of the 265-284 containing plasmids, lane 2 of

the 329-348 containing plasmids, lane 1 of the 393-412 containing plasmids, lane 2 of the 433-452 containing plasmids, and lane 3 of the 441-460 containing plasmids.

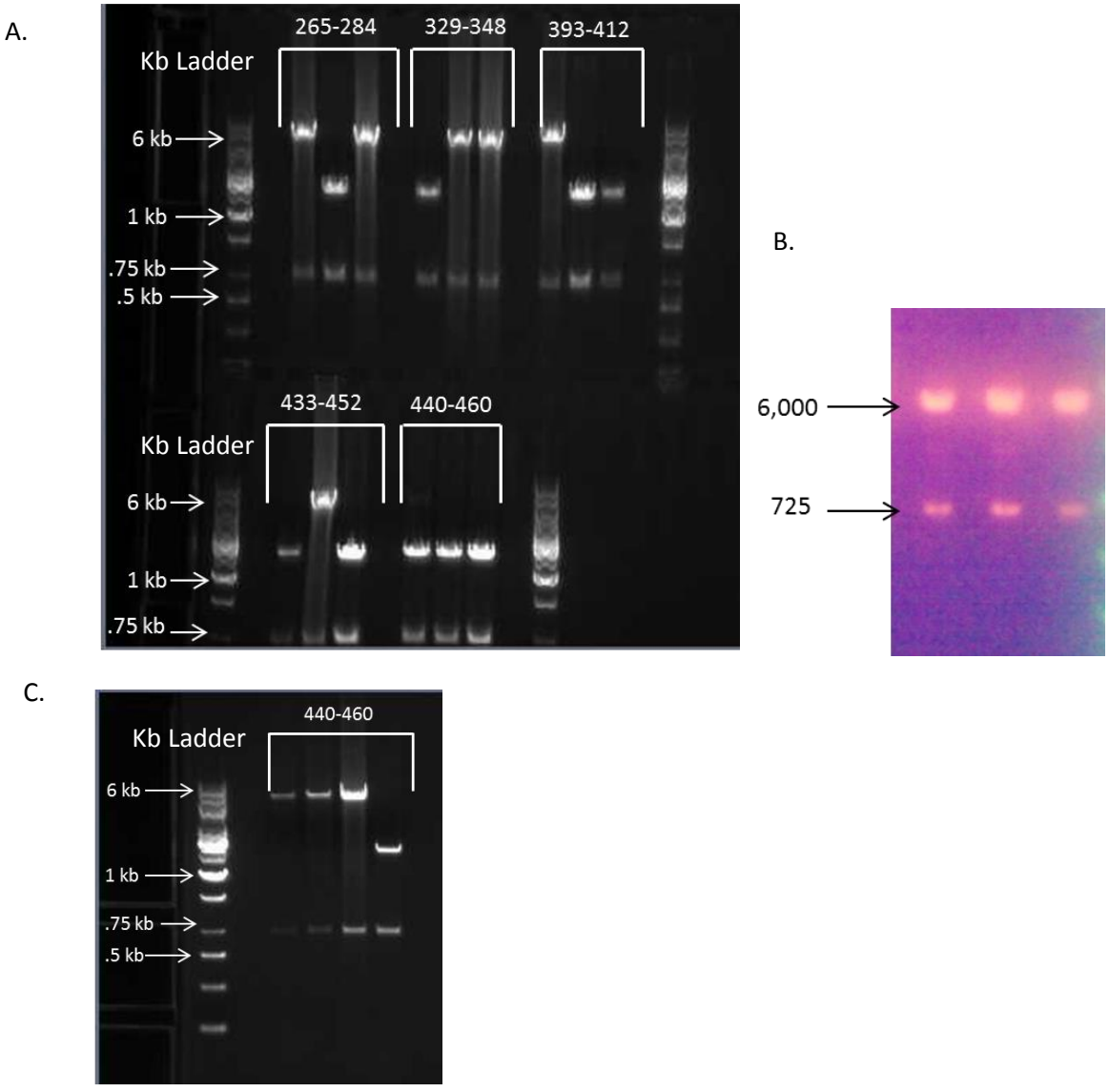

Figure 18. A. After digestion of P2 plasmids containing DR4β full length cDNA, Influenza peptide containing vector differences were noted. B. After digestion of Z4 plasmids containing DR4α full length cDNA. C. 441-460 Influenza peptide sequence containing vector after sub-cloning

When evaluating the 58α-β- mammalian cells for surface expression of DR MHC molecules we noted that all the peptide constructs had higher surface expression than the control 58α-β- cells. This assay tested the stability of the MHC class II molecules containing the different influenza peptides, in order to evaluate which molecules would be the most successful when in the insect cell system. Constructs encoding Influenza HA 329-348, 393-412, and 441-460 showed to have the highest surface expression, which is a marker of stability; however all of the peptides had an increase in the mean fluorescence intensity so we proceeded with all 5 peptide constructs.

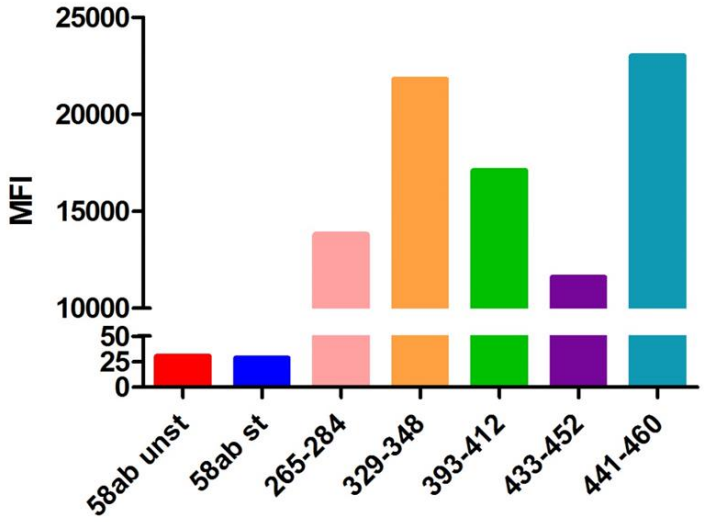

Figure 19. Mammalian Cell System Results

Cloning of Extracellular Domain of DR4 genes, PUC19, and DH5α cells

After the stability of the surface DR4 MHC Class II proteins was verified in the mammalian cell system, the extracellular domain (ECD) of both DR4α and DR4β were isolated. The ECD is purified from the pUC19 plasmids containing DR4α full length and DR4β full length, along with the influenza peptide sequences separately.

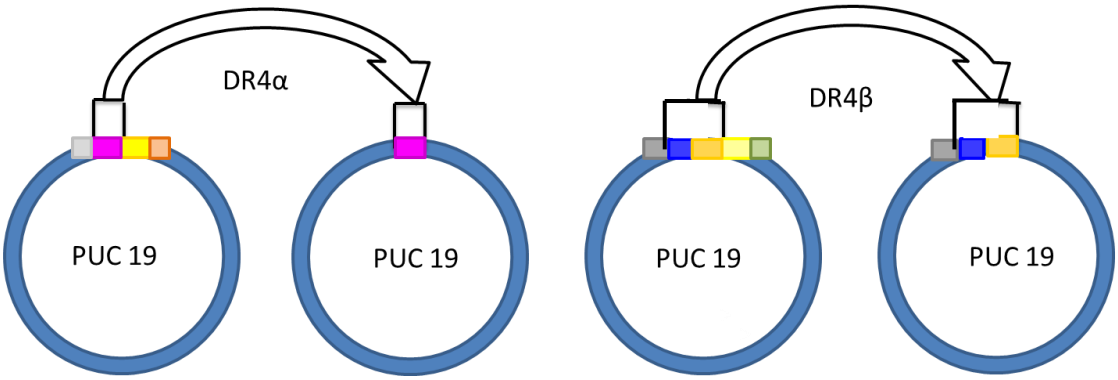

Figure 20. Shuttling of ECD/ECD+peptide into an pUC19

After cloning of the pUC19 plasmids in DH5α *E. coli* cells the plasmids were isolated using a Miniprep kit. The plasmids were then digested with BamHI and XbaI and run on a .8% agarose gel in order to verify that the insert was correctly incorporated into the plasmid.

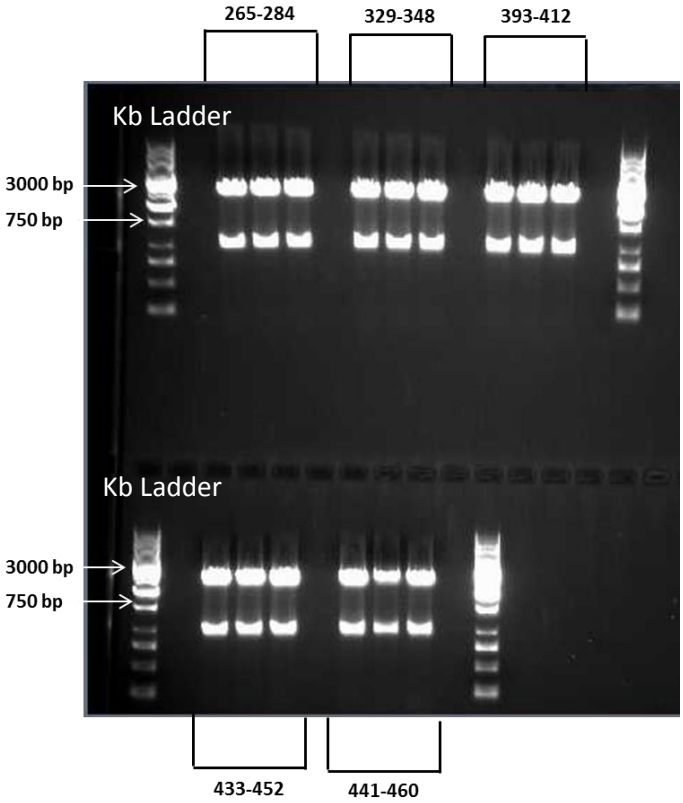

Figure 21. Gel migration of digestion samples, Influenza peptide sequences are noted

Cloning of ECD of DR4 genes, baculoviral vectors, and insect cell lines

After verification of insert size in the pUC19 plasmids, the ECD/ECD+peptide was digested with BamHI and XbaI restriction enzymes for sub-cloning. The pBV vectors were incorporated into baculoviruses that were used to infect insect cells. After infection, the insect cells contain the viral vector necessary for making the proteins and contain the proper machinery to secrete the proteins into the media.

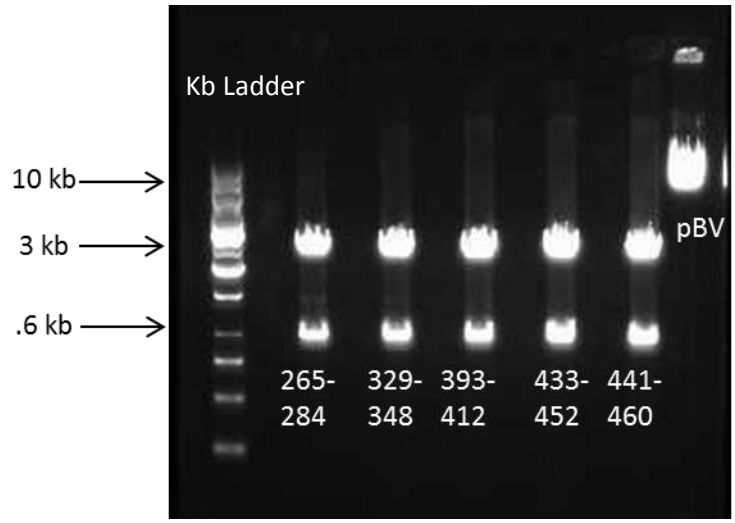

Figure 22. After digestion of pUC19 containing DR4β plus peptide sequences and pBV, Influenza peptide sequences are noted

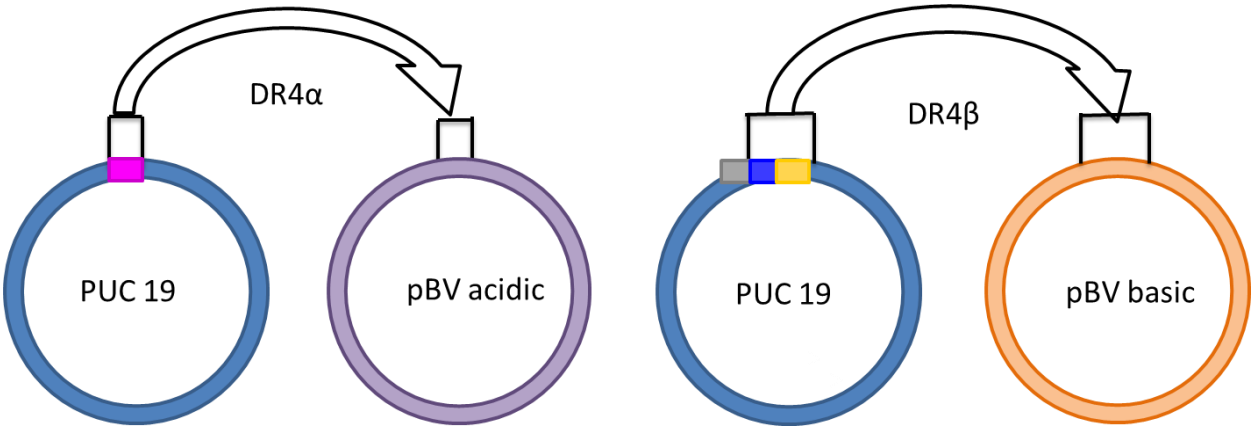

Figure 23. Shuttle of ECD and ECD+peptide into baculoviral vectors

After cloning of the pBV vectors in DH5α cells colonies were selected and the plasmid was isolated using a Miniprep kit. The plasmid was then digested and run on a .8% agarose gel in order to verify the incorporation of the DR4α ECD and DR4β ECD plus influenza peptide. The gel is evaluated on a UV light box, and **Figure 24** shows the plasmid backbone and DR4 inserts all migrated the expected length.

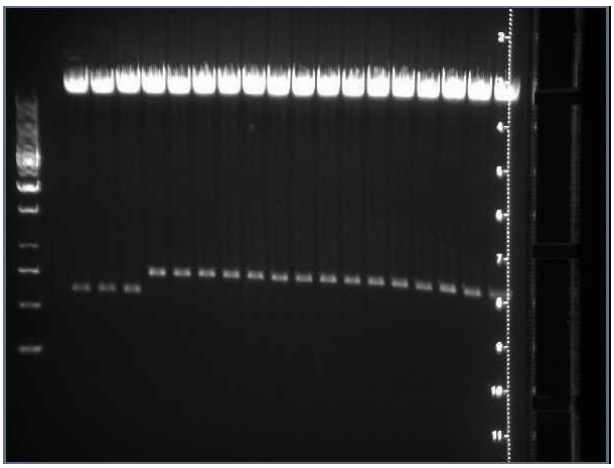

Figure 24. After digestion of isolated pBV plasmids.

The plasmids were then incorporated into p0 baculoviruses by SF9 insect cells. P1 baculovirus progeny were used to infect Hi5 cells. Infections were done with varying multiplicity of infection (MOI), meaning that there were different amounts of media containing p1 progeny added to the Hi5 cell lines. The purpose of this is to make sure that the Hi5 cells are receiving enough viruses to make protein, but not so much viruses that the cells die. After evaluation of protein output, an ideal amount of virus can be interpreted for future experiments. The protein concentration in the media was determined by FFLISA.

Evaluation of the FFLISA results for the media collection of the Hi5 insect cell system was done on the flow cytometer. Of the peptide constructs tested in the Hi5 insect cell system, only the construct containing 441-460 Influenza peptide sequence had detectable protein expression above the control

streptavidin beads (**Figure 25)**. This lack of expression indicates DR4 MHC class II with 441-460 influenza peptide was the only one that was stable enough when secreted into the media to maintain its structure and be detected by FFLISA.

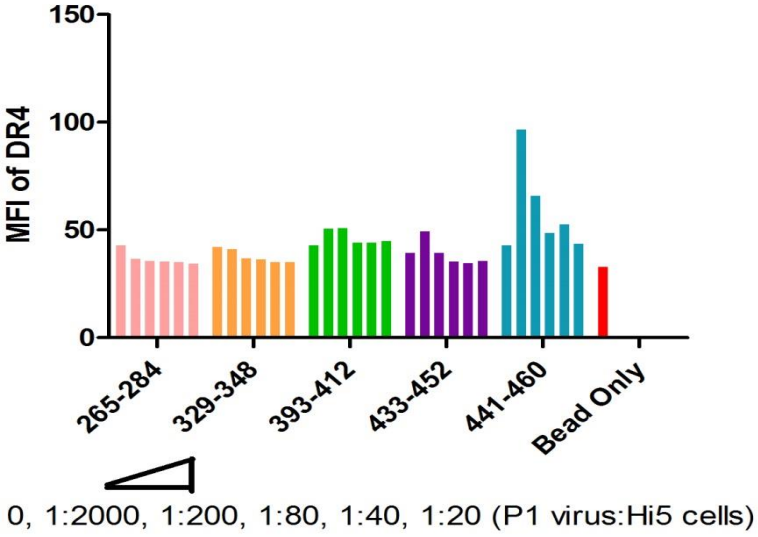

Figure 25. Insect Cell FFLISA Results

Peptide construct 441-460 had the highest mean fluorescent intensity when tested in the mammalian cell system. This result provides further evidence that the mammalian cell system is a good test for protein stability, efficacy and success when translated to the insect cell system.

Conclusions:

A DR4 tetramer containing an influenza peptide can be used as a tool to investigate numerous attributes of the immune system in response to influenza. Here to fore it has been difficult to measure the number of Influenza specific CD4+ T Cells in the aged population and how well these specific TCRs are able to recognize influenza peptide. We hypothesize that the CD4+ T cell TCR that recognizes influenza in the aged population is decreased in number when compared to adult population and as a result is not able to respond to infection of Influenza as well as younger adults. There is evidence of similar changes in the CD8+ T cell population (3). Further, the efficacy of influenza vaccination is decreased in older adults. The tetramer staining of a CD4+ T cell will allow the isolation of T cells with TCRs specific to influenza. This will allow for antigen specific examination of the CD4+ TCR repertoire, which will lead to further understanding of the TCR repertoire itself and how it changes with age.

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