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The Graduate School

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DEVELOPMENT OF SPECIFIC γδ T CELL POPULATIONS IS CRITICALLY AFFECTED BY RECOMBINATION SIGNAL SEQUENCE-ASSOCIATED RESTRICTION ON TCRδ GENE REARRANGEMENT

A Thesis in

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Abstract

Preferential localization of γδ T cells in the epidermis of the body suggests their roles in the first line of immune defense. However, little is known about the molecular mechanisms that regulate γδ T cell development and tissue distribution. In wild type mice, $V\gamma3^+$ T cells are exclusively found in the skin. However, in the absence of $V\gamma3^+$ T cells, other subsets of γδ T cells can substitute as skin γδ T cells. Previous studies found that the development of substitute skin γδ T cells vary among different genetic backgrounds. Substitute skin γδ T cells were found in both Cγ1 and Vγ3 knockout mice of C57Bl/6 but not 129/svj background. Similarly, transgenic $V\gamma2^+$ γδ T cells were found in the skin of $TCRV\gamma2TgC\gamma1^{-/-}$ mice of C57Bl/6 but not 129/svj background. The transgenic skin γδ T cells expressed Vγ2 along with endogenous Vδ7. As the Vδ7 gene is present in the 129/svj genome it is unclear why it is not expressed on the 129/svj skin. Our analysis found that this is, at least in part, due to reduced rearrangement of TCRVδ7 gene in the 129/svj strain. Furthermore, We found that recombination signal sequences (RSS) of the Vδ7 gene is different between the C57Bl/B6 and 129/svj strains. The Vδ7 RSS of 129/svj strain is identical to those of the other Vα genes of its family although the Vδ7 RSS of B6 strain is unique in itself, suggesting that the recombination signal sequence might be responsible for the reduced Vδ7 gene usage in TCRδ gene assembly in the 129/svj background.

There is a possibility that the non expression of Vδ7 in129/svj is due to the lack of a selection ligand. In order to investigate this possibility we have developed a reporter $V\gamma2V\delta7^+$ T cell-line. This $V\gamma2V\delta7^+$ reporter T cell-line has a construct containing the lac-Z gene under the control of the IL-2 promoter. Upon activation of the Vγ2Vδ7 TCR, NFAT should bind to the IL-2 promoter and lac-Z should be produced. Preliminary results show that the Vγ2Vδ7 reporter T cell line functions accordingly and can be applied in Vγ2Vδ7 ligand detection assays on C57Bl/6 and 129/svj mice.

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LIST OF ABBREVIATIONS

129	**129/svj mouse strain**
α	**Alpha**
AMP	**Ampicillin**
ATP	**Adenosine triphosphate**
β	**Beta**
B6	**C57Bl/6 mouse strain**
CPRG	**Chlorophenol red β-D-galactopyranoside**
δ	**Delta**
DETC	**Dendritic Epidermal T Cells**
DNA	**Deoxyribonucleic acid**
EDTA	**Ethylenediaminetetraacetic acid**
FP	**Forward Primer**
γ	**Gamma**
GFP	**Green Fluorescent Protein**
IgG	**Immunoglobulin G**
IEL	**Intraepithelial Lymphocytes**
IMGT	**International ImmunoGeneTics Information System**
IRES	**Internal Ribosome Entry site**
mAb	**Monoclonal Antibody**
MHC	**Major Histocompatibility Complex**
MSCV	**Murine Stem Cell Virus Retroviral Expression System**
PCR	**Polymerase Chain Reaction**
PE	**Phycoerythin**
RT-PCR	**Reverse Transcription Polymerase Chain Reaction**

RNA	**Ribonucleic Acid**
RAG	**Recombination Activation Gene**
RP	**Reverse Primer**
RSS	**Recombination Signal Sequence**
sIEL	**Skin Intraepithelial Lymphocytes**
TCR	**T Cell Receptor**
Tg	**Transgenic**
WT	**Wild Type**

Chapter 1

INTRODUCTION

T cells and T cell Receptors

T cells are generally considered to be part of the adaptive immune system. They exhibit such characteristics as antigenic specificity, diversity, immunologic memory and self/nonself recognition. Although T cell progenitors arise in the bone marrow, they mature in the thymus (thus the name T cells). T cells express antigen binding molecules on their surfaces known as T-cell receptors (TCRs) (Fig 1.1). The T-cell receptor is a heterodimer composed of either α and β or γ and δ chains. T cells with α and β chain TCRs, are known as αβ T cells. The majority of the T cells in the human and the mouse express αβ TCRs and recognize antigen bound to major histocompatibility molecules (MHC). MHC molecules assist in both humoral and cell mediated immune responses by presenting antigen to T cell receptors. γδ T cells can recognize antigen without the aid of MHC molecules. γδ T cells are found mainly in epithelial layers and have been suggested to play a role in innate immunity. Some proposed functions for γδ T cells include wound healing, surveillance against tumours and allergic responses. More is known about αβ T cell development and maturation than γδ T cells.

The skin is the body's first line of defense to the outside environment. Its large surface area creates a nonspecific anatomic barrier against many elements such as pH and forces due to friction. The skin also has specialized cells that participate in both innate and adaptive immune systems. For example, the epidermal layer of the skin is largely composed of epithelial cells called Keratinocytes. When under stress, these cells secrete a number of cytokines that function to recruit members of the adaptive immune system [1, 2]. The epidermis also contains Intraepithelial Lymphocytes (IELs). IELs have been found to be mainly composed of γδ T cells [1, 3]. Research shows that γδ T cells play a major role in such functions as down regulation of inflammation, wound healing, and immune surveillance against tumors [18, 19, 20]. IELs have been observed in epithelial sites such as the skin, intestine, urinary tracts and lungs. The specialized IELs found in the skin are known as sIELs (skin Intraepithelial Lymphocytes). These sIELs have been found to be mainly composed of Vγ3Vδ1 T cells [4, 5, 6]. In

order to understand how various diseases affect the skin, a good understanding of the biology of the skin as well as natural defenses provided for the skin is necessary.

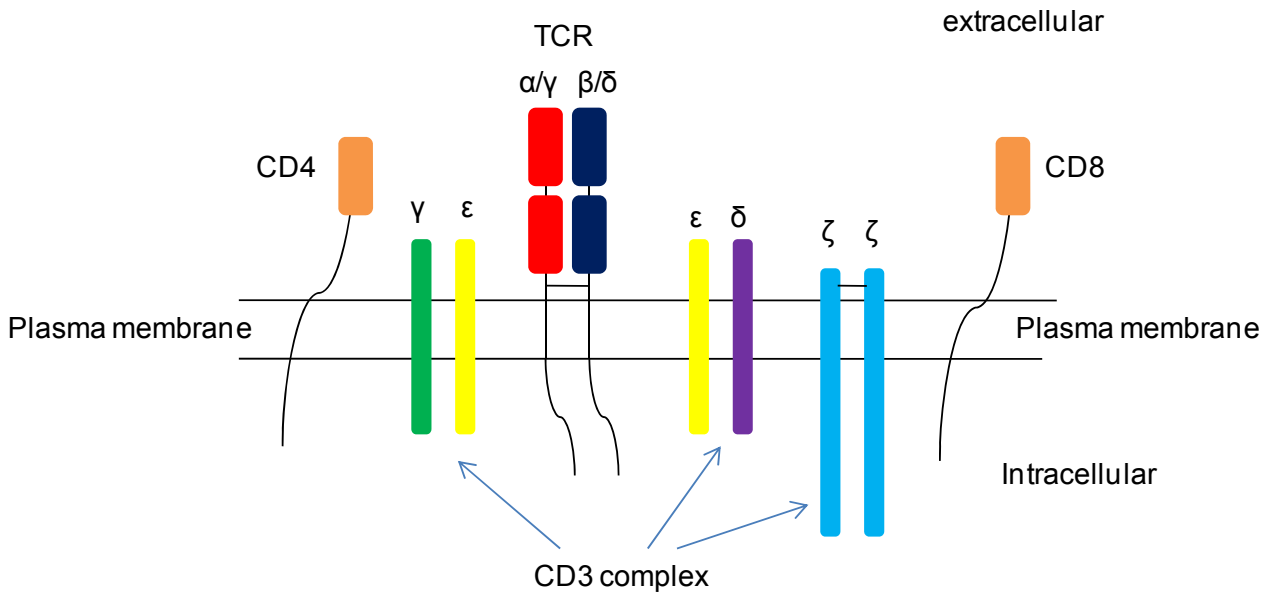


Fig1.1: Schematics of a T cell Receptor. The T Cell Receptor (TCR) is expressed on the surface of a T cell. CD4 and CD8 are co-receptors that play a role in recognition of antigen-MHC complexes and signal transduction. Unlike αβ Tcells, γδ T cells do not require CD4 or CD8 co-receptors for antigen recognition. After the αβ or γδ TCR binds an antigen, intracellular signaling for the appropriate response occurs via the CD3 complex.

T Cell Receptor Rearrangement (Importance of the Recombination Signal Sequence (RSS))

As mentioned earlier, T cells express antigen binding molecules on their surfaces known as T-cell receptors (TCRs). A T cell receptor is a dimer consisting of two chains. Each chain can be divided into two parts: a variable region which is the antigen specific region and a constant region which connects the variable region to the cell and signal transduction units. In order to produce functional T cell receptors, T cell gene segments that encode the variable and constant regions on each chain have to be productively rearranged. V and J gene segments rearrange in α and γ DNA to form the variable region of those chains while V, D and J gene segments rearrange in β and δ DNA. Transcription and translation of the rearranged genes eventually yield a monomer of a TCR. α chains dimerize with β chains and γ dimerizes with δ to form αβ or γδ TCR heterodimers.

Recombination signal sequences (RSS) (Fig 1.2) are sequences that flank the 3' end of the V gene segments, the 5' end of the J gene segments and the 5'and 3' end of the D gene segments. The sequence of each RSS consists of a conserved heptamer and a conserved AT rich nonamer separated by a sequence of 12 or 23 bps (known as a spacer). Rearrangement can only occur between an RSS with a 12 bp spacer (12-RSS) and another with a 23 bp spacer (23-RSS), this is known as the 12/23 rule. It is on the RSS that Recombination activating proteins (RAG1/2) bind in order for rearrangement to proceed [21, 22].Variations in the consensus sequence of the heptamer or nonamer have been shown to affect rearrangement efficiency [21, 22, 23]. A few reports have suggested that the sequence of the spacer also plays a role in rearrangement efficiency [21, 24], while others have suggested that there are certain conserved sequences within the spacer [25].

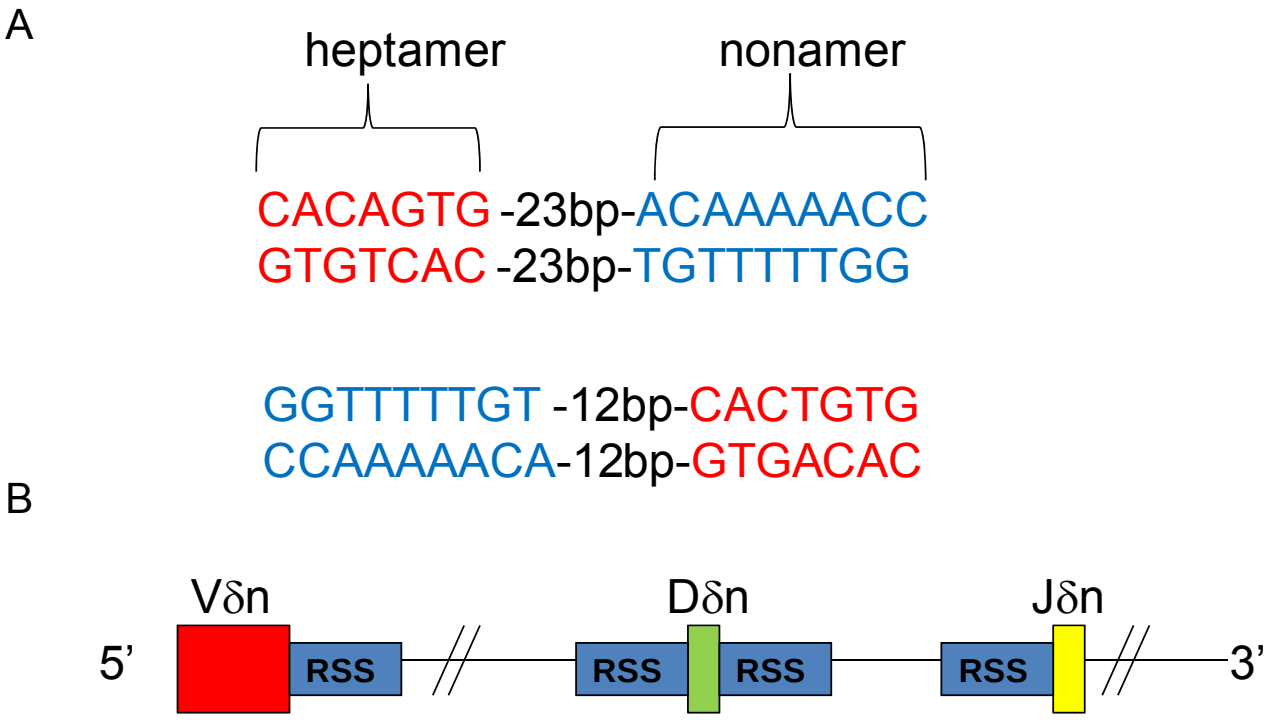


Fig 1.2: Position of RSSs on V, D and J regions. A) Recombination Signal Sequences (RSS) consists of a conserved heptamer and a conserved AT rich nonamer, separated by a sequence of 12 or 23 base pairs. B) RSSs flank the 3' end of the V region (3'), 5' and 3' end of the D region and the 5' end of the J region. Regions with RSS of 12bps can only join with regions of RSS of 23bps (12/23 rule). RAG enzymes (Recombination-activating genes) recognize RSS and facilitate rearrangement.

It is also important to consider the Beyond 12/23 rule postulated by Bassing et al (Fig1.3) [32]. This rule states that despite the 12/23 rule, some V regions with the appropriate 12 or 23 spacer RSS are incapable of joining with J regions with 23 or 12 spacer RSS respectively.

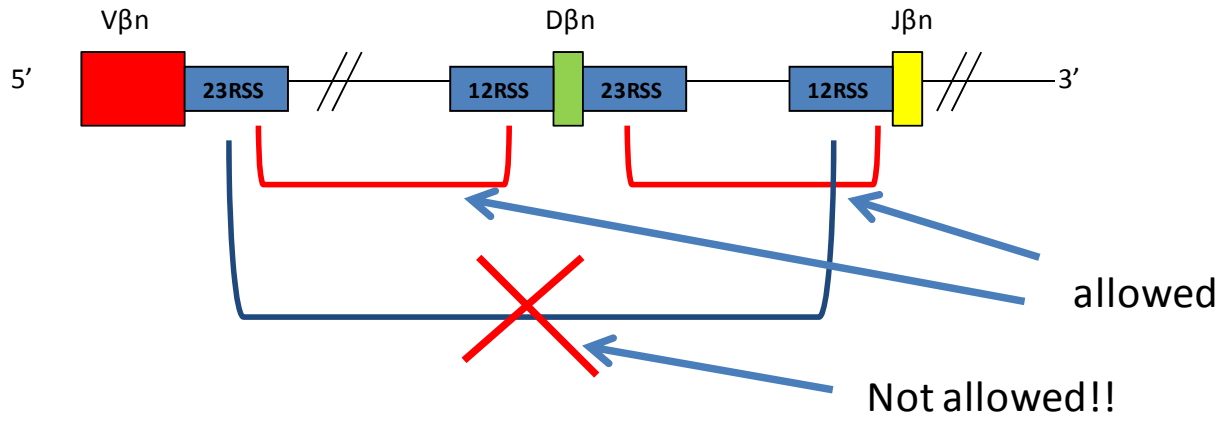


Fig 1.3: Depiction of the Beyond 12/23 Rule. Vβn is capable of joining to Jβn due to the 12/23 rule but cannot due to controlled joining to Dβn.

Fetal Thymic Selection of sIEL Precursors and Development of sIELs

It has been shown that sIEL development is dependent on generation of γδ T cells in the early fetal thymus [6, 7]. It has also been suggested that Vγ3 gene and Vδ1 gene rearrangements are favoured for the development of sIELs (Fig1.4) [5, 6, 7]. However, in the absence of Vγ3, other γδ T cells could substitute as sIELs (Fig 1.5) [1, 10, 11].

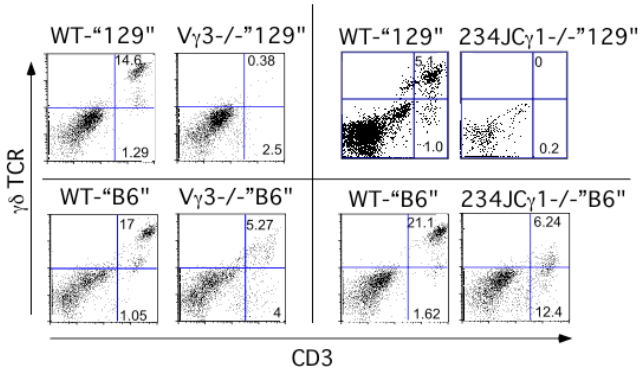


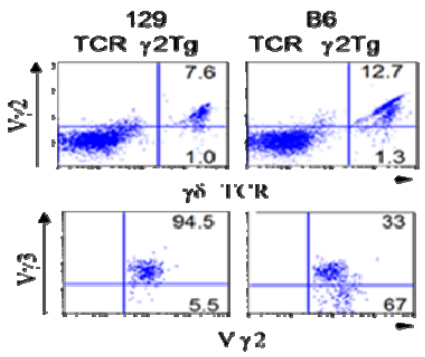
Fig1.4: Vγ3 sIELs are Favoured for the Development of Skin DETCs. As observed in wild type mice of C57Bl/6 and 129/svj background, Vγ3 sIELs are predominant in epidermal skin cells. However in the absence of Vγ3, sIELs are greatly reduced in B6 mice and almost absent in 129. WT :- wildtype, $V\gamma3^{-/-}$:-Vγ3 knockout, $234JC\gamma1^{-/-}$:-$C\gamma1^{-/-}$ cluster knockout (Vγ3 is part of this cluster).

	WT C57Bl/6	TCRγ2Tg234JCγ$^{-/-}$
%Vγ3	19.7	0.4
%Vγ2	0.5	12.4

Fig 1.5: Substitute γδ T cell sIELs Develop in the Absence of Vγ3^{+} sIELs. The Cγ1 cluster which includes the Vγ3 and Vγ2 genes was knocked out via homologous recombination with a neo cassette. A Vγ2 transgene present in Cγ1 knockout mice was observed to facilitate the presence of sIELs in the absence of Vγ3^{+} sIELs [1].

Xiong and co-workers also have data that suggests that development of the substitute skin γδ T cells in the absence of the native Vγ3^{+} skin γδ T cells in the mouse is dependent on the genetic background. The Cγ1 cluster of TCRγ locus encodes several TCRγ genes including Vγ3. When a Vγ2 transgene (Vγ2Tg) is placed on a Cγ1$^{-/-}$ background of C57Bl/6 and 129/svj mice, Vγ2^{+} skin cells are only observed on the C57Bl/6 background (Fig. 1.6). This is in contrast to Vγ2 skin cells observed on the skin of transgenic wildtype mice of both C57Bl/6 and 129/svj backgrounds.

A



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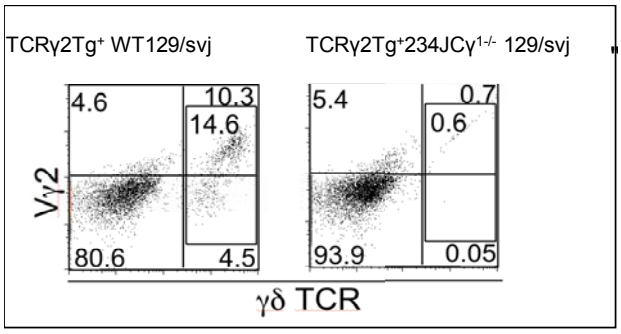


Fig 1.6: Vγ2 Transgene is Not Expressed in Cγ1$^{-/-}$ 129/svj mice. A) Both Vγ2^{+}Vγ3^{+} and Vγ2^{+}Vγ3^{-} sIEL populations developed in TCRγ2 transgenic mice of "B6" background while only Vγ2^{+}Vγ3^{+} sIELs developed in TCRγ2 transgenic mice of "129" background. Epidermal cell preparations of TCRγ2Tg^{+} mice were stained for detecting Vγ2^{+}, Vγ3^{+} and total γδ T cells. The Vγ2^{+} γδ T cells of TCRγ2Tg^{+} mice of 129 (left panel) and B6 backgrounds (right panel) from the top panel were gated for analysis of Vγ3 expression (bottom panel). **B)** TCRγ2^{+}234JCγ1$^{-/-}$ mice backcrossed on 129/svj strain lack TCRγδ^{+} sIELs (right panel). Left panel shows transgenic littermates that harbor the wild-type TCRγ locus.

Xiong and co-workers conducted further analysis of Vγ2 expression in wild type and Cγ1 knockout populations of C57Bl/6 and 129/svj background mice. RT-PCR analysis of TCRδ gene usage in Vγ2Tg wildtype and Vγ2Tg Cγ1$^{-/-}$ mice of both C57Bl/6 and 129/svj backgrounds showed that on C57Bl/6 mice, Vδ7 was expressed along with Vγ2 in wild type transgenic and knockout transgenic mice. However, Vδ7 was not expressed on any 129 mouse type (Fig 1.7) [1].

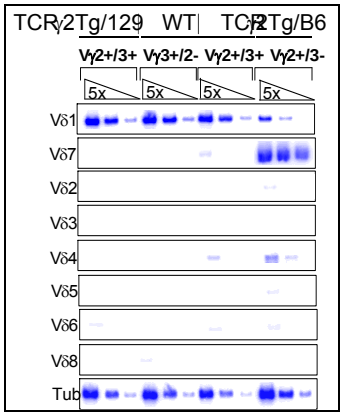


Fig 1.7: Vδ7$^+$ TCRδ gene is Predominantly used in C57Bl/6 Vγ2$^+$Vγ3$^-$ sIEL. Vδ7$^+$ TCRδ gene is predominantly used in Vγ2$^+$Vγ3$^-$ sIEL population of the TCRγ2Tg$^+$ mice of "B6" background while Vδ1$^+$ TCRδ gene is predominantly used in Vγ2$^+$Vγ3$^+$ sIEL populations of both "B6" and "129" backgrounds**.** RNA was prepared from sorted populations of skin gd T cells with different Vγ gene expressions and analyzed for Vδ gene usage by semi-quantitative RT-PCR.

Together, these data show that in the absence of Vγ3, the Vγ2 transgene is expressed on the C57Bl/6 background but not the 129/svj background. On the C57Bl/6 background, Vγ2 is expressed along with the endogenous Vδ7, suggesting that Vγ2/Vδ7$^+$ γδ T cells are selected for development into sIELs. In this project, we performed a set of experiments to determine whether a genomic difference in the Vδ7 genes of C57Bl/B6 and 129/svj is involved in the differential development of substitute transgenic Vγ2$^+$ sIELs. Our analysis found that compared to C57Bl/6 strain there is reduced rearrangement of TCR Vδ7 gene in the 129/svj strain. Furthermore, the Vδ7 gene belongs to a subgroup of genes known as the TRAV13 family. We found that the recombination signal sequences (RSS) of the Vδ7 gene in C57Bl/6 is quite unique compared to all other members of the TRAV13 family in both C57Bl/6 and 129/svj strains. On the other hand, the RSS of the Vδ7 gene for 129/svj strain is more identical to those of the other TRAV13 family members. Most of the TRAV13 family members have been associated with TCRα chain rearrangement. The unique RSS of Vδ7 in C57Bl/6 thus suggests that the recombination signal sequence might be responsible for the reduced Vδ7 gene usage in TCRδ gene assembly in the 129/svj background and that the RSS may play a role in determining alpha chain or delta chain cell fate.

Development of a Vγ2/Vδ7 Reporter T cell Line To Investigate Positive Selection of Vγ2/Vδ7 in 129/svj

Positive selection is an important step in development of αβ T cells. It is a process in which a ligand presented by the appropriate MHC selects the developing $CD4^+$ or $CD8^+$ αβ T cells that recognize it. This process eventually leads to production of αβ T cells useful in combating infections and cellular irregularities.

Whether positive selection occurs in γδ T cell development is currently under investigation. The antigen specificity of most γδ T cells is unknown making selection and specificity studies even more difficult. There have however been some suggestions that positive selection does occur in γδ T cells [44, 45, 46]. Thus, we considered the possibility that 129/svj mice do not express Vδ7 due to a lack of, or mutation of the selection ligand responsible for Vγ2/Vδ7 T cell development. To investigate this possibility, we designed a Vγ2/Vδ7 reporter cell line.

We have engineered a reporter cell that expresses Vγ2/Vδ7 TCRs that could be used to detect the presence of activating/selection ligands in the skin and/or thymi of 129/svj and C57Bl/6 mice. If the absence of Vγ2/Vδ7 in 129/svj skin is indeed due to non rearrangement of Vδ7, then the selection ligands necessary for Vγ2/Vδ7 migration to the skin should be present in 129/svj thymus and /or skin. Thus, if our theory on the RSS being responsible for non Vδ7 expression in 129/svj is right, a reporter cell should show activation in 129/svj and C57Bl/6 skin and thymus. The design of our reporter cell was based on the BWZ cell line.

The BWZ Cell Line

The BWZ cell line is a result of gene knock out studies carried out on two variants of the AKR thymoma BW5147 cell. The BW5147 cell is incapable of expressing functional TCR α and β chains. Using γ-irradiation, White et al. [17] generated mutants with the α gene or both α and β genes deleted. The BWZ cell line was generated by transfecting linearized NFAT-lacZ DNA plasmid by electroporation into the $\alpha^-\beta^-$BW5147 cell line.

For this project, using reverse transcription PCR and multistep PCR we generated Vγ2 and Vδ7 TCR chains which had CD28 and CD3 zeta intracellular portions for signal amplification (Fig 1.8). The two TCR chains were then transfected into the BWZ cell via the MSCV retroviral plasmid (Fig 1.9).

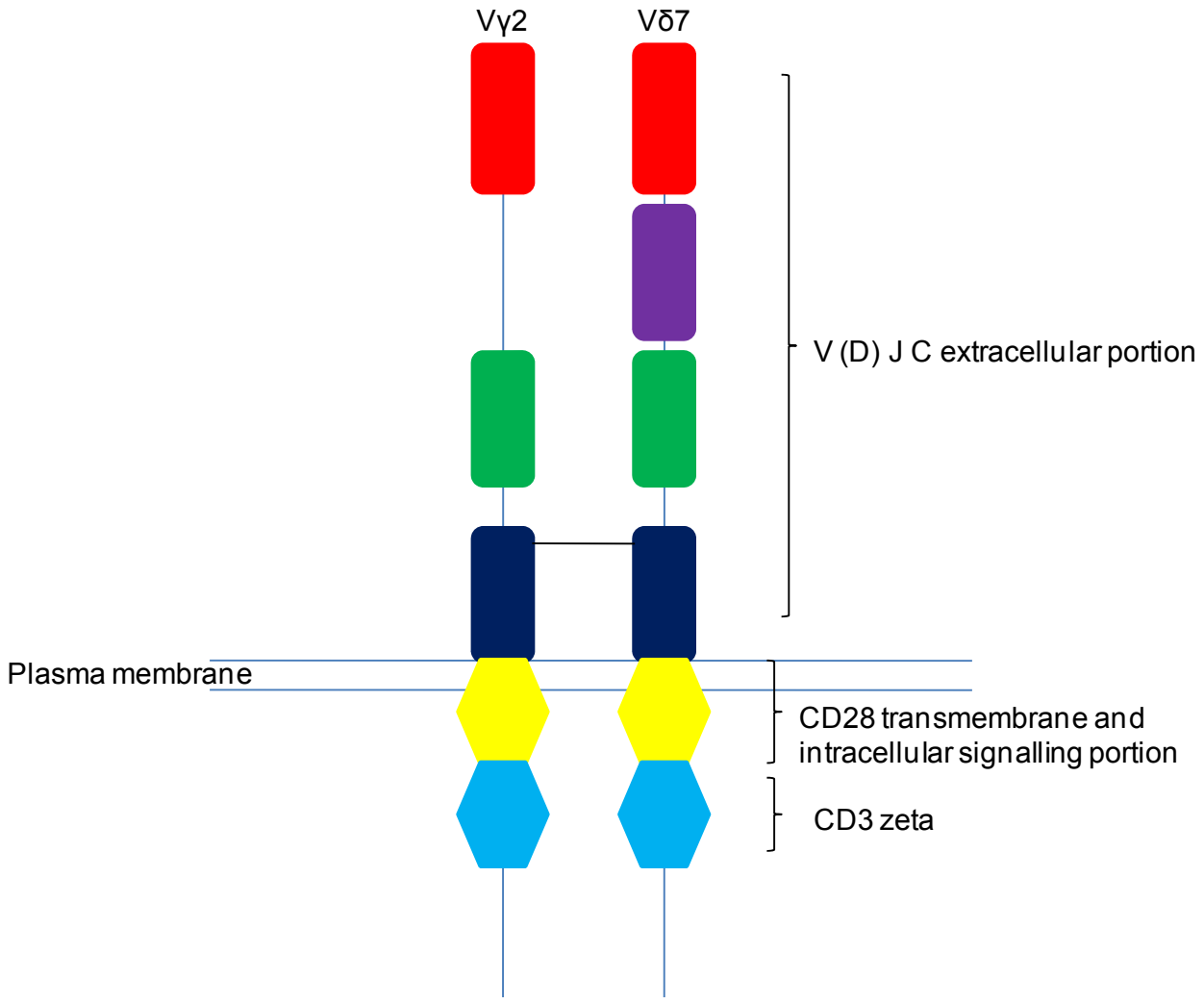


Fig 1.8: PCR Amplified TCR. Extracellular portions of the amplified TCR chains contain VDJC regions of the delta chain and VJC of the gamma chain. The intracellular portions contain the signal transducing units, CD28 and CD3. The CD28 transmembrane region was used as the transmembrane region in both chains.

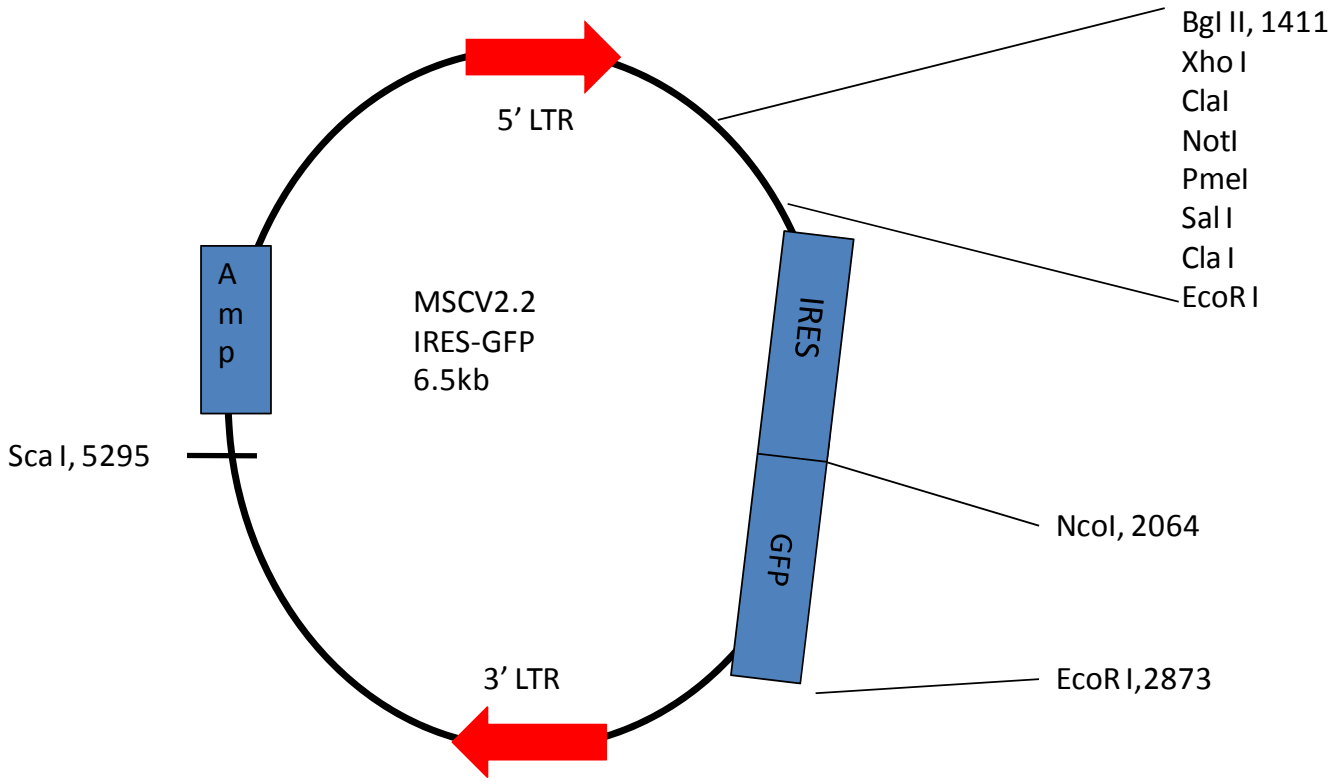


Fig 1.9: The MSCV IRES-GFP Retroviral Plasmid. MSCV was ligated with each TCR chain via NotI and PmeI. Viral products containing the MSCV-TCR were generated in 293 cells and used to infect BWZ cells.

The lacZ gene would be used in a lacZ assay to measure T cell activity. Based on the BWZ-lacZ construct, in an activated T cell, the nuclear factor in activated T cells (NFAT) influences the IL-2 promoter. The lacZ gene is under the control of the IL-2 promoter, thus, upon activation of a BWZ cell expressing functional TCRs, the NFAT binds to the IL-2 promoter and leads to the expression of the lacZ gene. The lacZ gene products would remain sequestered within the activated cells and thus activated cells can be detected via a chromogenic X-gal assay (Fig 1.10).

Our results show that the Vγ2CD28CD3/Vδ7CD28CD3 TCRs were successfully transfected and the BWZ cells express the TCRs. The Vγ2CD28CD3/Vδ7CD28CD3 TCRs are also shown to be functional and could be used in testing for ligands in a C57Bl/6 and 129/svj skin or thymus background.

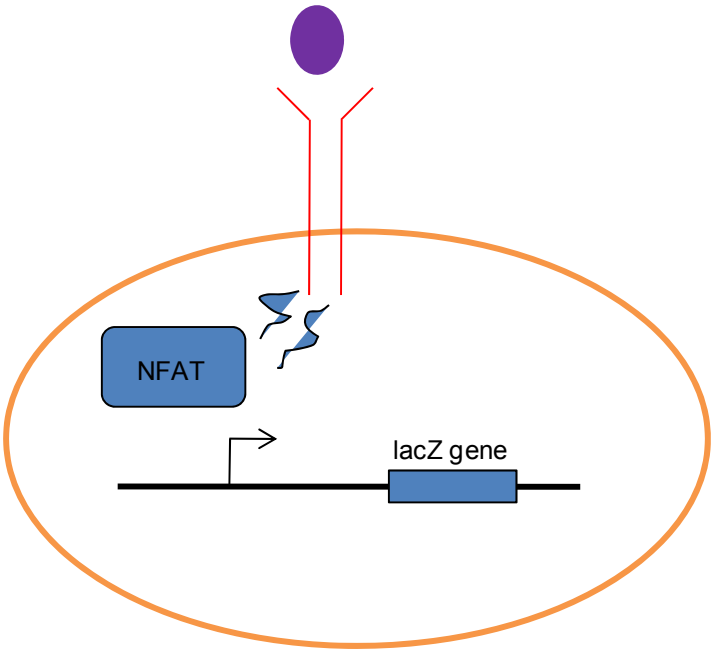


Fig 1.10: Schematics of the BWZ Cell Line. Upon activation of the functional TCRs by a ligand, NFAT binds to the IL-2 promoter. This leads to the transcription of lacZ gene and eventual production of lacZ within the cell. The lacZ can then be detected via a chromogenic assay.

Chapter 2

MATERIALS AND METHODS

Recombination Signal Sequence-Associated Restriction on TCRδ Gene Rearrangement

Extraction of DNA from Thymic Tissue

Thymi were obtained from Vγ2 transgenic Adults and day 17 fetuses of Vγ2 transgenic C57Bl/6 and 129/svj background. Genomic DNA was then extracted from thymic tissues as follows: thymi were resuspended in DMEM (10% FBS) and crushed in a homogenizer to obtain thymocytes. Thymocytes were then filtered through a nylon mesh and spun down at 1000-1300 rpm for 5 mins. The supernatant was removed and 300-500µl extraction buffer (100mM Tris-HCl pH 8.0, 2% SDS, 50mM EDTA) was added. From a stock of 10mg/ml, 50µl proteinase-K was added. The solution was then mixed by inversion and incubated at 55 °C for 12hrs with occasional vigorous mixing. Phenol-Chloroform procedure was then used to purify DNA. DNA was finally precipitated using ethanol and 3-5M Sodium acetate.

PCR Analysis of Rearrangement of Vδ7 Genomic DNA

For rearrangement studies, DNA from Adults and fetuses were serially diluted five-fold. In order to compare DNA concentration between 129/svj and C57Bl/6 samples, PCR was quantified using a GAPDH control (Fig 2.1). The conditions for the GAPDH PCR quantification reaction are as follows: First Denaturing Temperature: 94 °C for 5 min, Second Denaturing Temperature: 94 °C for 1min, Annealing Temperature: 59 °C for 30 sec, Initial Extension Temperature: 72 °C for 1 min, Number of Cycles: 30, Final Extension Temperature: 72 °C for 10 min. In order to confirm the presence of the Vδ7 gene in C57Bl/6 and 129/svj mice, the primers shown in Fig 2.2 were designed to amplify the variable region of each member of the Vα/δ TRAV13 family in both mouse strains.

mmGAPDHf	*CTGACGTGCCGCCTGGAGAAA*
mmGAPDHr	*TGTTGGGGGCCGAGTTGGGATAG*

Fig 2.1: GAPDH Primer. These two primers were used to amplify GAPDH in DNA samples. GAPDH was used as a quantification control between samples.

Mid-Vd7 FP: 5'-CTTGGTTCTGCAGGAGGGGGAGAACGCAGAGC-3'

End-Vd7 RP:5'- ATAGCACAGAGATAAGTGCCTGAGTCTGTGAT-3'

Fig 2.2: TRAV13 Primers. Mid-Vd7 FP and End-Vd7 RP are the forward and reverse primers respectively. The two primers are specific for every member of the TRAV13 family.

The above primer set was designed to yield a PCR product of 237nucleotides. Analysis of rearrangement of Vδ7 was carried out using the Mid-Vd7 forward primer discussed above and a reverse primer (Fig 2.3) that covered the end of J delta 1 and part of the J delta 1 intron. This rearrangement primer set was designed to yield a PCR product of 310 nucleotides. A touchdown PCR was employed for both the Vδ7 gene PCR and Vδ7 rearrangement PCR in order to avoid amplification of non-specific aspects of the genome. Conditions for the touchdown PCR are as follows: Step one (high temperature annealing)- First Denaturing Temperature: 94 °C for 5 min, Second Denaturing Temperature: 94 °C for 1 min, Annealing Temperature: 65 °C for 30 sec with a step down rate of -0.5 °C every cycle, Initial Extension Temperature: 72 °C for 1 min, Number of Cycles: 20; Step two (low temperature annealing)- Denaturing Temperature: 94 °C for 45 sec, Annealing Temperature: 55 °C for 30 sec, Initial Extension Temperature: 72 °C for 1 min, Final Extension Temperature: 72 °C for 10min, Number of Cycles: 35. The above PCR products were analysed on a 2% DNA agarose gel.

IntronJd1-RP: 5'-AATGACTTACTTGGTTCCACAGTCACTTGGGT-3'

Fig 2.3: Jδ1 reverse primer. This reverse primer was used along with the Mid-Vd7 FP to check for rearrangement. The Jδ1 reverse primer covers part of the 3'end of Jδ1 and its intrinsic region.

Sequence Analysis of C57Bl/6 Fetal Samples

Rearranged PCR product from C57Bl/6 and 129/svj fetal samples were cloned into TOPO TA cloning vector using the manufacturers procedure. Competent *E.coli* cells were then transformed and plated on ampicillin or kanamycin resistant plates. After overnight growth, several individual colonies were picked and grown in LB media plus antibiotics (ampicillin or kanamycin) overnight. Plasmids were extracted from the overnight LB growth via boiling lysis. Plasmid DNA was analyzed for the correct length insert (310nt) in Topo by cutting

with EcoRI and running the digestion products on a 0.8% DNA agarose gel. Plasmids bearing the correct insert length were sent for sequencing at Macrogen (http://dna.macrogen.com). Sequences were evaluated for TRAV13 family relationships as well as translational competence using NCBI blast, Expasy and Clustal W software.

Boiling Lysis

Overnight *E.coli* LB cultures were centrifuged @ 3000rpm for 5min at room temperature. Supernatant was decanted and the cell pellets were resuspended in 200µl of STET buffer. After transferring the suspension into 1.5ml tubes, 10µl of lysozyme/RNase mix was added and the tubes were heated in a 100 °C water bath for 3 min. Tubes were then centrifuged for 10 min at 14000rpm. Supernatant was transferred to new micro tubes and 200µl of 5M ammonium acetate and 400µl of isopropanol was added to each tube. After mixing, the tubes were centrifuged for 2 mins at 14000rpm. After discarding the supernatant, the pellets were washed twice with 70% ethanol and dried. Pellets were then dissolved in 50-70µl water or TE.

Identification of TRAV13 TCRα/δ Loci on C57Bl/6 and 129/svj Chromosome 14

In order to find the loci of TRAV13 on chromosome 14 of C57Bl/6 and 129/svj mouse strains, a probe was used to blast the mouse genome on build 37 on the NCBI server. The probe was obtained by sequencing Vδ7 from DETC of Vγ2Tg C57Bl/6. The probe covers parts of VDJ of rearranged Vδ7 and is shown below:

5'-CCAACGTCCTGGGGGAAGCCTCGTCAGCCTGTTGTCCAATCCTTCTGGGA

CAAAGCACACTGGAAGACTGACATCCACCACAGTCACTAAAGAACGTCGC

GGCTCTTTGCACATTTCCTCCTCCCAGATCACAGACTCAGGCACTTATCT

CTGTGCTATCGGAGGGATACGAGCTACCGACAAACTCGTCTTTGGACAAG

GAACCCAAGTGACTGTGGAACCAAAAAGCCAGCCTCCGGCCAAACCATCT-3'

Fig 2.4: Vδ7 Blast Probe. This probe (obtained by sequencing Vδ7 in DETC of Vγ2 transgenic C57Bl/6) is specific for the variable region of Vδ7.

The call number for C57Bl/6 is NT_039606 and for 129/svj is NT_039614. The identified families of TRAV13 on both mouse strains were analyzed via Clustal W, Expasy and the NCBI sequence alignment program.

Development of a Vγ2/Vδ7 Reporter T cell Line To Investigate Positive Selection of Vγ2Vδ7 in 129/svj

RT-PCR of RNA obtained from Vγ2 transgenic C57Bl/6 skin

RNA was extracted from the skin of a Vγ2Tg C57Bl/6 mouse and reverse transcription PCR was carried out on the RNA sample in order to obtain cDNA for Vγ2/Vδ7 TCR. The conditions for the reverse transcription PCR are as follows: 1µl oligo dT_{15} was added to 10µl of RNA and the reaction mixture was made up to 12µl total volume with water. The mixture was heated to 70 °C for 10 min and then chilled on ice for 2 min. After chilling the mixture was centrifuged and 4 µl 5X first strand buffer, 2 µl of 0.1 M DTT and 1 µl of 10 mM dNTP were added. The mixture was incubated at 42 °C for 2 min and 1µl (200 units) of Superscript II Reverse Transcriptase was added. The reaction was then incubated for 50 min at 42 °C after which the reaction was inactivated at 70 °C for 15 min.

Multistep PCR to Obtain CD28-CD3 TCR Chains

We employed the use of a multistep PCR technique in order to obtain Vγ2 and Vδ7 TCR chains that were complete with V (D) J C regions, the transmembrane of CD28, the intracellular portion of CD28 and the intracellelular CD3 zeta chain.

To amplify the extracellular variable and constant domains of each TCR chain, the forward primer for each chain contained (from the 5’ to 3’ end)- a random 6 nucleotide sequence, a restriction enzyme digestion sequence, a kozak sequence (optimal sequence for initiation by ribosomes) and a nucleotide sequence specific for the variable region of interest. The reverse primer for the extracellular portion of each TCR chain contained (from 3’ to 5’ end) - a primer specific for the constant region of interest. The V, (D) and J domains were

amplified along with the constant (C) domains to include the regions coding for the disulphide bond that would bridge the two TCR chains.

For the intracellular signaling units of each TCR chain, a forward primer was designed containing part of the 3' end of the constant domain of the extracellular portion and part of the transmembrane region of CD28. The reverse primer for the intracellular portion, from 3' to 5' end, contained a random 6 nucleotide sequence, a restriction enzyme digestion sequence, and a nucleotide sequence specific for the CD3 zeta chain. All primers used in the multistep PCR are shown in Fig 2.5.

The template for the amplification of the extracellular portion was cDNA obtained from reverse transcription PCR of skin DETC from Vγ2 transgenic C57Bl/6 mouse. The template for the amplification of the intracellular portion was a plasmid containing the CD28 (a costimulatory receptor) and the zeta chain of CD3. Two separate PCR reactions were carried out using the following conditions: Denaturing Temperature: 94 °C for 1 min, Annealing Temperature: 55 °C for 30 sec, Extension Temperature: 72 °C for 1 min, Number of cycles: 30, Final Extension Temperature: 72 °C for 10 min.

A third PCR reaction was carried out to link the extracellular portion of the TCR chains to the intracellular portion. The forward primer used in the extracellular portion and the reverse primer used in the intracellular portion of each TCR chain type were used as primers in the third step PCR. For DNA template, the products of the extracellular and intracellular portion PCRs were used. The conditions for the third step PCR are as follows: Denaturing Temperature: 94 °C for 1 min, Annealing Temperature: 55 °C for 30 sec, Extension Temperature: 72 °C for 1 min, Number of cycles: 30, Final Extension Temperature: 72 °C for 10 min. The whole amplified Vγ2 TCR chain has an expected length of 1500 nucleotides while the whole amplified Vδ7 TCR chain has an expected length of 1383 nucleotides. All PCR product sizes were confirmed on 0.8% DNA agarose gels.

Vg2-FP: 5'-CAA CCG CTC GAG GCC ACC ATG AAG AAC CCT GGC TCA CAA-3'

Cg1-RP: 5'-TTG CCA GCA AGT TGT AGG CTTG-3'

Cg1-CD28: 5'-CCT ACA ACT TGC TGG CAA-AAA ATT GAG TTC ATG TAC-3'

3'Of SacII: 5'-GGG AGG GAG AGG GGC GGA TC-3'

Vd7-FP: 5'-CAA CCG CTC GAG GCC ACC ATG AAG AGG CTG CTG TGC TCT-3'

Cd-RP: GCC ATA GCA AGG CTC TGA AAT TTG-3'

Cd-CD28: TCA GAG CCT TGC TAT GGC-AAA ATT GAG TTC ATG TAC-3'

Fig 2.5: Multistep PCR Primers. The above primers were designed for the multistep PCR. Vg2-FP and Vd7-FP are forward primers for the Vδ7 and Vγ2 V(D)J rearranged regions. Cg1-RP and Cd-RP are reverse primers for the constant regions of gamma and delta chains respectively. Cg1-CD28 and Cd-CD28 are primers used to join the TCR chains to the CD28 signaling unit. The 3'Of SacII primer was used as a reverse primer for the CD3 zeta unit.

Sequence Verification of TCR chains

In order to verify that the amplified Vγ2 and Vδ7 TCR chains were of the correct sequence, PCR products were cloned into TOPO TA cloning vector using the manufacturers procedure. Competent *E.coli* cells were then transformed and plated on ampicillin or kanamycin resistant plates. After overnight growth, several individual colonies were picked and grown overnight in LB media plus antibiotics (ampicillin or kanamycin). Plasmids were extracted from the overnight LB growth via boiling lysis. Plasmid DNA was analyzed for the correct length insert (1500nt for Vγ2 and 1383nt for Vδ7) in Topo by cutting with EcoRI and running the digestion products on a 0.8% DNA agarose gel. Plasmids bearing the correct insert length were sent for sequencing at the Pennsylvania State University Nucleic Acid Facility (http://tanager.biotec.psu.edu/). Sequences were compared to the respective reference sequences using the nucleotide alignment tool on the NCBI website. The samples containing the correct sequences were identified and subjected to further colony growth.

Ligation of TCR Chains into MSCV Plasmid

After confirming the correct sequence for both Vγ2 and Vδ7 TCR chains, each chain was cut from Topo TA vector by digestion with PmeI and NotI. The MSCV plasmid was also cut with Not I and Pme I.

Digested products were separated on a 0.8% DNA agarose gel. The Vγ2 and Vδ7 TCR chain inserts and the MSCV cut vector were cut from the agarose gel and DNA was extracted using a Qiagen DNA gel purification kit. Upon obtaining the purified inserts and vector, two ligation reactions were set up (cut MSCV + insert Vγ2 and cut MSCV + insert Vδ7). Ligation was carried out under the following conditions: 3 mols insert, 1 mol cut vector, ligation enzyme buffer and ligation enzyme, overnight ligation at room temperature. 5µl of ligation reaction mix was used to transform competent one-shot cells. The cells were grown overnight on ampicillin LB plates. After overnight growth, several individual colonies were picked and grown in LB media plus ampicillin overnight. Plasmids were extracted from the overnight LB growth via boiling lysis. To obtain the plasmids that had been inserted with the TCR chains, plasmids were digested with Not I and Pme I.

Preparation of Plasmids for Viral Particle Generation

The following plasmids were used for the generation of viral particles: pCL-Eco and MSCV (containing either Vγ2 or Vδ7 TCR. The pCL-Eco plasmid is a 12.6kb plasmid that contains GAG and ENV genes. The GAG gene is necessary for the assembly of the viral particle and the ENV genes help in the envelop formation and viral particle penetration ability. The MSCV plasmids contained either Vγ2 or Vδ7 TCR under the control of the 5’ LTR and a green fluorescence protein under the control of IRES. The IRES-GFP gene occurred after the insertion site for the TCRs. All plasmids were prepared by midi prep, extracted by lysis and precipitation and purified with phenol/chloroform. The plasmids used for viral particle generation are shown in Fig 2.6 and Fig 2.7.

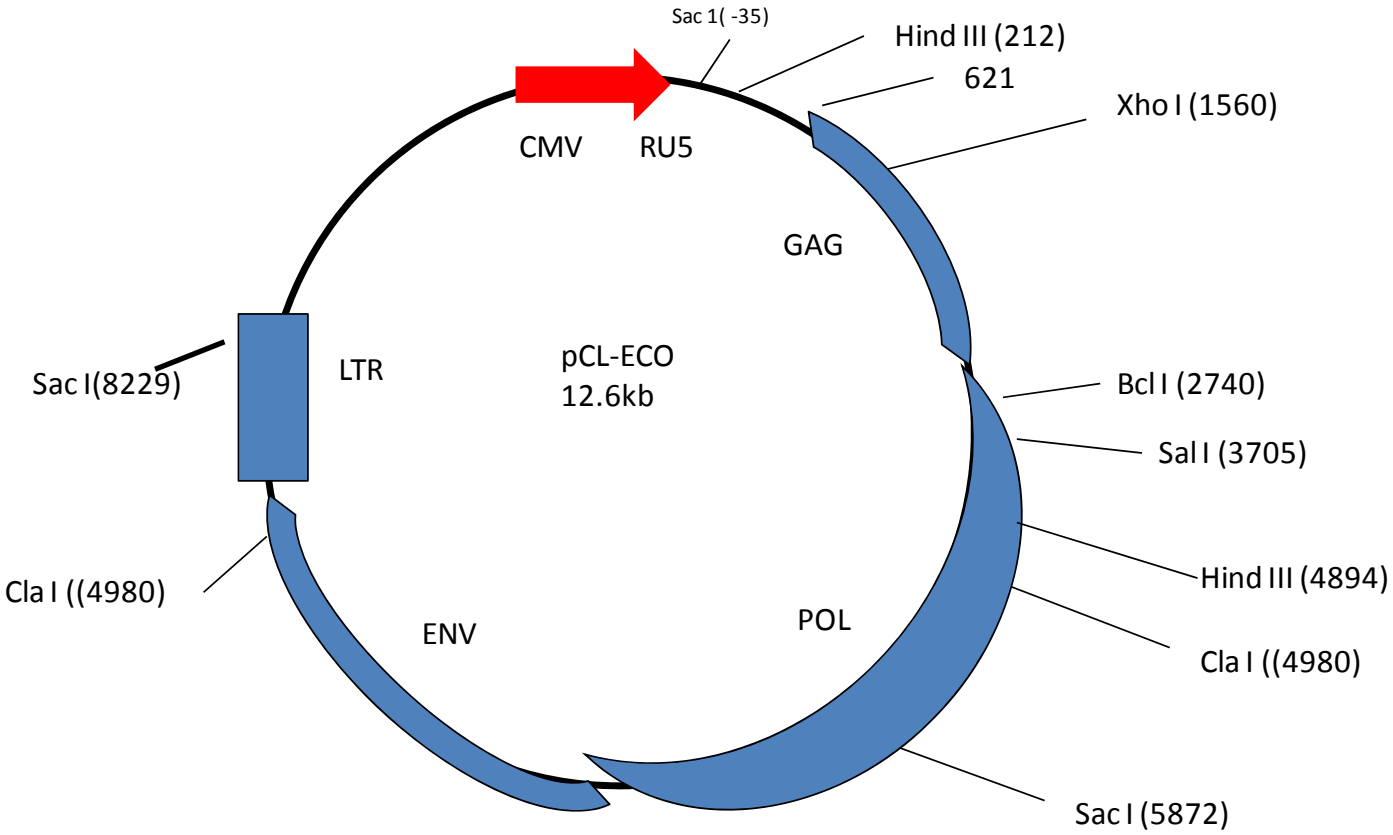


Fig 2.6: The pCL-Eco Plasmid. This plasmid contains GAG and ENV genes necessary for viral particle envelop formation and assembly.

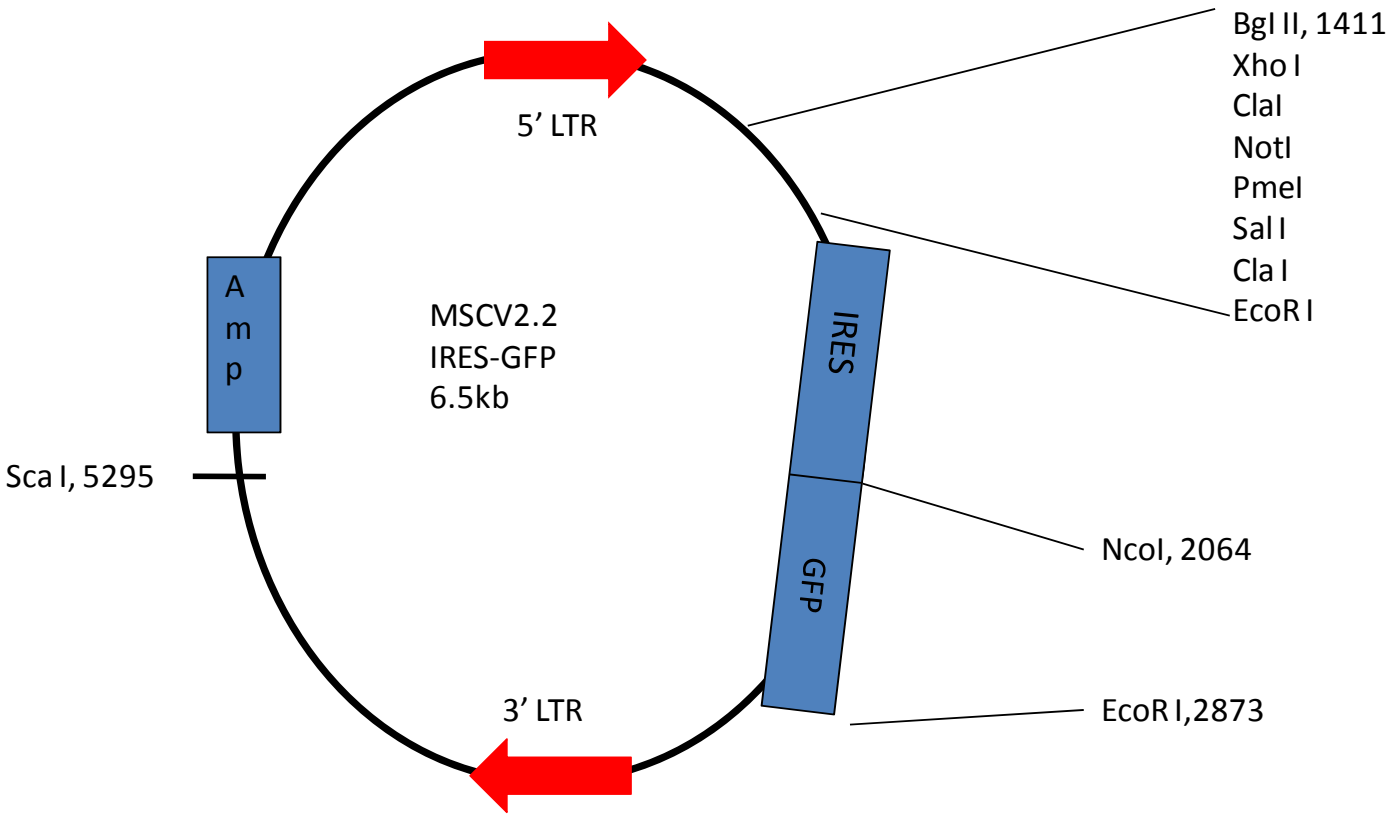


Fig. 2.7: The MSCVGFP Plasmid. The insertion sites are indicated after the 5'LTR. The TCR chains were inserted between NotI and PmeI. GFP was used as an indication of successful transfection (in 293 cells) and infection (in BWZ cells).

Tissue Cell Culture

For the infection of the BWZ cell line, viral particles containing the Vγ2 and Vδ7 TCR chains were generated in 293 cells. The 293 cells were grown in DMEM medium containing 5% fetal bovine serum and antibiotics. Cell growth was at 37 °C in a 5% CO_2 incubator.

The BWZ cells were grown in RPMI medium containing 5% fetal bovine serum and antibiotics. Cell growth was at 37 °C in a 5% CO_2 incubator.

Transfection of 293 cells for Production of Viral Particles

The 293 cells were transfected with the MSCV (containing Vγ2 or Vδ7 TCR) and pCL-Eco using a Fugene-6 or lipofectamine transfection agent. Typically, for each transfection, Fugene 6 reagent (9µl) was mixed with 91µl of Opti-mem and then incubated for 5 min at room temperature. Respective MSCV TCR

plasmids and pCL-Eco (10 μg each) were then added to the mixture and the complex was incubated for 30 mins. The complex was added to the 293 cells in a dropwise manner and the cells were rocked gently to distribute the complex evenly. Cells were then incubated at 37 °C for 6 hours. After 6 hours, the transfection medium was removed and DMEM was added. Successful transfection was observed approximately 24-48 hours after with the expression of GFP in transfected cells. Viral particles were harvested 48 and 72 hours after transfection and filtered through a 0.45μm filter. Aliquouts were frozen in a -80 °C freezer until use.

Infection of BWZ Cell Line with Vγ2/Vδ7 TCR Viral Particles

A two step infection process was carried out in order to express both Vδ7 and Vγ2 TCRs in the same cell. In the first step, cells were infected with viral particles containing either Vδ7 or Vγ2. Confirmation of successful infection was done by observing GFP expression on a flow cytometer. These singly infected cells were then sorted on GFP expression and stable transfectants were obtained. In the second step, Vδ7 singly infected cells and Vγ2 singly infected cells were infected with Vγ2 and Vδ7 viral particles respectively. After the second infection, a γδTCR monoclonal antibody and a Vγ2 monoclonal antibody were used to confirm expression of both TCR chains in the infected cells. Cells were sorted on expression of GFP and Vγ2 mAb and then GFP and γδTCR mAb. The main process of infecting cells is described below.

BWZ cell lines were infected with the viral particles via two main methods- spinfection and non-spinfection. In the non-spinfection method, 3ml of RPMI (10% FBS) was mixed with 1ml of viral supernatant and 64 μl of 500 μg/ml polybrene (final conc of polybrene was 8 μg/ml). BWZ cells were resuspended in the 3:1 viral mixture (RPMI : viral supernatant) for 1hr in a 37 °C incubator. After 1hour, 2 ml of RPMI was added and cells were incubated at 37 °C. Cells were observed to have GFP expression on a flow cytometer indicating successful infection.

In the spinfection method, 3ml of RPMI (10% FBS) was mixed with 1ml of viral supernatant and 64 μl of 500 μg/ml polybrene (final conc of polybrene was 8 μg/ml). BWZ cells were then resuspended in the 3:1 viral mixture (RPMI : viral supernatant). The plates containing the BWZ cells were sealed with parafilm and the cells

were spun while resuspended in viral soup at 1000 rpm for 90min at room temp. After spinfection, 2ml of RPMI (10% FBS) was added and cells were incubated at 37 °C for 72 hrs. Successful transfection was confirmed as described earlier.

Cell Cytometery Analysis of BWZ Cell TCR and GFP Expression

After the first step (single TCR) infection with the viral particles, the BWZ cells were incubated for 72 hours and then analysed on a flow cytometer for GFP expression (525nm). Cells expressing GFP were sorted and cultured. Stably infected single TCRs were then infected with the alternate TCR (e.g. Vδ7 single infected cells were infected with Vγ2 viral particles) and the double infected cells were cultured. A γδ TCR monoclonal antibody (GL3) conjugated with biotin and secondarily stained with PE along with a Hamster anti-mouse Vγ2Ab Fitc secondarily stained with anti-hamster IgGPE were used to detect BWZ TCR expression in double infected cells.

Stimulation Assay for Infected BWZ Cells

CPRG stimulation assay: In the CPRG stimulation assay, GL4 mAb (5µg/ml in 50µl PBS) was incubated in 96 well plates overnight at 37 °C in a humidified incubator. Following incubation, BWZ cells were added ($1*10^5$/well). A positive control was also set-up on the same plate whereby BWZ cells ($1*10^5$/well) were stimulated for 4 hours by PMA/ionomycin (medium containing 10 ng/ml PMA and 1µM calcium ionophore). After stimulation of cells, the plate was centrifuged and cells were resuspended in 100 µL of 1×CPRG buffer (90 mg/L chlorophenol-red-β-D-galactopyranoside (Roche Diagnostics, Indianapolis, IN), 9 mM MgCl2, 0.1% NP-40, in PBS). Plates were incubated at 37 °C or at room temperature, then analyzed at OD of 560nm and 625nm using a microplate reader.

Chapter 3

RESULTS

Recombination Signal Sequence-Associated Restriction on TCRδ Gene Rearrangement

Identification of Vδ7 and Its Homologs in the Genomic Database of 129/svj and C57Bl/6 Mice

The NIH NCBI mouse database contains contributions to the whole genomic sequence of several mouse strains. I blasted the mouse database of NCBI with a Vδ7 sequence in order to identify the location of the Vδ7 genes in 129/svj and C56Bl/6 strains. The probe was obtained by sequencing Vδ7 from DETC of Vγ2Tg C57Bl/6. The blast yielded 13 homologs of Vδ7 in the C57Bl/6 mouse database and 9 homologs of Vδ7 in the 129/svj mouse database, all on chromosome 14 (Fig 3.1). The call number for C57Bl/6 is NT_039606 and for 129/svj is NT_039614. This result was in agreement with the report on the mouse T cell receptor alpha and delta variable genes by Bosc and Lefranc [38], wherein it was suggested that Vδ7 belonged to a family of variable genes termed TRAV13 most of which are capable of alpha gene rearrangements. Nine members of the TRAV13 family were also observed in 129/svj at the time of publication of the Bosc and Lefranc paper.

It was observed that the TRAV13 family occurred upstream of the Dδ1, Dδ2 and Jδ genes (Fig 3.1). In order to classify the degree of similarity between every homolog in both C57Bl/6 and 129/svj genome, a homology sequence analysis was carried out by aligning all the sequences and grouping them based on their sequence alignment scores (Fig 3.2). The homology analysis revealed that the 13 members of TRAV13 present on the C57Bl/6 genome could be divided into 3 groups (Fig 3.1). Each group contains 4 homologs and each homolog of each group appears to have an identical sequence in a neighbouring group. This pattern was also observed in the 129/svj genome whereby the 9 members of the 129/svj genome could also be divided into 2 groups with each group comprising 4 homologs that had identical sequences in the other group. The thirteenth homolog in B6 and the ninth homolog in 129 did not have identical sequences in any of the other groups but also yielded an interesting observation as discussed below.

The Vδ7 homologs were termed according to the terminology used by IMGT (Bosc and Lefranc [38]). While Bosc and Lefranc observed 9 homologs in 129/svj, I observed 13 homologs in C57Bl/6. According to the IMGT

nomenclature, duplicate genes are termed TRAVnD-1 (n being the number associated with that gene), we have termed the double duplicate genes observed in C57Bl/6 as TRAVnDD-1 (Fig 3.2). Members of the 2 groups of TRAV13 present on 129/svj genome were observed to have sequence similarity to 12 members of the C57Bl/6 genome (Fig 3.2). For example, TRAV13-1 of C57Bl/6 is more similar in sequence to TRAV13-1 of 129 than it is to TRAV13-2 of 129. As mentioned above, TRAV13-5 of C57Bl/6 genome was found to be quite similar sequentially to TRAV13-5 of 129/svj even though both had less homologs within the groups occurring upstream. However, most interesting is the observation that TRAV13-4/DV7 of C57Bl/6 is the only homolog that aligned 100% with the cDNA probe from the C57Bl/6 Vγ2Tg mice. This latter finding suggests that there is a unique factor within the TRAV13-4/DV7 of C57Bl/6 sequence that allows its rearrangement and selection to the skin over other TRAV13 genes on C57Bl/6. Also, the absence of this unique factor in any of the TRAV13 genes in 129/svj prevents the rearrangement or the selection of Vδ7 in 129/svj mice.

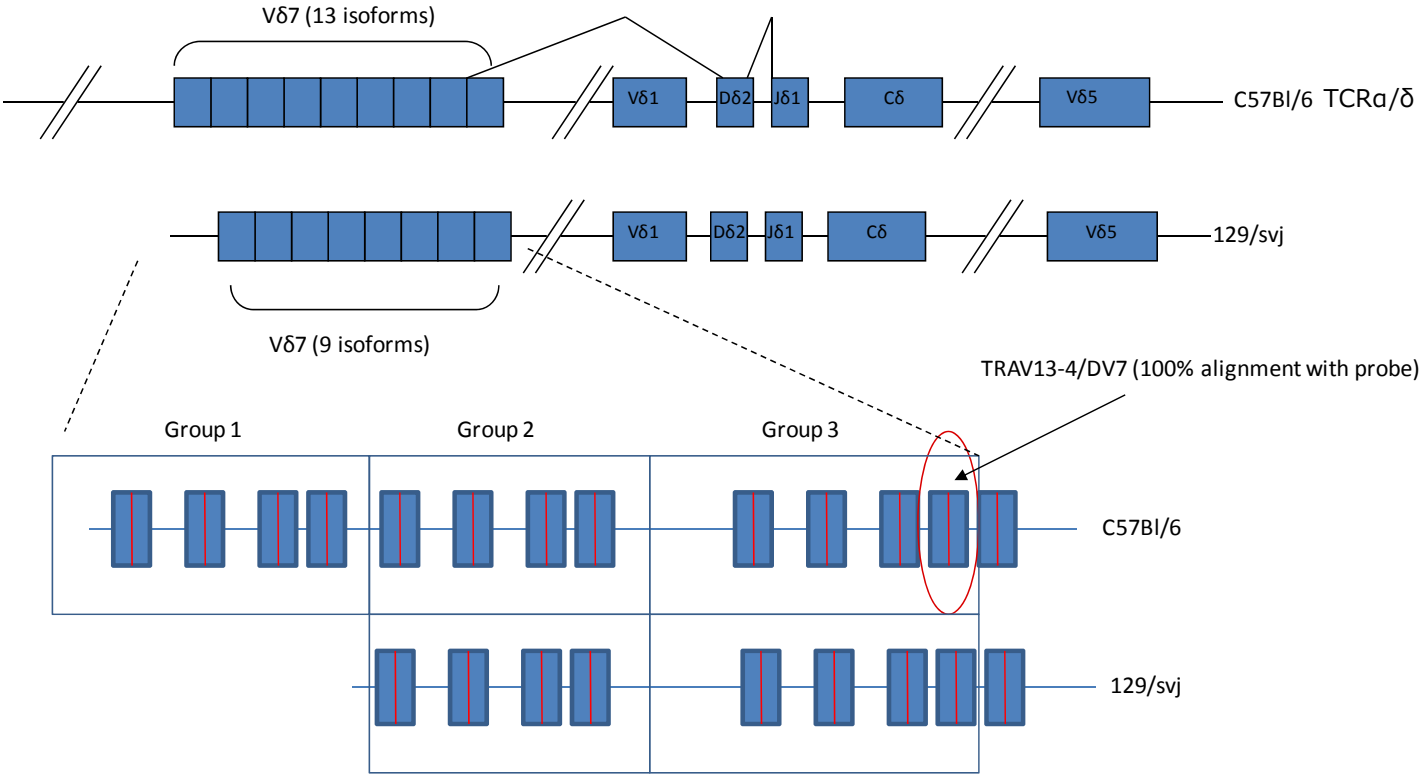


Fig 3.1 Vδ7 Homologs present in the C57Bl/6 and 129/svj Genomes. Top panel shows approximate orientation of the TRAV13 family members along with other delta chain genes on chromosome 14 of C57Bl/6 and 129/svj. Bottom panel shows the groups into which the TRAV13 family members can be divided in C57Bl/6 and 129/svj genomes. TRAV13-4/DV7 was the only gene that aligned 100% with the Vγ2Vδ7 DETC probe.

A

C57Bl/6	Region	C57Bl/6													129/svj									
		TRAV13D-1	TRAV13D-2	TRAV13D-3	TRAV13D-4	TRAV13D-1	TRAV13D-2	TRAV13D-3	TRAV13D-4	TRAV13-1	TRAV13-2	TRAV13-3	TRAV13-4/DV7	TRAV13-5	TRAV13D-1	TRAV13D-2	TRAV13D-3	TRAV13D-4	TRAV13-1	TRAV13-2	TRAV13-3	TRAV13-4/DV7	TRAV13-5
	TRAV13D-1	100	91	90	91	99	91	90	92	96	91	91	92	86	96	91	91	92	97	91	91	92	85
	TRAV13D-2	91	100	88	96	90	100	88	91	92	96	88	97	87	92	96	88	91	91	98	89	97	87
	TRAV13D-3	90	88	100	87	90	88	99	88	90	87	99	88	82	90	87	98	88	91	87	98	88	81
	TRAV13D-4	91	96	87	100	90	96	87	91	91	97	87	97	87	92	97	88	91	91	96	88	98	87
	TRAV13D-1	99	90	90	90	100	90	90	92	96	91	90	91	84	96	90	91	92	96	90	91	91	85
	TRAV13D-2	91	100	88	96	90	100	88	91	92	96	88	97	87	92	96	88	91	91	99	89	97	87
	TRAV13D-3	90	88	99	87	90	88	100	88	90	88	99	88	82	90	87	98	88	91	87	98	88	81
	TRAV13D-4	92	91	88	91	92	91	88	100	92	91	88	92	87	92	91	89	99	92	91	89	93	87
	TRAV13-1	96	92	90	91	96	92	90	92	100	92	91	91	86	99	91	91	92	97	91	91	91	85
	TRAV13-2	91	96	87	97	91	96	88	91	92	100	88	96	87	92	99	88	91	91	96	88	97	87
	TRAV13-3	91	88	99	87	90	88	99	88	91	88	100	88	82	90	87	98	88	91	87	98	88	81
	TRAV13-4/DV7	92	97	88	97	91	97	88	92	91	96	88	100	86	92	96	89	92	91	96	89	98	86
	TRAV13-5	86	87	82	87	84	87	82	87	86	87	82	86	100	85	87	83	87	85	87	83	88	99
129/svj	TRAV13D-1	96	92	90	92	96	92	90	92	99	92	90	92	85	100	92	90	92	97	91	91	92	84
	TRAV13D-2	91	96	87	97	90	96	87	91	91	99	87	96	87	92	100	88	97	90	96	88	96	87
	TRAV13D-3	91	88	98	88	91	88	98	89	91	88	98	89	83	90	88	100	89	91	88	98	89	82
	TRAV13D-4	92	91	88	91	94	91	88	99	92	91	88	92	87	92	97	89	100	92	91	89	93	87
	TRAV13-1	97	91	91	91	96	91	91	92	97	91	91	91	85	97	90	91	92	100	90	92	91	85
	TRAV13-2	91	98	87	96	90	99	87	91	91	96	87	96	87	91	96	88	91	90	100	88	96	87
	TRAV13-3	91	89	98	88	91	89	98	89	91	88	98	89	83	91	88	98	89	92	88	100	89	82
	TRAV13-4/DV7	92	97	88	98	91	97	88	93	91	97	88	98	88	92	96	89	93	91	96	89	100	87
	TRAV13-5	85	87	81	87	85	87	81	87	85	87	81	86	99	84	87	82	87	85	87	82	87	100

B

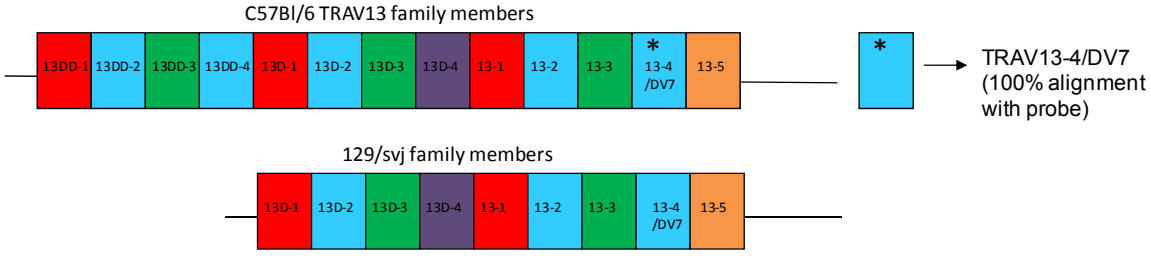


Fig. 3.2 Homology Sequence Analysis. A) Sequences of all TRAV13 family members were aligned and compared against each other. Alignment scores between each family member are indicated. B) Sequences with an alignment score of 96% and above were colour coded similarly.

Analysis of Vδ7 Gene Rearrangement in Fetal Thymi of both 129/svj and C57Bl/6 Mice

A number of factors could be responsible for the absence of Vδ7 expression in 129/svj skin. It is possible that mutation(s) present in all the variable region sequences of the TRAV13 family members of the 129 genome prevents eventual recognition of selection ligands and thus no Vδ7 is expressed in 129/svj skin. It is also possible that the selection ligand is absent or mutated in 129/svj mice and so no selection is occurring. Before investigating these theories, it was important to show that TRAV13 homologs were indeed present in the experimental mice stock. To investigate the presence of Vδ7, a 32 nucleotide forward primer (Mid-Vd7 FP) capable of annealing to all variable regions of TRAV13 family of C57Bl/6 and 129/svj was designed. The 32 nucleotide reverse primer (End-Vd7 RP) was also capable of annealing to all variable regions of TRAV13

family and was designed from the common end of the variable regions of TRAV13 family (Fig 3.3). A PCR reaction with these two primers should produce a 237 nucleotide fragment. DNA was obtained from day 17 fetuses and adult thymi of both 129/svj and C57Bl/6 mice. Day 17 fetal thymi were used in order to detect rearrangement at the developmental stage. In order to appropriately compare DNA quantity, a five-fold serial dilution of DNA was carried out and quantified using a GAPDH control. The results (Fig 3.4) show that TRAV13 family members are indeed present in the experimental 129/svj and C57Bl/6 mice genomes. In both C57Bl/6 and 129/svj samples, the adult and fetal samples were observed to contain amplified Vδ7 regions in significant amounts.

The first step in investigating the absence of Vδ7 expression in 129/svj strain is to determine if rearrangement of Vδ7 is indeed occurring. Rearrangement of the V, D and J regions must occur in order for the delta chain to form and TCRs on T cells to eventually get selected. The same forward primer that was used in identifying variable region presence in the experimental mice was used (Fig 2.2). To amplify rearranged delta chains from the Jδ1 region, we designed a 32 nucleotide reverse primer (IntronJd1-RP) that covered the end of Jδ1 along with part of the intron 3' of Jδ1. A PCR reaction using these primers should produce a 310 nucleotide fragment to indicate rearranged VDJ Vδ7 chains. Our results produced evidence that the problem of non-expression of Vδ7 in 129/svj transgenic skin is due to a lack of rearrangement of the Vδ7 chain in the 129/svj genome at the developmental stage. In the adult samples, rearranged Vδ7 was observed in greater quantity in C57Bl/6 mice than in 129/svj mice. This same observation was made in the fetal samples (Fig 3.4).

Mid-Vd7 FP: CTTGGTTCTGCAGGAGGGGGAGAACGCAGAGC

End-Vd7 RP: ATAGCACAGAGATAAGTGCCTGAGTCTGTGAT

IntronJd1-RP: AATGACTTACTTGGTTCCACAGTCACTTGGGT

Fig 3.3: Rearrangement and Vδ7 Gene Confirmation Primers. The above primers were used for Vδ7 gene and Vδ7 TCR rearrangement studies. Mid-Vd7 FP is the forward primer that was specific for all TRAV13 family members.

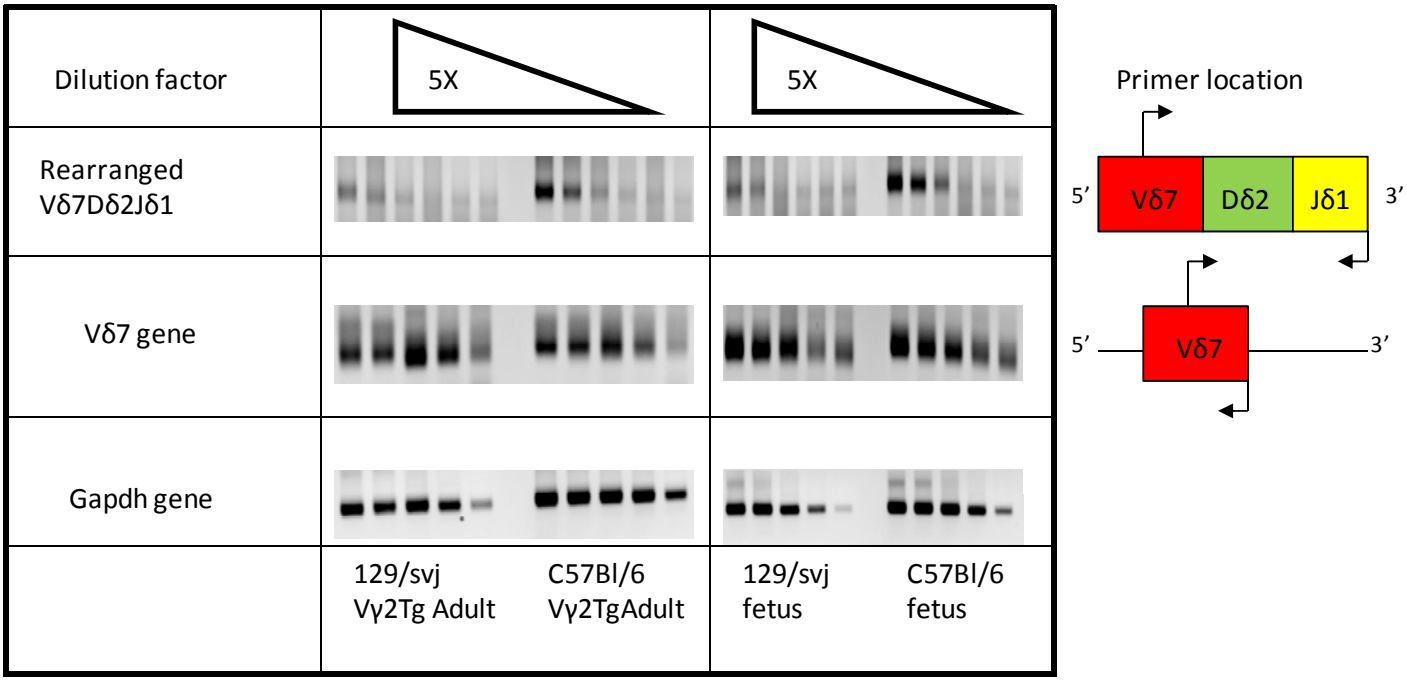


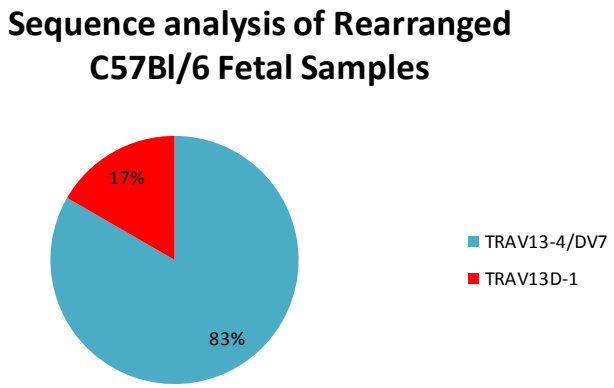
Fig 3.4: Analysis of Vδ7 Gene Rearrangement in C57Bl/6 and 129/svj Strains. Primers were used to successfully amplify the Vδ7 gene indicating the presence of Vδ7 in both mouse strains at the fetal and adult stages. Primers could only amplify rearranged Vδ7TCR in C57Bl/6 fetal and adult stages and not in any stage of the 129/svj strain.

Sequence Analysis of Rearranged Fetal Thymic TRAV13 Delta Chains of C57Bl/6 Mice.

To determine what members of the TRAV13 family are used in assembly of TCRδ genes, we subcloned the rearranged Vδ7-Jδ1 PCR products into plasmids and sampled them for frequencies of the different TRAV13 members.

Sequencing results show that 83% of the rearranged DNA had a variable region identical to TRAV13-4/DV7. Only one other TRAV13 family member was identified in the results. TRAV13-1 comprised 17% of the variable regions identified in the rearranged DNA (Fig 3.5). This result suggested that TRAV13-4/DV7is predominantly used in TCRδ gene assembly, consistent with previous reports.

A



B

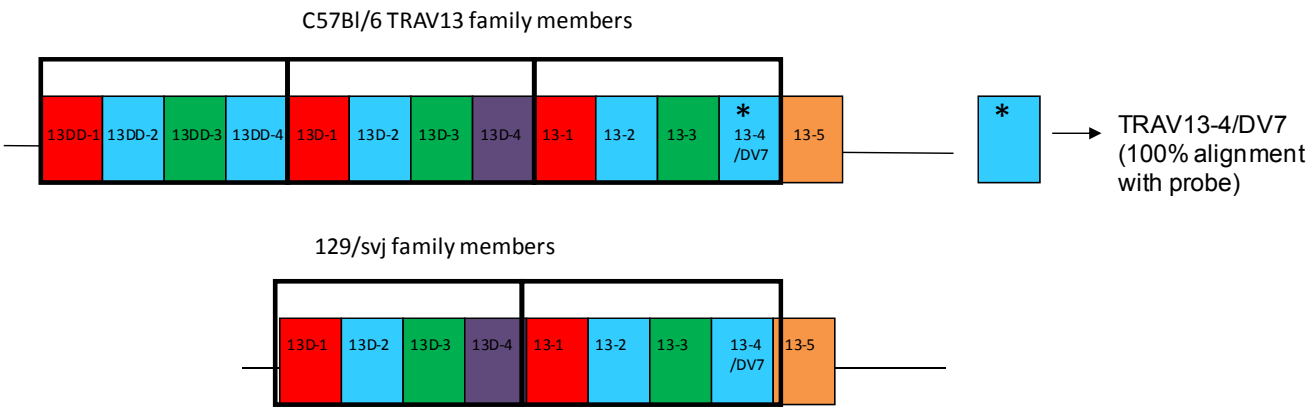


Fig 3.5 Sequence Analysis of Rearranged C57Bl/6 Fetal Samples. A) Pie chart indicating TRAV13 family members present in rearranged C57Bl/6 fetal samples. B) Colour coded reference of TRAV13 family members. Members with the same colour code have a high percentage of sequence homology.

To ensure that we were not observing already selected rearrangement products from the thymus, we analysed the TRAV13-4/DV7 rearranged sequences for frequencies of in-frame and out-of-frame rearrangements. Using the clustal-W software, the sequences were translated to amino acid sequences. The resulting amino acids of each TRAV13-4/DV7 sequence was compared to the reference translation and also analysed for early stop codons. Only 33% of the TRAV13-4/DV7 rearranged sequences were in frame (Fig 3.6). This result suggests the rearrangement of Vδ7 is a random process without influences of cellular selection, which would have resulted in higher percentages of in-frame rearrangements.

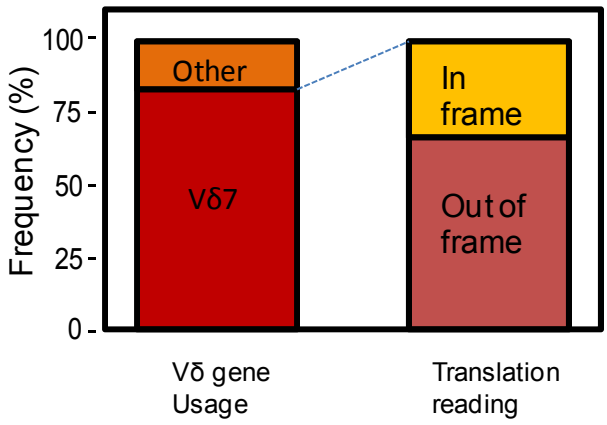


Fig. 3.6 Translational Comparison of TRAV13-4/DV7 Rearranged Sequences. Rearranged sequences of C57Bl/6 TRAV13-4/DV7 were translated and analysed for being in-frame or out of frame with respect to the reference sequence. Only 33% of the rearranged sequences were in-frame suggesting that selection was yet to occur when these samples were obtained.

Analysis of the Recombination Signal Sequences 3' of the Variable Region of TRAV13 Family Members in C57Bl/6 and 129/svj Genome

Rearrangement of TCR chains is dependent on the RSS. The RSS as earlier mentioned is a DNA sequence consisting of a conserved nonamer and heptamer between which lies a 23 or 12 base pair non-conserved sequence. All regions capable of rearrangement have one or two RSS. Each TRAV13 family member should have an RSS at the 3' end. Thus, using the NCBI database we analysed the RSS of the 13 and 9 TRAV13 family members of C57Bl/6 and 129/svj respectively. It was found that the RSS of TRAV13-4/DV7 of C57Bl/6 (the only member of the TRAV13 family that was expressed on the skin of Vγ2Tg C57Bl/6 mice) was quite unique when compared with all other TRAV13 members of both C57Bl/6 and 129/svj strains (Fig 3.7). Particularly, the first nucleotide of the 23 base pair spacer of the C57Bl/6 TRAV13-4/DV7 RSS is adenine (A), whereas the corresponding site in the 129/svj TRAV13-4/DV7 RSS and all other TRAV13 family members is a cytosine (C). Furthermore, the fourth nucleotide of the nonamer of the C57Bl/6 TRAV13-4/DV7 RSS is a thymine (T) while the corresponding site is a cytosine (C) in the 129/svj TRAV13-4/DV7 RSS and all

other TRAV13 family members. Indeed, the RSS of the 129/svj TRAV13-4/DV7 gene, while significantly different from the RSS of the C57Bl/6 TRAV13-4/DV7 gene, is nearly identical to the RSS of the other family members that rearrange preferentially to TCRα chain. This implies that the RSS in 129/svj TRAV13-4/DV7 gene may be the reason for its reduced rearrangement in TCRδ chain assembly.

A

C57Bl/6 Region	Heptamer-Spacer-Nonamer
TRAV13DD-1	CACAGTG CTCCCCACACACCTGCAGCCCGC ACACAAACC
TRAV13DD-2	CACAGTG CTCCCCACACACCTGCAGCCTGT ACACAAACC
TRAV13DD-3	CACAGTG CTCCCCACACACCTGCAGCCCAA ACACAAACC
TRAV13DD-4	CACAGTG CTCCCCACACACCAGCAGCCCGC ACACAAACC
TRAV13D-1	CACAGTG CTCCCCACACACCTGCAGCCCGC ACACAAACC
TRAV13D-2	CACAGTG CTCCCCACACACCTGCAGCCTGT ACACAAACC
TRAV13D-3	CACAGTG CTCCCCACACACCTGCAGCCCGA ACACAAACC
TRAV13D-4	CACAGTG CTCCCCACACACCTGCAGCCCGC ACACAAACC
TRAV13-1	CACAGTG CTCCACACACACCTGCAGCCCGA ACACAAACC
TRAV13-2	CACAGTG CTCCCCACACACCTGCAGCCTGT ACACAAACC
TRAV13-3	CACAGTG CTCCCCACACACCTGCAGCCCGA ACACAAACC
TRAV13-4/DV7(skin C57Bl/6-Vδ7)	CACAGTG ATCCCCACACACCTGCAGCCTGC ACATAAACC
TRAV13-5	CACAGTG CTCCCCAGGCACCTGCAGCCTGT ACACAAACC
Dδ2 (same in C57Bl/6 and 129/svj)	**GGTTTTTGC AAAGCTCTGTAG CACCGTG**

B

129/svj	Heptamer-Spacer-Nonamer
TRAV13D-1	CACAGTG CTCCCCACACACCTGCAGCCCGC ACACAAACC
TRAV13D-2	CACAGTG CTCCCCACACACCTGCAGCCTGT ACACAAACC
TRAV13D-3	CACAGTG CTCCCCACACACCTGCAGCCCGC ACACAAACC
TRAV13D-4	CACAGTG CTCCCCACACACCTGCAGCCCGC ACACAAACC
TRAV13-1	CACAGTG CTCCACACACACCTGCAGCCCGC ACACATACC
TRAV13-2	CACAGTG CTCCCCACACACCTGCAGCCTGT ACACAAACC
TRAV13-3	CACAGTG CTCCCCACACACCTGCAGCCCGA ACACAAACC
TRAV13-4/DV7	CACAGTG CTCCCCACACACCAGCAGCCCGC ACACAAACC
TRAV13-5	CACAGTG CTCCCCAGGCACCTGCAGCCTGT ACACAAACC
C57Bl/6 TRAV13-4/DV7	CACAGTG ATCCCCACACACCTGCAGCCTGC ACATAAACC
Dδ2 (same in C57Bl/6 and 129/svj)	**GGTTTTTGC AAAGCTCTGTAG CACCGTG**

Fig 3.7: RSS Comparison of TRAV13 Family Members in C57Bl/6 and 129/svj Strains. A) RSS of TRAV13 family members present in the C57Bl/6 mouse strain. B) RSS of TRAV13 family members present in the 129/svj mouse strain. Differences between the RSSs of all other TRAV13 members and TRAV13-4/DV7 RSS are highlighted in red. The TRAV13-4/DV7 RSS is highlighted in yellow.

Sequence Analysis of Rearranged Fetal Thymic TRAV13 Delta Chains of 129/svj Mice

Based on results obtained from the comparative analysis of Vδ7 rearrangement in 129/svj and C57Bl/6 mice, I was interested in observing what members of the TRAV13 family (if any) were rearranged to D delta in 129/svj mice. I hypothesized that the difference in RSS between Vδ7 (TRAV13-4/DV7) in C57Bl/6 and its corresponding homolog in 129/svj would lead to less rearrangement or none at all of the 129/svj Vδ7 homolog. To determine what members of the TRAV13 family are used in assembly of TCRδ genes in 129/svj, we subcloned the rearranged Vδ7-Jδ1 PCR products into plasmids and sampled them for frequencies of the different TRAV13 members.

Sequencing results showed that while 58% of the rearranged DNA had a variable region identical to TRAV13D-2, TRAV13-2 or TRAV13-4/DV7 of 129/svj. Further analysis revealed that only 0.5% of the rearranged sequence was TRAV13-4/DV7. The other 42% of rearranged DNA had variable regions identical to either TRAV13D-1 or TRAV13-1 (Fig 3.8). This result further supports the hypothesis that a specific sequence of RSS controls rearrangement to delta or alpha chain. It is possible that TRAV13-4/DV7 in 129/svj is a functional RSS that is capable of rearranging to the alpha chain and not the delta chain.

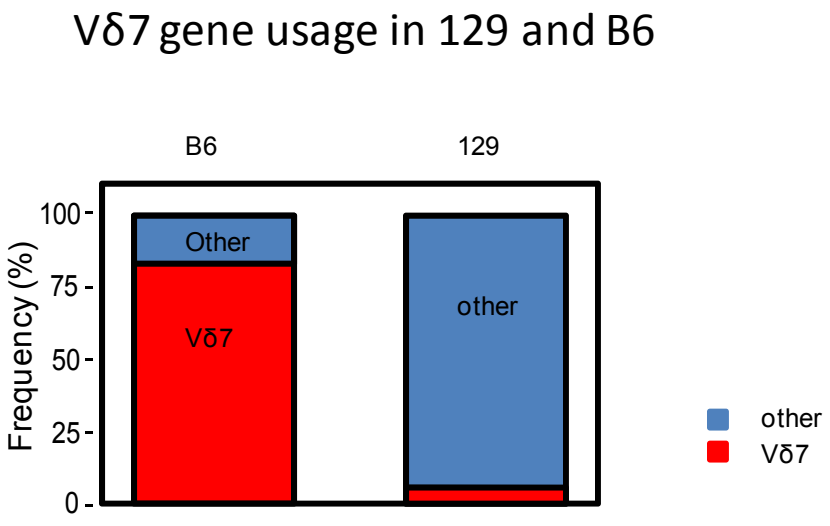


Fig. 3.8: Vδ7 (TRAV13-4DV/7) Gene Usage in 129/svj and C57Bl/6 Mice. Using primers specific for all TRAV-13 family members and Jδ1, rearranged sequences of C57Bl/6 and 129/svj fetal thymocytes were sequenced and analysed for variable chain usage. While 83% of the delta chain rearranged samples from C57Bl/6 were TRAV13-4/DV7 sequences, only 0.5% of the rearranged samples in 129/svj were TRAV13-4/DV7.

Development of a Vγ2/Vδ7 Reporter T cell Line

Multistep PCR to obtain Vγ2CD28CD3 and Vδ7CD28CD3 TCR Chains

Using the primers shown in Fig 2.5, Vγ2 and Vδ7 TCR chains were amplified using a multistep PCR. The expected length of the Vγ2 TCR chain including CD28 and CD3 is 1500 nucleotides and the expected length of the Vδ7 TCR chain including CD28 and CD3 is 1383 nucleotides. After the multistep PCR, a 0.8% DNA agarose gel was run to confirm production of the TCR chains containing CD28 and CD3. Results are shown in Fig 3.9

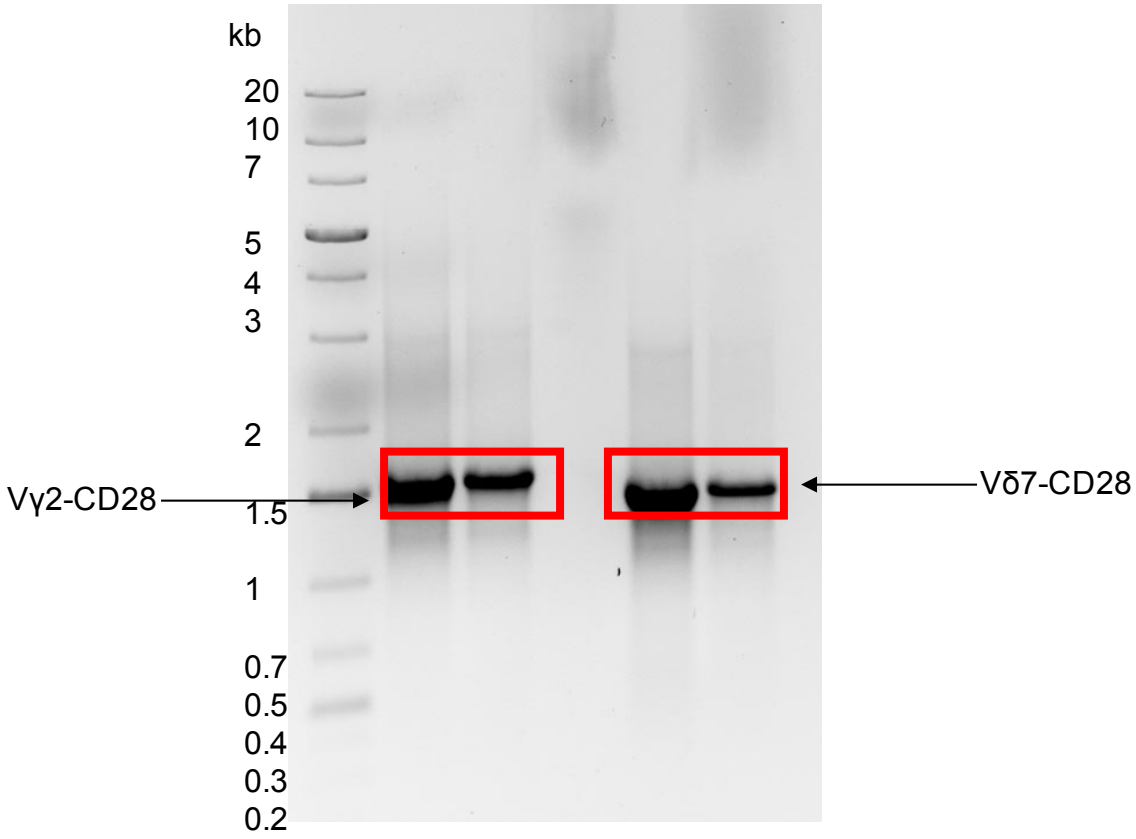


Fig 3.9: Preparation of Vγ2CD28CD3 and Vδ7CD28CD3 TCR Chains. The Vδ7 and Vγ2 TCR chains containing CD28 and CD3 zeta were prepared via a 3 step PCR. After the 3rd step PCR, samples were run on a 0.8% agarose gel to confirm the DNA length. Expected length for Vγ2CD28CD3 was 1500nt and for Vδ7CD28CD3 was 1383nt.

In order to obtain the correct sequence for each chain, the 3rd step PCR products were ligated with Topo TA cloning vector and sequenced. Fig 3.10 shows uncut and cut Topo plasmids with Vγ2CD28CD3 and

Vδ7CD28CD3 TCR inserts. Several plasmids with the correct insert lengths were obtained for both Vγ2CD28CD3 and Vδ7CD28CD3 and sent for sequencing at the Pennsylvania State University Nucleic Acid Facility. Topo plasmids containing the correct sequence for Vγ2CD28CD3 or Vδ7CD28CD3 were identified and the Vγ2CD28CD3 and Vδ7CD28CD3 TCR chains were excised by cutting with NotI and PmeI.

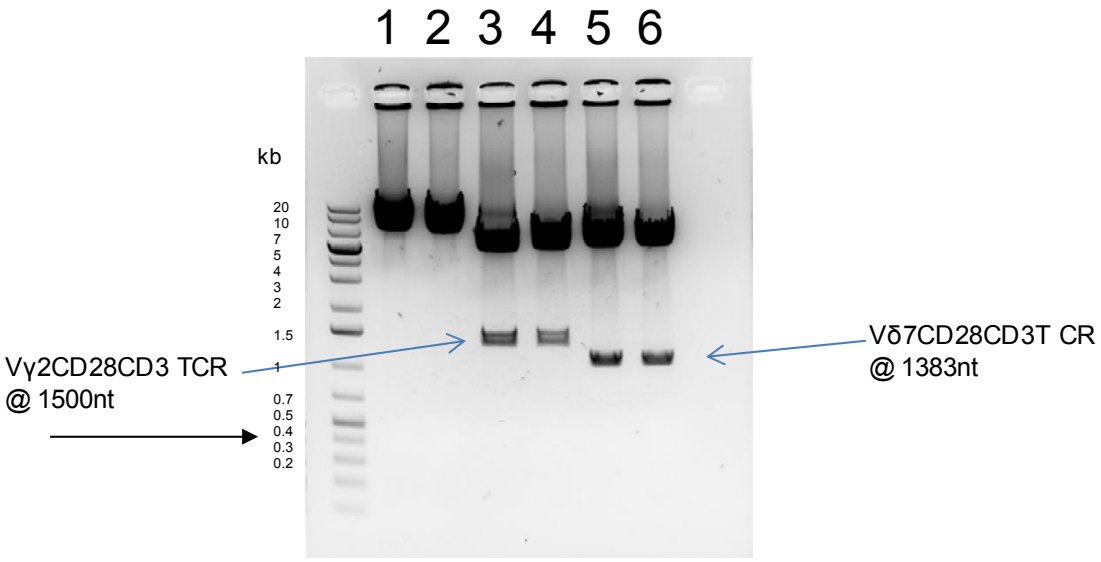


Fig 3.10: Topo Plasmids Containing Vγ2CD28CD3 and Vδ7CD28CD3 TCR Amplified Chains. Lanes 1 and 2 show undigested Topo plasmid containing Vγ2CD28CD3 and Topo plasmid containing Vδ7CD28CD3 respectively. To check for inserts, each plasmid was digested with NotI and PmeI. Topo plasmid containing Vγ2CD28CD3 digested with NotI+PmeI (Lanes 3 and 4) and Topo plasmid containing Vδ7CD28CD3 digested with NotI+PmeI (lanes 5 and 6).

Ligation of TCR chains into MSCV Plasmid

The Vγ2CD28CD3 and Vδ7CD28CD3 TCR chains were excised from the Topo TA vector by digestion with NotI and PmeI. The MSCV vector was cut with NotI and PmeI to enable ligation of the Vδ7CD28CD3 and Vγ2CD28CD3 TCR inserts. The cut MSCV vector was ligated with the cut Vγ2CD28CD3 and Vδ7CD28CD3 TCR inserts. Competent cells were transformed and plasmids were obtained from these clones. To confirm insertion of the TCR chains, plasmids were digested with NotI and PmeI and also XhoI and EcoRI. The resulting products were run on a gel to assess quality and quantity. Fig 3.11 shows the plasmids extracted from the clones.

NotI and PmeI digestion yielded the expected fragments of 1500 nucleotides and 1383 nucleotides for Vγ2CD28CD3 and Vδ7CD28CD3 respectively along with the MSCV vector fragment of 6.5kb. Digestion with XhoI and EcoRI further confirmed correct plasmid insertion. There are two EcoRI sites in the Vδ7CD28CD3 TCR at positions 514 and 588. Thus, when Vδ7CD28CD3 TCR was digested with XhoI and EcoRI, 3 fragments were typically obtained- The MSCV vector fragment at 6.5kb, a 514 nucleotide fragment and a 796 nucleotide fragment. There are no EcoRI or XhoI sites within the Vγ2CD28CD3 TCR sequence. Thus, after XhoI and EcoRI digestion, only 2 fragments (1500 nucleotide Vγ2CD28CD3 insert and 6.5kb MSCV vector) were obtained.

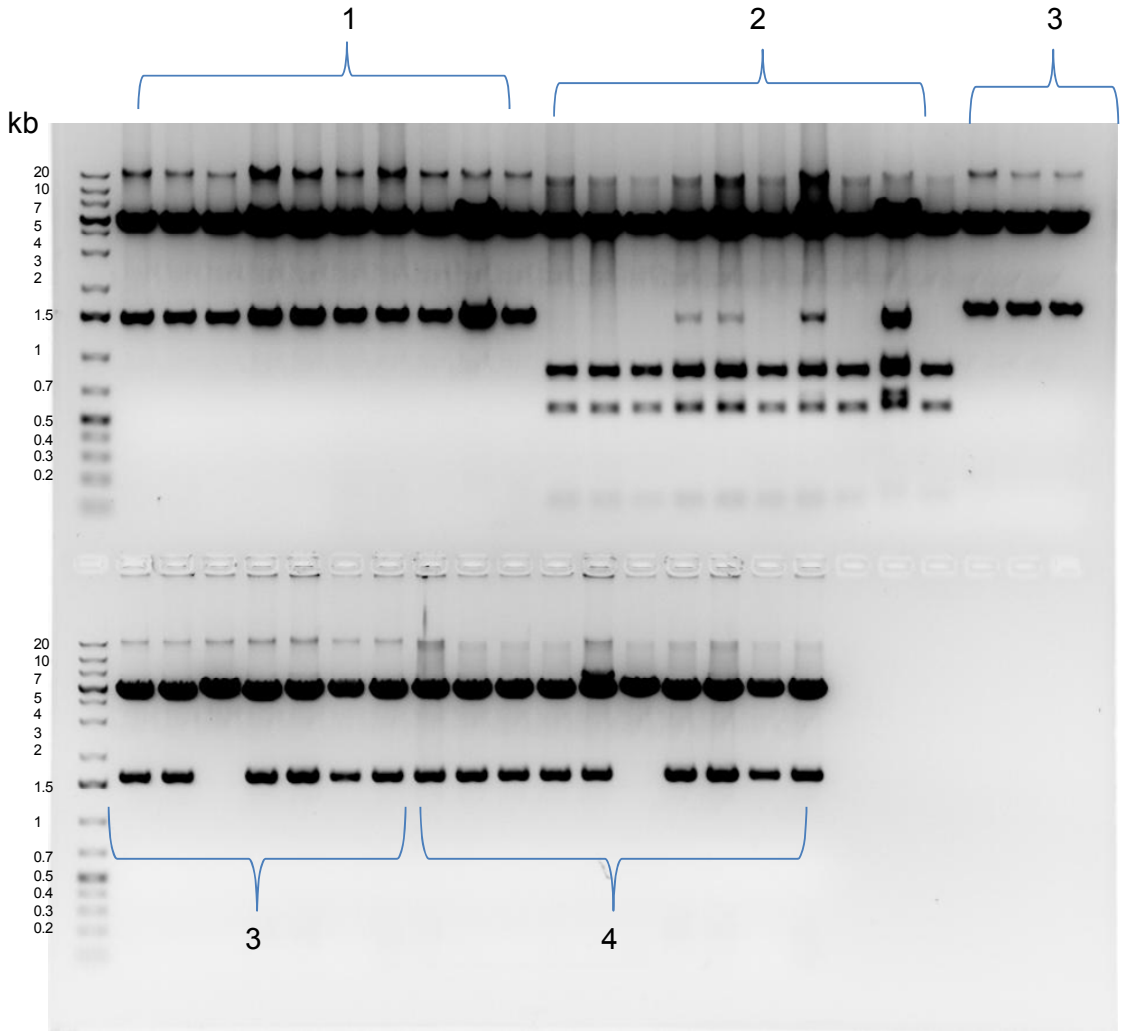


Fig 3.11: Restriction Enzyme Check for Correct Insert Size. MSCVGFP plasmids inserted with Vδ7CD28CD3 TCR (groups 1 and 2) and Vγ2CD28CD3 (groups 3 and 4) were digested with NotI + PmeI (groups 1 and 3) and XhoI + EcoRI (groups 2 and 4). Plasmids containing the correct insert size showed the expected number of fragments.

Preparation of Plasmids for Viral Particle Generation

Prior to transfection of the 293 cell line, MSCV vector containing Vγ2CD28CD3 and Vδ7CD28CD3 TCRs along with pCL-Eco plasmid (has Gag and Eco required for viral particle assembly and packaging) were prepared by midi-prep. To make sure that the appropriate plasmids were obtained, MSCV plasmids from the midiprep were digested with NotI and PmeI and pCL-Eco plasmids were digested with XhoI and Sal I as well as XhoI and Hind III (see pCL-Eco plasmid map Fig 2.6). Fig 3.12 shows the result of the midiprep.

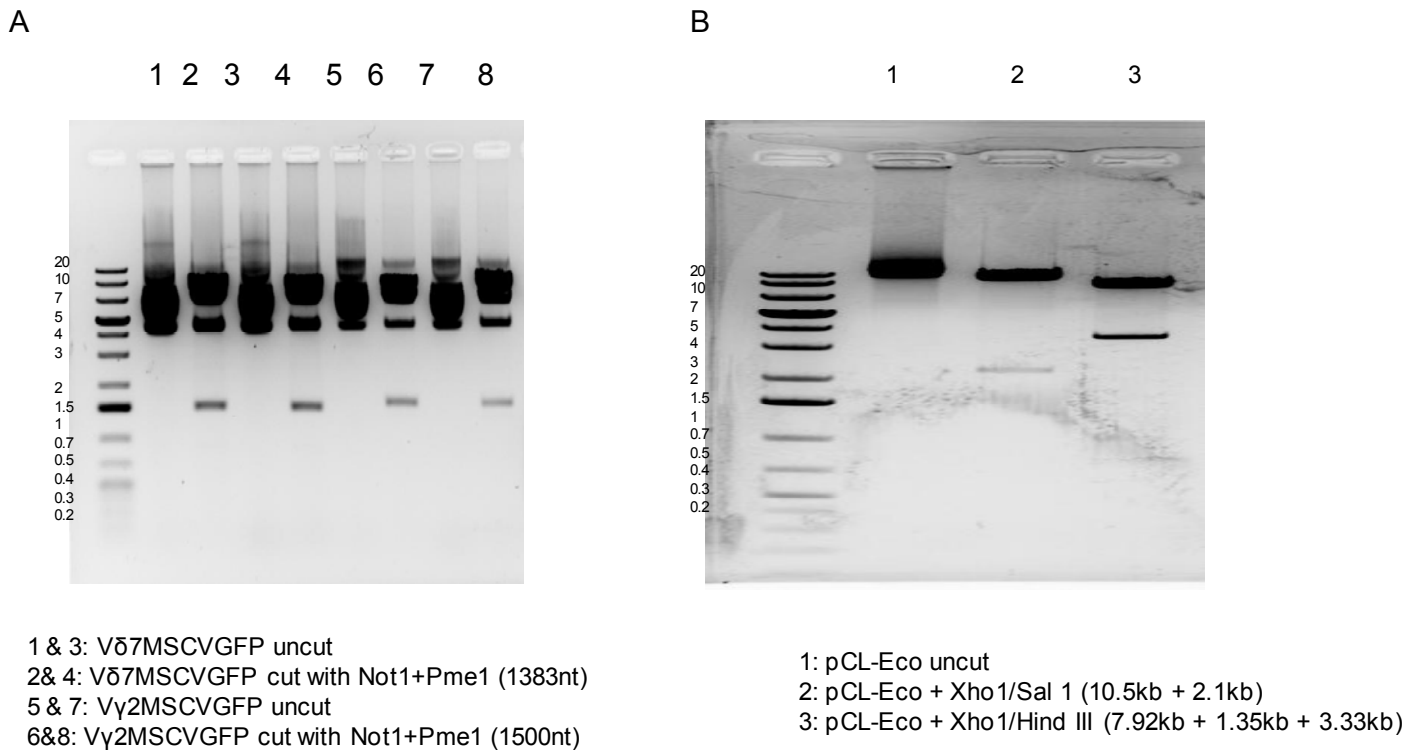


Fig 3.12 Preparation of Plasmids for Viral Particle Generation. A) Two preparations of Vδ7CD28CD3-MSCVGFP plasmid- undigested (lanes 1 and 3) and digested with NotI+PmeI (lanes 2 and 4) and two preparations of Vγ2CD28CD3-MSCVGFP plasmid- undigested (lanes 5 and 7) and digested with NotI+PmeI (lanes 6 and 8) were prepared via midiprep. B) Undigested midi prep of pCL-Eco plasmid (lane1), digested with XhoI+SalI (lane2) and XhoI+HindIII (lane3).

Production of Viral Particles from Transfection of 293 Cells

293 cells were transfected with pCL-Eco along with either Vδ7CD28CD3-MSCVGFP or Vγ2CD28CD3-MSCVGFP using a Fugene 6 reagent. 48 hours after transfection, the cells were viewed under a fluorescent microscope to observe GFP expression as an indication of successful transfection. GFP expression was observed in transfected cells. Fig 3.13 shows transfected 293 cells expressing GFP.

A) B) C)

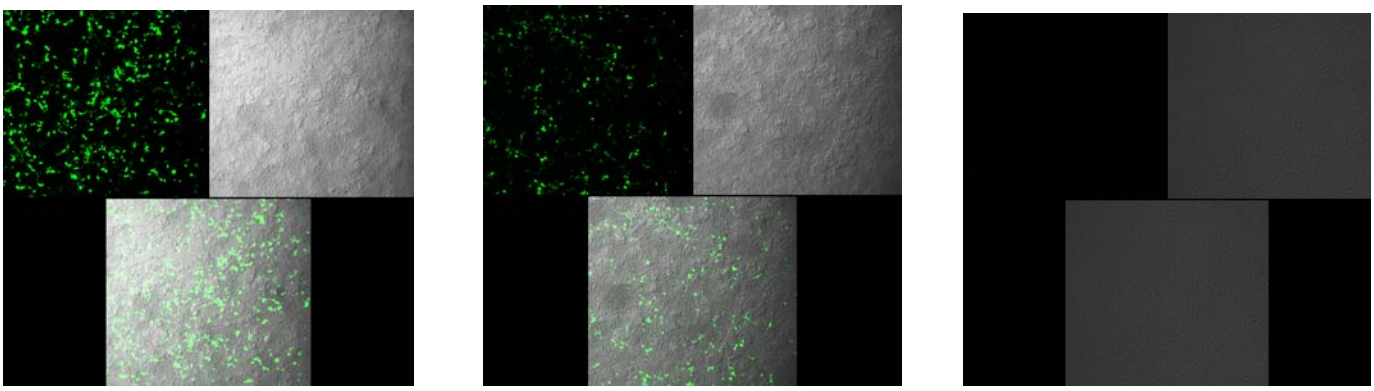


Fig 3.13: 293 cells Transfected with Vγ2CD28CD3-MSCVGFP and Vδ7CD28CD3-MSCVGFP. A) 293 cells transfected with the Vδ7CD28CD3MSCVGFP plasmid. B) 293 cells transfected with the Vγ2CD28CD3MSCVGFP plasmid. C) 293 cells with medium plus Fugene 6. 293 cells were transfected with either a Fugene 6 or lipofectamine reagent. Panels clockwise- Cells expressing GFP; all cells with green fluorescence light off; GFP expressing cells merged with non-GFP cells.

Infection of BWZ cell line with Vγ2CD28CD3-MSCVGFP and Vδ7CD28CD3-MSCVGFP TCR Viral Particles

Two rounds of infection were carried out on BWZ cells. BWZ cells were initially infected with supernatant obtained from 293 cells transfected with either Vγ2CD28CD3-MSCVGFP or Vδ7CD28CD3-MSCVGFP. After 48-72 hours, the infected BWZ cells were examined on a flow cytometer for GFP expression. Compared to the uninfected cells that showed no GFP expression, Vγ2CD28CD3-MSCVGFP and Vδ7CD28CD3-MSCVGFP infected cells showed a significant percentage of GFP positive cells. In order to obtain a high percentage of infected cells, GFP positive cells were separated by sorting. It was observed that Vδ7CD28CD3-MSCVGFP infected cells had a higher degree of GFP expression than Vγ2CD28CD3-MSCVGFP infected cells. This difference in GFP expression could have been as a result of a difference in plasmid quality. Fig 3.14 shows the results of the first round of infection for BWZ cells.

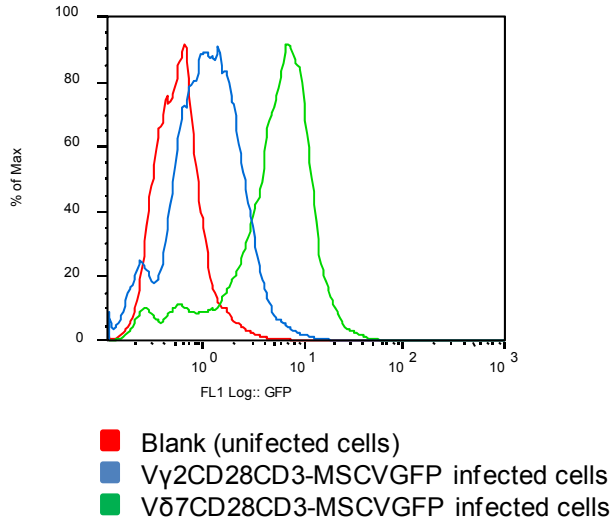
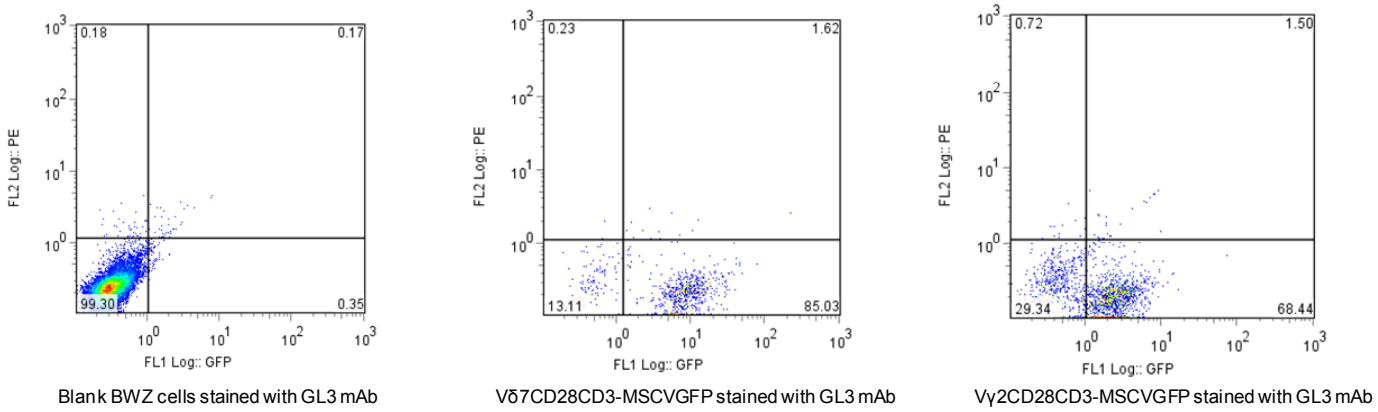


Fig 3.14: GFP Expression in Single Infected Cells. BWZ cells infected with Vγ2CD28CD3 and Vδ7CD28CD3 retroviral plasmids show evidence of successful infection by expression of GFP. Vδ7CD28CD3 infected cells have a higher degree of expression of GFP than do Vγ2CD28CD3 infected cells.

Single T cell receptor chains cannot be expressed without being associated to a respective T cell receptor chain. For example, a gamma chain needs a delta chain in order to be expressed on the T cell surface. To make sure that our system was working as a T cell should, we stained infected cells with GL3 monoclonal antibody. This antibody is specific for gamma delta TCRs and recognizes the C delta domain in particular. Thus if we had correctly infected the BWZ cells, the Vδ7CD28CD3-MSCVGFP singly infected cells should not stain positively with the GL3 mAb. This was found to be the case as can be observed in figure 3.15A. Likewise, when the Vγ2CD28CD3-MSCVGFP singly infected cells were stained with Vγ2 mAb, no positive staining was observed (Fig 3.16).

A



B

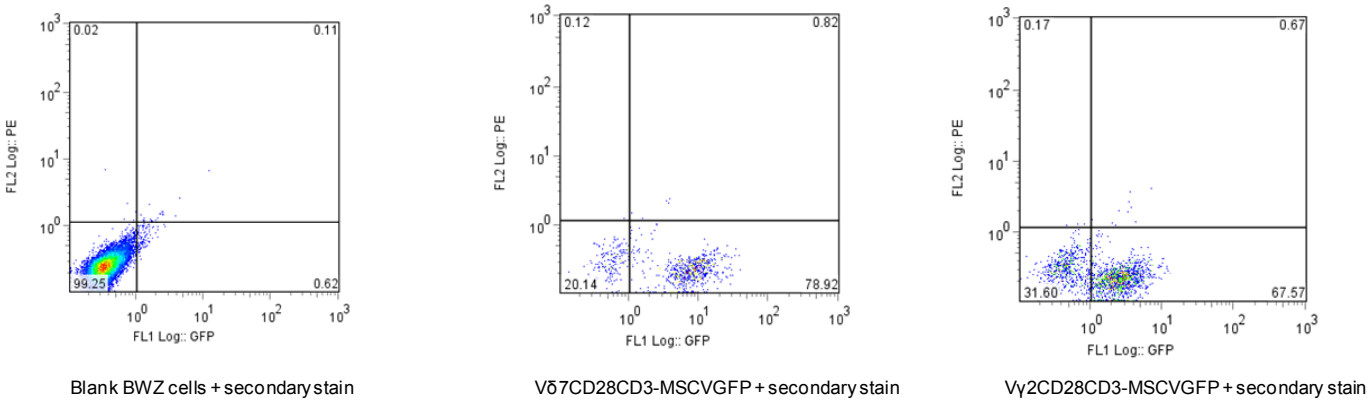


Fig 3.15: No δ chain Expression in Single Chain Infected Cells. A) Primary staining of blank, Vδ7CD28CD3-MSCVGFP and Vγ2CD28CD3-MSCVGFP infected cells with GL3 mAb (conjugated with biotin) and secondarily stained with streptavidin PE. Vδ7CD28CD3-MSCVGFP infected cells show no significant positive staining for δ chain TCR. B) Secondary control staining of blank, Vδ7CD28CD3-MSCVGFP, Vγ2CD28CD3-MSCVGFP infected cells with streptavidin PE. Blank cells and Vγ2CD28CD3-MSCVGFP infected cells were used as controls in this experiment.

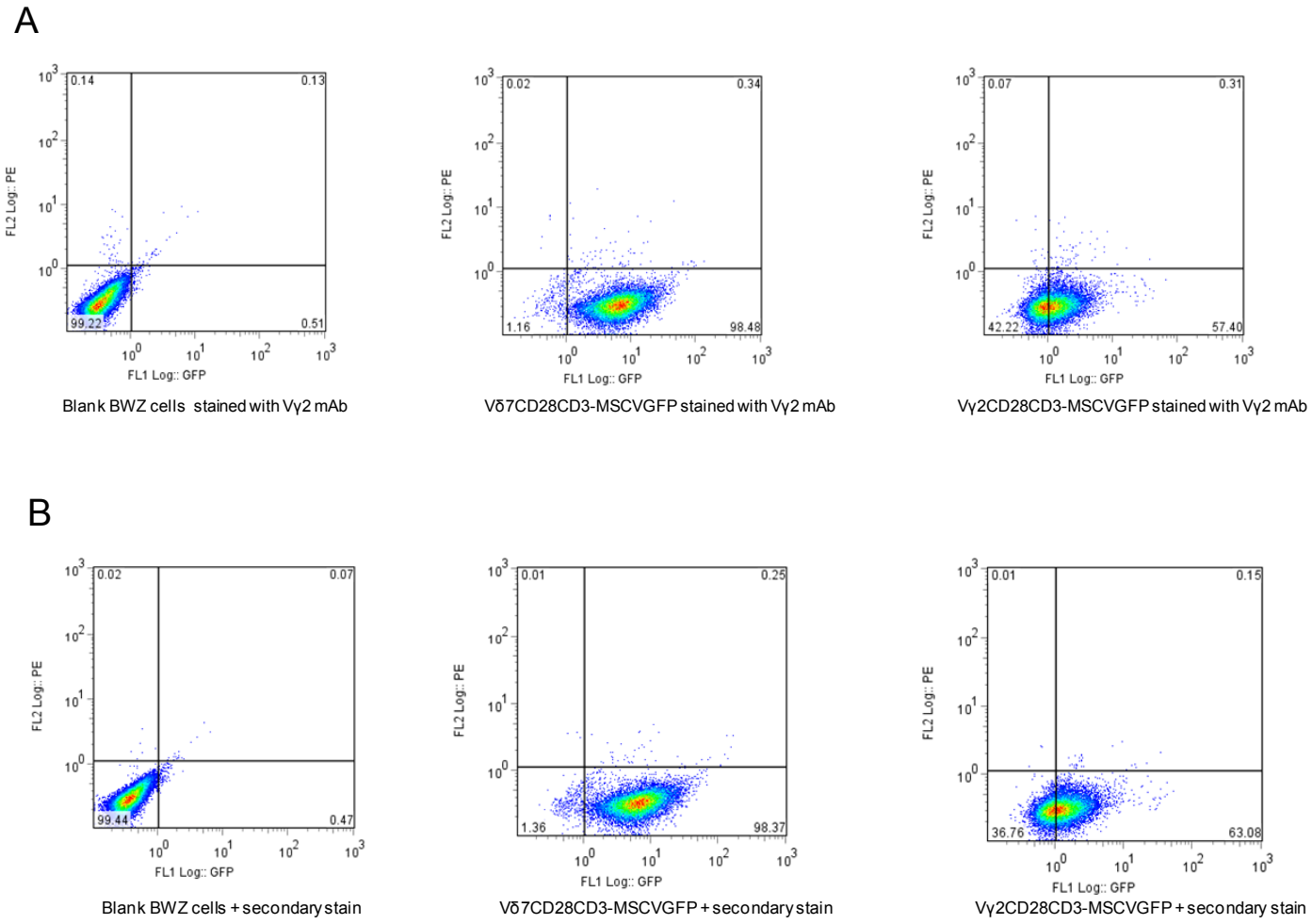


Fig 3.16: No Vγ2 chain Expression in Single Chain Infected Cells. A) Primary staining of blank, Vδ7CD28CD3-MSCVGFP and Vγ2CD28CD3-MSCVGFP infected cells with hamster anti-mouse Vγ2 mAb conjugated with FITC and secondarily stained with IgG goat anti-hamster PE. In order to observe Vγ2 staining, Vγ2 mAb was secondarily stained with IgG goat anti-hamster PE. No significant positive staining for Vγ2 chain was observed in the Vγ2CD28CD3-MSCVGFP single infected cells. B) Secondary control staining of blank, Vδ7CD28CD3-MSCVGFP and Vγ2CD28CD3-MSCVGFP infected cells with IgG goat anti-hamster PE. Blank cells and Vδ7CD28CD3-MSCVGFP infected cells were used as controls in this experiment.

In the second round of infection of BWZ cells, cells previously infected with Vδ7CD28CD3-MSCVGFP were infected with Vγ2CD28CD3-MSCVGFP viral supernatant and vice versa. These doubly infected cells were then stained with GL3 and Vγ2 mAbs and analysed on a flow cytometer. We observed that cells initially infected with Vγ2CD28CD3-MSCVGFP and then infected with Vδ7CD28CD3-MSCVGFP , had two populations of GFP expressing cells. One population had a higher GFP intensity (comparable to that in Vδ7CD28CD3-MSCVGFP singly infected cells) and the other population had a lower GFP intensity

(comparable to that in Vγ2CD28CD3-MSCVGFP singly infected cells). Vδ7CD28CD3-MSCVGFP cells that were infected with Vγ2CD28CD3-MSCVGFP were of one population of GFP intensity (Fig 3.17). All double infected cells showed positive staining for GL3 and Vγ2 mAbs. The (Vγ2+Vδ7)CD28CD3-MSCVGFP-Hi cells, showed the highest degree of Vγ2 mAb staining. The doubly infected cells were sorted first based on GFP expression and GL3 staining and then were subjected to a second sort of GFP expression and Vγ2mAb staining. Fig 3.17B shows flow cytometer results after the second sort. These results indicate that Vγ2CD28CD3 and Vδ7CD28CD3 TCRs were successfully expressed in the BWZ cell line. The two TCR chains had successfully formed a disulphide bridge and the presence of CD3 complex in the BWZ cell line allowed the expression of the two TCR chains. Thus, our method of multistep PCR generation of TCR chains for T cell expression worked.

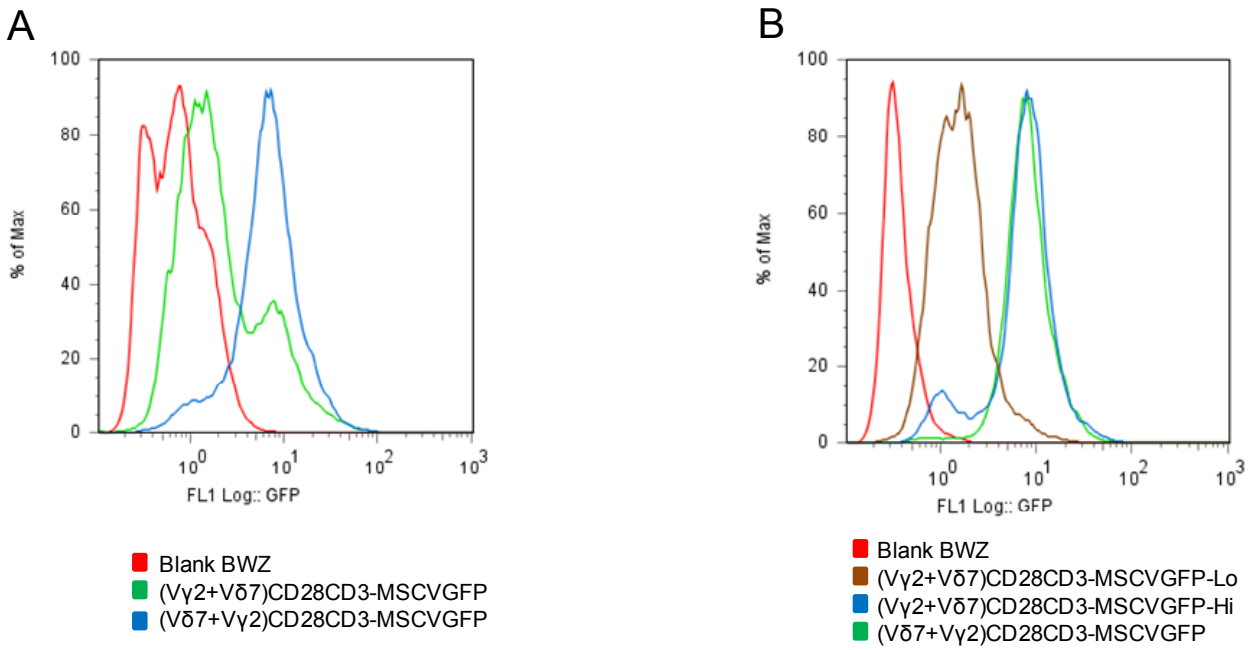


Fig 3.17: GFP Expression in Double Chain Infected Cells. A) All GFP double infected cells maintain GFP expression. Two populations of GFP expressing cells are observed in (Vγ2+Vδ7)CD28CD3-MSCVGFP infected cells. B) After sorting, three types of double infected cells were obtained -two populations of (Vγ2+Vδ7)CD28CD3-MSCVGFP infected cells and one population of (Vδ7+ Vγ2)CD28CD3-MSCVGFP cells.

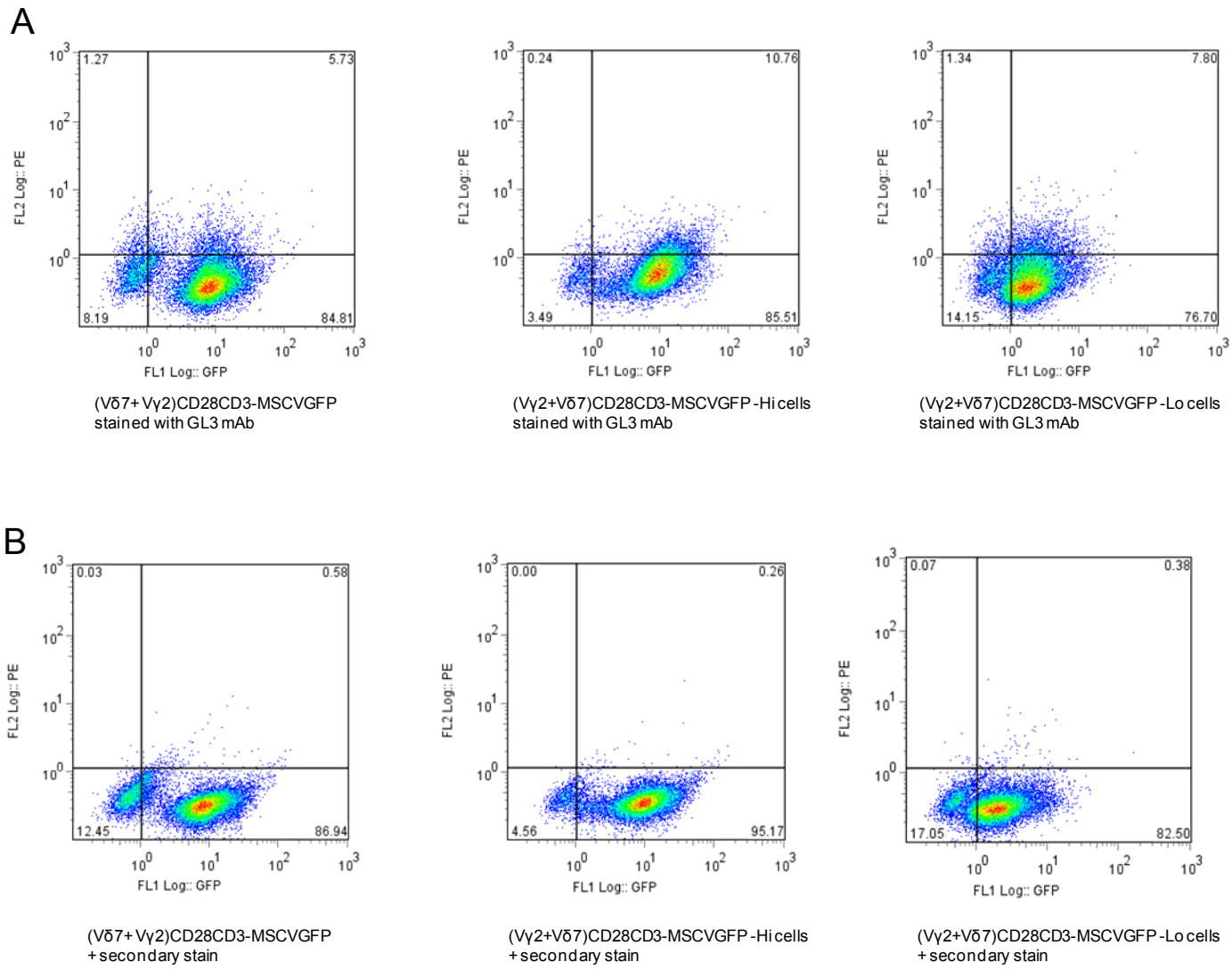


Fig 3.18: δ Chain Expression Observed in Double Chain Infected Cells. A) Primary staining of (Vδ7+ Vγ2)CD28CD3-MSCVGFP, (Vγ2+Vδ7)CD28CD3-MSCVGFP-Hi and (Vγ2+Vδ7)CD28CD3-MSCVGFP-Lo infected cells with GL3 mAb (conjugated with biotin) and secondarily stained with streptavidin PE. All double infected cells showed significant positive staining for δ chain (compared to Fig 5.7). B) Secondary control staining of (Vδ7+ Vγ2)CD28CD3-MSCVGFP, (Vγ2+Vδ7)CD28CD3-MSCVGFP-Hi and (Vγ2+Vδ7)CD28CD3-MSCVGFP-Lo infected cells with streptavidin PE.

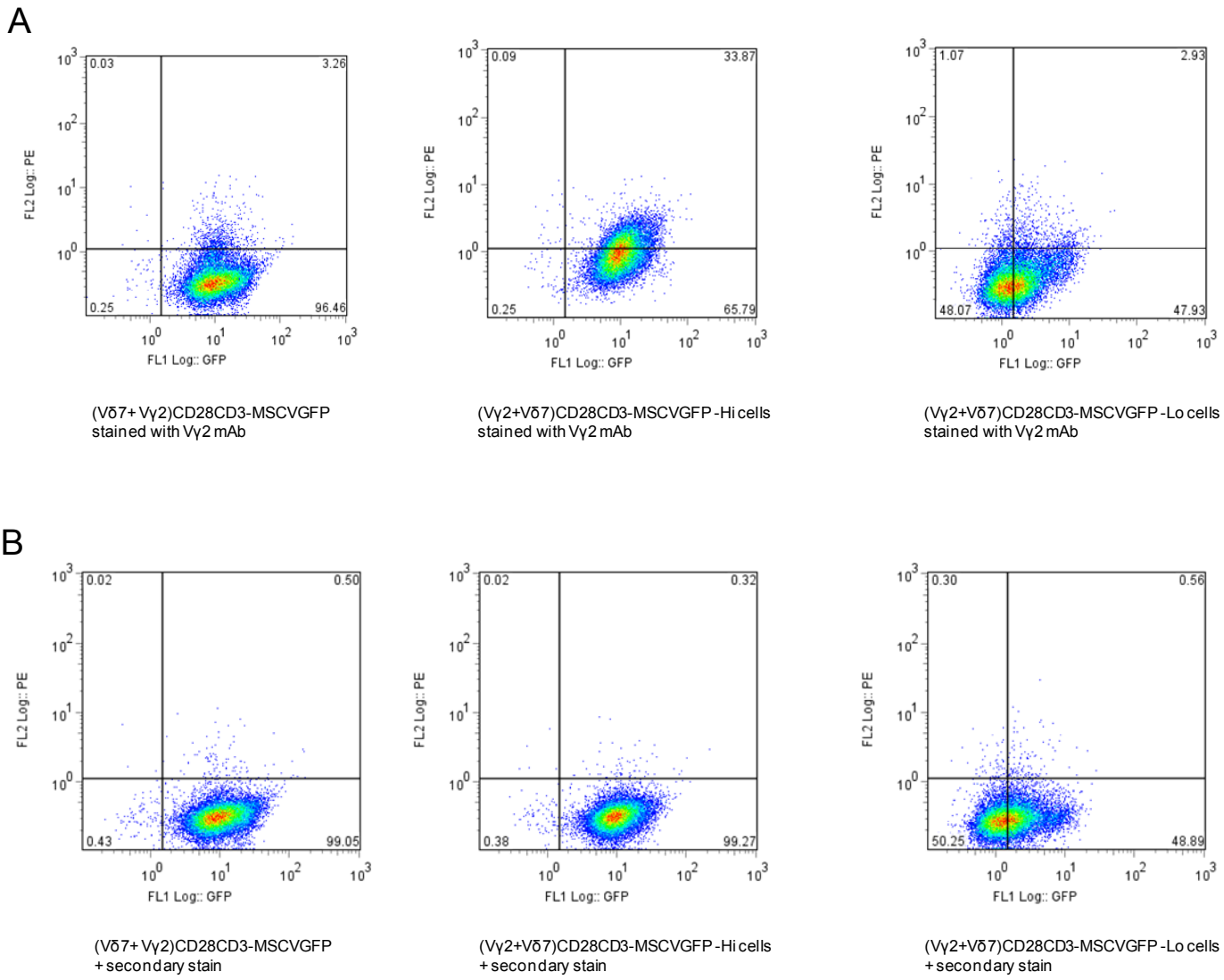


Fig 3.19: Vγ2 chain Expression in Vγ2+Vδ7 Double Infected Cells. A) Primary staining of (Vδ7+ Vγ2)CD28CD3-MSCVGFP, (Vγ2+Vδ7)CD28CD3-MSCVGFP-Hi and (Vγ2+Vδ7)CD28CD3-MSCVGFP-Lo infected cells with hamster anti-mouse Vγ2 mAb conjugated with FITC and secondarily stained with IgG goat anti-hamster PE. In order to observe Vγ2 staining, Vγ2 mAb was secondarily stained with IgG goat anti-hamster PE. All double infected cells showed significant positive staining for Vγ2 chain (compare to Fig 5.8). B) Secondary control staining of (Vδ7+ Vγ2)CD28CD3-MSCVGFP, (Vγ2+Vδ7)CD28CD3-MSCVGFP-Hi and (Vγ2+Vδ7)CD28CD3-MSCVGFP-Lo infected cells with IgG goat anti-hamster PE.

Stimulation Assay for Infected BWZ Cells

The next step of this project was to test the functionality of the Vγ2CD28CD3/Vδ7CD28CD3 TCR chains expressed on the infected BWZ cells. We used 6 types of BWZ cells for this experiment-1) uninfected (blank) cells; 2) Vδ7CD28CD3 infected cells; 3) Vγ2CD28CD3 infected cells; 4) (Vδ7+ Vγ2)CD28CD3

infected cells; 5) (Vγ2+ Vδ7)CD28CD3-HiGFP infected cells; 6) (Vγ2+ Vδ7)CD28CD3-LoGFP infected cells. Functionality experiments were conducted via TCR stimulation. As explained earlier, upon activation of the TCR, NFAT binds to the IL-2 promoter and the LacZ gene is then transcribed and LacZ protein is eventually produced within the cell. PMA and Ionomycin which can be used to stimulate the production of LacZ without the necessity of a TCR were used as positive controls. To test for TCR activity, we employed the use of GL4 mAb for stimulation (as described in chapter 2). The blank cells and single infected cells were used as controls to test for cell line self activation as they should not be stimulated by the GL4 mAb. All cells were tested for LacZ activity by incubating with GL4 or PMA/ionomycin and then addition of CPRG. Results were read on a plate reader.

The results show that the Vγ2CD28CD3 single infected cells were activated by PMA/Ionomycin but not by GL4. The Blank and Vδ7CD28CD3-MSCV cells were not activated by PMA/Ionomycin due to an error in addition of PMA+Ionomycin to the samples. The overall results from the PMA/Ionomycin stimulation implies that the BWZ cell line used in this project is not constitutively producing LacZ.

All double infected cells ((Vδ7+Vγ2)CD28CD3, (Vγ2+ Vδ7)CD28CD3-HiGFP and (Vγ2+ Vδ7)CD28CD3-LoGFP) were activated by PMA/Ionomycin. However, only the (Vγ2+ Vδ7)CD28CD3-HiGFP cells were activated by GL4 (Fig 3.20). Thus, we have successfully produced functional Vγ2CD28CD3/Vδ7CD28CD3 TCR expressing BWZ cells. Overall, the (Vγ2+ Vδ7CD28CD3HiGFP cells were shown to be stained by GL3 and Vg2 mAb and were stimulated by GL4. Thus, these cells could be used in identifying ligands specific for Vγ2Vδ7 TCR.

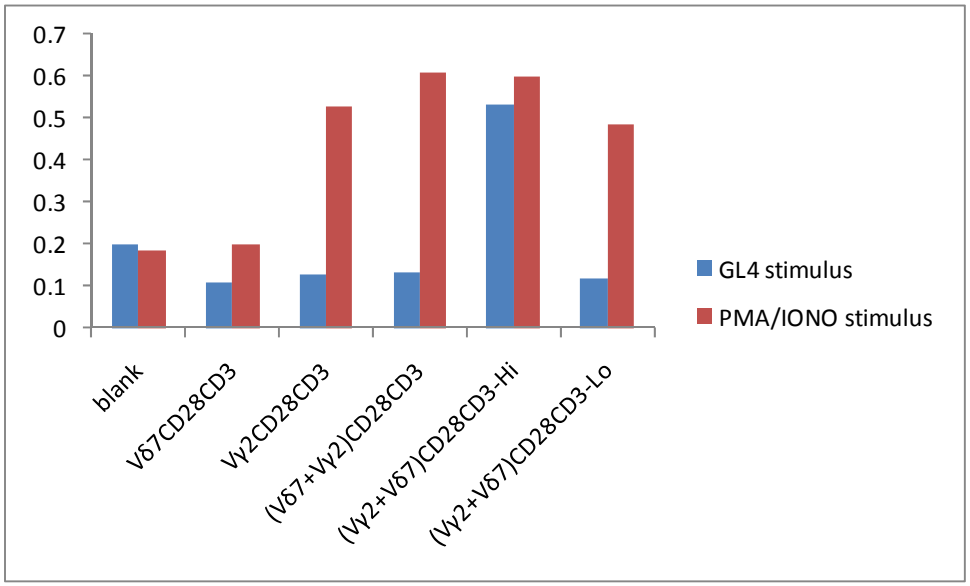


Fig 3.20: Stimulation Assay for Infected BWZ Cells. PMA+Ionomycin was used as a positive control to produce LacZ within all cells. Blank and Vδ7CD28CD3-MSCV cells do not show activation with PMA+Ionomycin due to experimental error in addition of PMA. Cells with functional TCRs should be stimulated by GL4 to produce LacZ. Only (Vγ2+Vδ7)CD28CD3-MSCVGFP-Hi cells showed activation by GL4.

Chapter 4

CONCLUSSION

Recombination Signal Sequence-Associated Restriction on TCRδ Gene Rearrangement

In our previous research, we observed that 129/svj mice did not express the Vδ7 chain unlike what could be observed in the C57Bl/6 mice. The Vδ7 gene is a member of a family of alpha-delta variable genes known as TRAV13 family. We show here that the TRAV13 genes are indeed present in the129/svj genome but rearrangement studies indicate that rearrangement to the delta chain by TRAV13 members within the 129/svj genome is very low. Sequence analysis and genome comparison studies suggest that only one member of the TRAV13 family of variable genes on the alpha-delta locus is capable of rearranging to a delta chain. This theory correlates with statistical data on the IMGT database that indicates that of 9 identified TRAV13 members, only one (TRAV13-4/DV7) has been observed to be capable of rearranging to form a delta chain [38, 39]. We have found that this TRAV13 member capable of rearranging to form a delta chain is only located on the C57Bl/6 genome. This TRAV13 member is termed TRAV13-4/DV7 based on the nomenclature in the IMGT database. We considered a number of factors that could be responsible for the high potential of TRAV13-4/DV7 rearranging to the delta chain compared to other TRAV13 members. 1) Proximity of TRAV13-4/DV7 to the D delta 2 region, 2) genetic mutation in the other TRAV13 genes prevents selection of other rearranged Vδ7 chains other than TRAV13-4/DV7 delta chains, 3) differences in the recombination signal sequences (RSS) of the TRAV13 members preventing or allowing delta chain rearrangement, 4) accessibility of recombination factors to the variable genes during rearrangement.

Proximity: When the TRAV13 family members on C57Bl/6 are arranged according to the order in which they occur in the genome, TRAV13-4/DV7 is not the most proximal to the D delta regions. TRAV13-5 is 3' of TRAV13-4/DV7. Likewise, on the 129/svj genome, TRAV13-4/DV7 has a homolog which is also not the most proximal to the D delta region as TRAV13-5 is also 3' of the TRAV13-4/DV7 homolog on 129/svj. This

observation suggests that baring the presence of a nonsense mutation on TRAV13-5 gene on the C57Bl/6, TRAV13-4/DV7 is not rearranged based on its proximity to the D delta region.

Selection Prevented by Genetic Mutation in Other TRAV13 Genes: Our experiments on the translation of TRAV13-4/DV7 rearranged sequences obtained from fetal C57Bl/6 mice show that not all rearranged sequences are in-frame, with only 33% of the rearranged samples observed to be in-frame. This indicated that the samples being observed were collected prior to selection (out of frame sequences would not be selected). At the stage at which the samples were observed, only 2 TRAV-13 members (one of which was TRAV13-4/DV7) in C57Bl/6 were observed to undergo rearrangement. This implies that other TRAV13 members are incapable of rearrangement to D delta 2 and therefore, these unrearranged TRAV13 members would be unavailable for selection. Interestingly, the other TRAV13 family member (TRAV13D-1) had an RSS that differed in the spacer sequence by 2 nucleotides compared to the RSS of TRAV13-4/DV7. In contrast, TRAV13 members that were less homologous to TRAV13D-1 sequences differed by 4 nucleotides compared to the TRAV13-4/DV7 sequence.

Accessibility of Recombination Factors to the Variable Genes: it is possible that TRAV13 genes that did not rearrange to D delta 2 in the rearrangement assay were not accessible to recombination factors at the time of rearrangement. Thus, only loci that were opened would be rearranged. This factor could be somewhat ruled out as it does not explain why there is no Vδ7 rearrangement in 129/svj. If, as mentioned earlier there are no mutations to prevent Vδ7 rearrangement in 129/svj mice, then there should be an open Vδ7 loci for rearrangement in 129/svj. Therefore, another factor apart from accessibility of Vδ7 genes to rearrangement is responsible for non-expression of Vδ7 in 129/svj.

Differences in the Recombination Signal Sequences (RSS): We observed that the RSS of TRAV13-4/DV7 was quite unique when compared to the RSS of other TRAV13 family members. While all TRAV13 RSSs of both

C57Bl/6 and 129/svj have the same conserved heptamer region, there are differences in the spacer sequence and nonamer region. The nonamer region of the TRAV13-4/DV7 RSS differs from other TRAV13 RSS at the 4th nucleotide in both C57Bl/6 and 129/svj genomes. The 4th nucleotide of TRAV13-4/DV7 is a Thymine whereas the 4th nucleotide of all other TRAV13 genes is a Cytosine. It is possible that binding of the RAG genes to an incorrect sequence on the nonamer could prevent eventual RAG gene recognition of the heptamer sequence [41, 42]. However, the IMGT evidence that other TRAV13 genes do rearrange to alpha chains suggests that this may not be a case of "non-functional" RSS but rather a case of "selective" RSS preferential to certain rearrangements [38, 39]. That is, RAG proteins are capable of binding to the nonamer sequence of the other TRAV13 genes but the nonamer sequence of these TRAV13 genes prevents rearrangement to D delta regions.

Although a number of nucleotide differences can be observed between the spacer sequences of the RSS of TRAV13-4/DV7 and other TRAV13 genes, there is one common difference. The first nucleotide of the TRAV13-4/DV7 spacer sequence is an Adenine compared to a Cytosine at the first nucleotide position in all other spacer sequences of other TRAV13 genes in both C57Bl/6 and 129/svj genomes. RAG1 and RAG2 have previously been shown to directly interact with the spacer sequence [41, 42] and recent work has suggested that changes in the spacer sequence affect the rate of rearrangement [20, 25]. Correlating directly with our results, Montalbano et al. have shown that V gene usage in vivo can be dictated by the spacer sequence [20].

Our present results suggest that development of T cells into gamma-delta or alpha-beta T cells is highly dependent on the spacer and nonamer sequence. The heptamer sequence of the variable region RSS appears to be highly conserved in all TRAV13 family members and thus may be the main determinant of whether an RSS is functional or not. The spacer and nonamer sequences appear to dictate what genes the variable region would rearrange to. This selective mechanism may also be dependent on the RSS of the D delta region or the J alpha regions in question. It appears that certain nucleotides in the spacer and/or nonamer are determinants in selecting for alpha or delta rearrangements. Future experiments will look at rearrangement of the TRAV13 family members (in both 129/svj and C57Bl/6) to J alpha genes. Based on our results, we predict that TRAV13-4/DV7 in 129/svj genome would exhibit a higher degree of rearrangement to J alpha than TRAV13-4/DV7 in

C57Bl/6 genome. Other future experiments will look at the effect exchanging the RSS of TRAV13 members in 129/svj with the RSS of TRAV13-4/DV7 from C57Bl/6 would have on rearrangement.

Development of a Vγ2/Vδ7 Reporter T cell Line

We have used a multistep PCR method to amplify Vγ2 and Vδ7 TCR chains each containing CD28 and CD3 zeta signal transducing units. MSCV retroviral plasmid along with the pCl-Eco plasmid were successfully used to generate viral particles containing our TCR chain DNA. Using these viral particles we were able to express the Vγ2 and Vδ7 TCR chains simultaneously on the BWZ cell surface. The design of the TCR chains considered the extracellular portions of C delta and C gamma 1 that contained the coding region for the disulphide bridge. This bridge is important for the joining of the two TCR chains in order for appropriate extracellular expression. Our design also included a CD28 and CD3 zeta chain. The CD28 portion acted both as a transmembrane region and as part of the intracellular signal transducing unit. The CD3 zeta chain was added for enhanced signal proliferation after activation of the TCR.

The results from the staining of single transfected cells with Vγ2 mAb or GL3 mAb shows that single TCR chains were not expressed on the cell surface alone. This observation is in tandem with what is observed in vivo where single TCR chains cannot be expressed without disulphide bonding to a co-TCR chain [2].When the two TCR chains were present within the BWZ cells (after double infection), we observed positive staining for GL3 mAb and Vγ2 mAb. This shows that our design of the TCR chains disulphide bridge as well as the CD28 transmembrane region allowed for the expression of the TCRs on the membrane of the cells. These extracellular Vγ2/Vδ7 CD28-CD3 modified TCRs are also shown to be functional in the (Vγ2+Vδ7)CD28CD3-MSCVGFP-Hi cells.

It is unclear why the other infected cells, (Vδ7+Vγ2)CD28CD3-MSCVGFP and (Vγ2+Vδ7)CD28CD3-MSCV-Lo cells, are non functional in the CPRG assays. We speculate that the second infection in these two cell types was not as efficient as in the (Vγ2+Vδ7)CD28CD3-MSCVGFP-Hi cells. This may have resulted in cells not expressing a lot of disulphide bound TCR chains and therefore reduced extracellular expression of the Vγ2/Vδ7 CD28-CD3 TCR. This is evidenced in Fig 3.18 and Fig 3.19 where, compared to the number of positively stained cells in the (Vγ2+Vδ7)CD28CD3-MSCVGFP-Hi cells infected cells, a low number of

(Vδ7+Vγ2)CD28CD3-MSCVGFP and (Vγ2+Vδ7)CD28CD3-MSCV-Lo cells are positively stained for GL3 and Vγ2 mAbs.

The functional (Vγ2+Vδ7)CD28CD3-MSCVGFP-Hi cells can now be utilized in the detection of ligands specific for Vγ2/Vδ7 TCR. The high degree of activation observed in this cell (Fig 3.20) due to its dual intracellular signaling units (CD28 and CD3) suggests that it could be applied in detecting ligands present in low concentration. We intend to utilize this artificial T cell line in testing for the presence of Vγ2Vδ7 selection ligands in 129/svj mice. While it presently appears that the RSS is the main criteria in selection of a Vδ7 chain, it may be possible that 129/svj mice may still lack the selection ligands necessary for eventual selection of rearranged in frame Vγ2/Vδ7 TCRs.

Also, as Vγ2 cells have been observed to substitute for Vγ3 cells in murine skin [3] it would be interesting to investigate the possibility that both Vγ3 and Vγ2 TCRs share the same type of selection ligand.

Chapter 8

MEDICAL AND SCIENTIFIC SIGNIFICANCE

Intraepithelial lymphocytes (IELs) which consist mainly of γδ T cells are found in epithelial parts of the body. The intestine, reproductive tracts, skin and lungs are all lined with epithelial tissues that are comprised of IELs. As T cells, γδ T cells have been implicated in the adaptive immune response just like αβ T cells. However, more is known about αβ T cells than is known about γδ T cells. Recent research has brought to light the possibility of greater functions by γδ T cells in the body's immune system especially as participants in innate immunity . Studies have shown that γδ T cells may play major roles in wound healing, allergic responses, malaria resistance and tumour growth surveillance [1,2,,43, 17].

In this project, we observe that out of two strains of mice that possess a skin specific Vδ gene, only one strain appears to rearrange and express the gene. Research such as ours could aid in understanding why some people are susceptible to a certain type of disease and others are not. In the case of irregularities caused by non-rearrangement of essential immune T cell receptors, more comfortable and effective treatments can be developed. For example, adoptive transfer of the immune T cells in question may greatly alleviate problems.

The second part of this project demonstrates the bioengineering of a reporter cell line as a means of ligand detection. We have successfully expressed functional γδ TCRs on the BWZ cell line. We intend to use this reporter cell line to detect ligands of Vγ2Vδ7 TCRs. Identification of Vγ2Vδ7 TCRs ligands could give the scientific community an idea of the nature of ligands specific to DETCs

Most importantly, this project, has the potential of improving our present knowledge of the mechanism involved in the generation of γδ T cell receptors and thus our overall knowledge of the immune system.

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