

**THE ROLE OF GAMMA-DELTA TCR+ T-CELLS IN THE PATHOGENESIS
OF SYSTEMIC SCLEROSIS**

A Dissertation
Submitted to
the Temple University Graduate Board

in Partial Fulfillment
of the Requirements for the Degree
DOCTOR OF PHILOSOPHY

by
Adaobi I. Nwaneshiudu
May, 2010

©

by

Adaobi I. Nwaneshiudu

2010

All Rights Reserved

ABSTRACT

The Role of Gamma-delta TCR+ T-cells in the Pathogenesis of Systemic Sclerosis

Adaobi I. Nwaneshiudu

Doctor of Philosophy

Temple University, 2010

Doctoral Advisory Committee Chair: Chris D. Platsoucas, PhD

The human gamma-delta ($\gamma\delta$) TCR+ T-cell subset may undergo specific antigen-driven activation and clonal expansion, in the context of systemic sclerosis (SSc) pathogenesis. The purpose of this study was; 1) To determine whether $\gamma\delta$ TCR+ T-cells are clonally expanded in skin biopsies and peripheral blood from patients with SSc; and 2) To develop approaches for identification of the antigens recognized by these clonally-expanded $\gamma\delta$ TCR+ T-cells.

Total RNA was isolated from the skin biopsies and peripheral blood of patients with SSc (n=8). After cDNA synthesis, the γ - and δ -chain TCR transcripts were amplified by PCR, cloned and sequenced for analysis. Full length copies of the TCR transcripts were constructed, expressed in a TCR-negative Jurkat T-cell line using retroviral gene transduction, and verified by RT-PCR and flow cytometry for $\gamma\delta$ TCR expression. Putative antigen recognition, by the transduced $\gamma\delta$ TCR+ Jurkat T-cell lines, was assessed via; 1) Measuring intracellular calcium flux in the transduced cells after stimulation with putative SSc antigens, including DNA topoisomerase I, centromere proteins A and B, hsp 27, hsp 90 and the viral lysate of human cytomegalovirus; and 2) Cytotoxicity against human endothelial cell lines (HUVEC and HLMVEC) via measurement of lactate dehydrogenase release from the targets.

We report the presence of substantial, statistically-significant, proportions of identical γ - and δ -chain transcripts in skin biopsies and PBMC of patients with SSc, demonstrating the presence of antigen-driven clonal expansions. Jurkat T-cells, transduced with the clonally-expanded $\gamma\delta$ TCR transcripts from a patient, showed no evidence of cytotoxicity against the human endothelial cell lines, or calcium flux in response to stimulation with the putative SSc antigens assessed.

In conclusion, extensive clonal expansions of γ -and δ -chain TCR transcripts were identified in skin biopsies and peripheral blood of patients with SSc, demonstrating the presence of oligoclonal populations of $\gamma\delta$ TCR+ T-cells in these patients. These $\gamma\delta$ TCR+ T-cells have undergone proliferation and clonal expansion *in vivo* in response to as yet unidentified antigens. Furthermore, an approach has been developed for the identification of the antigens recognized by the clonally-expanded $\gamma\delta$ TCR transcripts, which can be expanded to additional patients with SSc.

ACKNOWLEDGMENTS

My biggest acknowledgements go to my family, especially my parents, Godwin C. Nwaneshiudu, MD, and Ifeyinwa C. Nwaneshiudu, MD PhD (Department of Microbiology and Immunology), for their tireless encouragement, patience and love; my brothers, Chinedu Nwaneshiudu, Okechukwu Nwaneshiudu, PhD (civil engineering), Ifeanyi Nwaneshiudu, MD, Emeka Nwaneshiudu, MS, Ikechukwu Nwaneshiudu, PhD candidate (chemical engineering); and my sisters, Chinwe Nwaneshiudu (MD PhD candidate, year 4, TUSM) and Chioma Nwaneshiudu. I would like to acknowledge my thesis advisory committee members; Chris D Platsoucas, PhD (advisor), Alex Y. Tsygankov, PhD (co-advisor), Marc Monestier, MD PhD, Earl Henderson, PhD, Emilia Oleszak, PhD, Barrie Ashby, PhD (Department of Pharmacology), Parkson Lee-Gau Chong, PhD (Department of Biochemistry), and Allen Myers, MD (Professor of Medicine/ Rheumatology) who also provided the study samples from patients with SSc. My acknowledgements also go to my fellow lab members, including Weon-Ju Jung, PhD who began the $\gamma\delta$ TCR clonal expansion study in patients with SSc, Nikolaos Zacharakis, Kyle Evans, and especially Fatima Whitfield, PhD. I would like to acknowledge the other students, faculty and staff in the microbiology and immunology department, with a special thanks to Doina Ganea, PhD (Chairperson) and members of her lab for creating a space for me in the new lab to complete my thesis studies as well as for important feedback on my thesis presentation. Finally, and most importantly, I would like to acknowledge and ascribe all thanks and praise to the Almighty God, through whom none of this would be possible. Specifically, for providing such intriguing questions and avenues for scientific discovery into the ways of nature and the human body, as well as giving me the opportunity to be a part of the minds that answer these questions.

This is dedicated to the Almighty God and to my current and future family.

TABLE OF CONTENTS

	PAGE
ABSTRACT	iv
ACKNOWLEDGMENTS	vi
DEDICATION.....	vii
LIST OF TABLES.....	ix
LIST OF FIGURES	xiii
CHAPTER	
1. INTRODUCTION.....	1
2. HYPOTHESIS AND SPECIFIC AIMS.....	24
3. MATERIALS AND METHODS	26
4. RESULTS	51
5. CONCLUSIONS AND DISCUSSION.....	151
REFERENCES CITED.....	163

LIST OF TABLES

Table		Page
1.	THE AMERICAN COLLEGE OF RHEUMATOLOGY SSc DIAGNOSTIC CRITERIA.....	1
2.	PRIMERS USED IN RT-PCR PROTOCOL FOR CLONAL EXPANSION STUDY	29
3.	CLINICAL DEMOGRAPHICS OF THE STUDY SSc PATIENTS	52
4.	PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ I SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-3)	54
5.	PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ I SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-4)	55
6.	PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ I SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE SKIN BIOPSY TISSUE FROM A SYSTEMIC SCLEROSIS PATIENT (SS-6)	57
7.	PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ I SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE SKIN BIOPSY TISSUE FROM A SYSTEMIC SCLEROSIS PATIENT (SS-16).....	58
8.	PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ I SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-16).....	59
9.	SUMMARY TABLE OF CLONAL EXPANSION OF V γ I-CHAIN TRANSCRIPTS IN SSc PATIENT SAMPLES	61
10.	PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ II (V γ 9) SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-4).....	64
11.	PRODUCTIVELY-REARRANGED CDR3 & CONSTANT REGION SEQUENCES OF V γ II (V γ 9) SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-7)	66
12.	PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ II (V γ 9) SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE SKIN BIOPSY TISSUE FROM A SYSTEMIC SCLEROSIS PATIENT (SS-16)	68
13.	PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ II (V γ 9) SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-16)	69

14.	SUMMARY TABLE OF CLONAL EXPANSION OF V γ II (V γ 9)-CHAIN TRANSCRIPTS IN SSc PATIENT SAMPLES	70
15.	PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-1).....	73
16.	PRODUCTIVELY REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-2).....	74
17.	PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-3).....	75
18.	PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-4).....	77
19.	PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE SKIN BIOPSY TISSUE FROM A SYSTEMIC SCLEROSIS PATIENT (SS-7).....	79
20.	PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-7).....	80
21.	PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE SKIN BIOPSY TISSUE FROM A SYSTEMIC SCLEROSIS PATIENT (SS-11)	81
22.	PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE SKIN BIOPSY TISSUE FROM A SYSTEMIC SCLEROSIS PATIENT (SS-16)	82
23.	PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-16)	84
24.	SUMMARY TABLE OF CLONAL EXPANSION OF V δ 1-CHAIN TRANSCRIPTS IN SSc PATIENT SAMPLES	86
25.	PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-1).....	90
26.	PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-2).....	92

27.	PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-3).....	94
28.	PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE SKIN BIOPSY TISSUE FROM A SYSTEMIC SCLEROSIS PATIENT (SS-4).....	96
29.	PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-4).....	98
30.	PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-6).....	100
31.	PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-7).....	102
32.	PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE SKIN BIOPSY TISSUE FROM A SYSTEMIC SCLEROSIS PATIENT (SS-16).....	104
33.	PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD OF SYSTEMIC SCLEROSIS PATIENT (SS-16).....	105
34.	SUMMARY TABLE OF CLONAL EXPANSION OF V δ 2-CHAIN TRANSCRIPTS IN SSc PATIENT SAMPLES	106
35.	COMPILED SUMMARY TABLE OF CLONAL EXPANSION PROFILE OF V γ - AND V δ -CHAIN TCR TRANSCRIPTS IN SSc SAMPLES FROM THE PREVIOUS AND CURRENT STUDIES	107
36.	COMPILED TABLE OF PRESENCE OR ABSENCE OF V γ - AND V δ -CHAIN TCR TRANSCRIPTS CLONAL EXPANSIONS IN SSc SAMPLES FROM THE PREVIOUS AND CURRENT STUDIES.....	108
37.	PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ I SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE SKIN SAMPLE RNA FROM A NORMAL DONOR (NS-1).....	116
38.	PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V γ II (V γ 9) SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN SKIN SAMPLE RNA FROM A NORMAL DONOR (NS-1) ...	119
39.	PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE SKIN SAMPLE RNA FROM A NORMAL DONOR (NS-1).....	120

40.	PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN SKIN SAMPLE RNA FROM A NORMAL DONOR (NS-1)	121
41.	PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ I SUBGROUP γ - CHAIN TCR TRANSCRIPTS IN THE SKIN FROM A NORMAL 18-20week FEMALE FETAL DONOR (FNS-2).....	122
42.	PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ II (V γ 9) SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE SKIN FROM A NORMAL 18-20week FEMALE FETAL DONOR (FNS-2).....	123
43.	PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE SKIN FROM A NORMAL 18-20week FEMALE FETAL DONOR (FNS-2).....	124
44.	PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE SKIN FROM A NORMAL 18-20week FEMALE FETAL DONOR (FNS-2).....	125

LIST OF FIGURES

Figure	Page
1. Genetic locus of the human γ - and δ -chain TCR locus showing the multiple gene segment	17
2. Concept of antigen-driven clonal expansion of T-cells	19
3. Summary diagram of cloning procedure of the cDNA copies of isolated TCR transcripts.....	31
4. Summary of full length construction of the TCR V γ - or V δ - chain	35
5. Summary of insertion of transcripts to expression vector plasmid.....	36
6. MSCV expression vector plasmid	37
7. pCR2.1 TOPO TA cloning vector showing restriction enzyme sites flanking gene insertion position.....	38
8. Set-up of cytotoxicity assay	46
9. General summary of full length construction, depicting the 3 overlapping segments spanning the full length gene of the TCR chain.....	129
10. Example of generation of full length TCR chain, specifically the full length copy of the most clonally-expanded V γ 9 chain from SS16 skin biopsy.....	130
11. Sequence analysis of the V γ 9 gene segments obtained.....	132
12. Color-coded depiction of the different regions of the TCR regions of the error-free, complete full length copy of the most clonally-expanded V γ 9 chain of SS16.....	133
13. Restriction digest check (NEBcutter) to determine restriction enzyme optimal for expression vector construction.	133
14. Example of generation of full length TCR chain, specifically the full length copy of the most clonally-expanded V δ 2 chain from SS16 skin biopsy.....	134
15. Sequence analysis of the V δ 2 gene segment obtained.	135
16. Color-coded depiction of the different regions of the TCR regions of the error-free, complete full length copy of the most clonally-expanded V δ 2 chain of SS16.....	136

17. Restriction digest check (NEBcutter) to determine restriction enzyme optimal for expression vector construction	137
18. First step of expression vector construction	138
19. Fourth step of pMSCV expression vector construction.....	139
20. Transfection of the packaging cell line with different expression vector constructs.....	140
21. Gel picture depicting post-transfection results, after RT-PCR using primers specific for either the V γ 9 and V δ 2 chain gene.....	141
22. Gel picture of V γ 9 gene RT-PCR of transduced Jurkat T-cell lines	142
23. Gel picture of V δ 2 gene RT-PCR of transduced Jurkat T-cell lines	143
24. Surface expression of the $\gamma\delta$ T-cell receptor of transduced cell lines.....	144
25. Calcium flux in a wildtype Jurkat T-cell line in response to positive control OKT3 stimulation.....	146
26. Calcium flux, depicted as % F _{max} , in the wildtype Jurkat and the transduced mock vector only and 16TMCE mutant Jurkat T-cells, with 5 μ g/ml of OKT3 for CD3 stimulation.	147
27. Calcium flux of 16TMCE (blue bars) compared to the mock vector only control (red bars) in response to the positive control stimulants and the putative SSc antigens.....	148
28. Initial trial without ionomycin normalization.....	149
29. Percent human endothelial cell cytotoxicity after co-incubation of 16TMCE.....	150

**CHAPTER 1
BACKGROUND INTRODUCTION**

1.1 INTRODUCTION TO SCLERODERMA

Scleroderma, a Greek word meaning “skin hardening”, is a rare, clinically-heterogeneous disorder with symptoms that result in considerable morbidity and mortality in affected individuals. Scleroderma, as with some other autoimmune diseases such as systemic lupus erythematosus, shows a predominance in women, being 3-8 times more prevalent than in men, especially during the childbearing years (Chen, See et al. 2003; Valentini and Black 2002). Progression of clinical skin disease is typically marked by an initial edematous phase, a sclerotic phase and an atrophic phase, with significant disability resulting from chronic inflammation, microvascular destruction and fibrosis (Rose and Leskovsek 1998).

TABLE 1 – THE AMERICAN COLLEGE OF RHEUMATOLOGY SSc DIAGNOSTIC CRITERIA (1980)

Major criterion for SSc diagnosis	Minor criteria for SSc diagnosis
Scleroderma (skin tightness, thickening, non-pitting induration) proximal to the metacarpophalangeal (MCP) joint	1) Sclerodactyly, 2) Digital pitting scars, 3) Bibasilar pulmonary fibrosis

Diagnosis of SSc is made by the presence of the major criterion or two or more minor criteria (97% sensitivity, and 98% specificity). Scleroderma represents a disease spectrum that includes prescleroderma, systemic sclerosis (SSc), and scleroderma sine scleroderma. Prescleroderma describes the clinical features of scleroderma that precedes skin changes. The most characteristic feature is Raynaud’s phenomenon; a transient digital ischemia characterized by pallor, cyanosis, and hyperemia of affected fingers and toes on exposure to cold, stress or emotion (Black 1996). Systemic sclerosis (SSc) is a manifestation of both skin and visceral organ pathologies. There are two subsets, the limited cutaneous SSc (lcSSc) and

the diffuse cutaneous SSc (dcSSc), which are distinguished by the extent of skin involvement, autoantibody profile, timing and the internal organ involvement (Denton and Black 2004). LcSSc is characterized by a longstanding presclerodermal period, i.e. Raynaud's phenomenon persisting for years before skin changes, skin involvement limited to the face and distal limbs, gastrointestinal manifestations, anti-centromere autoantibody (in 70-80% of patients), and a late incidence of pulmonary arterial hypertension in 10-15% of patients. DcSSc is more severe, characterized by rapid onset of skin changes within a year of Raynaud's phenomenon, trunk and proximal limb involvement, anti-DNA topoisomerase I autoantibody, and early visceral organ complications. Finally, scleroderma-sine-scleroderma is described as dcSSc without any skin involvement (Black 1996; Denton and Black 2004). There are also SSc-like diseases that may be, 1) immune-mediated, including eosinophilic fasciitis and graft-versus-host disease; 2) deposition disorders including scleromyxedema, systemic amyloidosis and nephrogenic systemic fibrosis; 3) occupational and iatrogenic toxic exposures including polyvinyl chloride, bleomycin, silica, and organic solvents; or 4) genetic syndromes including stiff-skin syndrome (Boin and Hummers 2008).

1.2 SYSTEMIC SCLEROSIS EPIDEMIOLOGY

SSc is distributed worldwide, and ethnic factors may influence the presentation, serologic expression and immunogenetics of SSc (Reveille, Fischbach et al. 2001). In the United States, the general prevalence of SSc has been reported to range from 28-286 cases/million, while the annual incidence may range from 4-19 cases/million/year (Valentini and Black 2002). Various studies have suggested higher mortality rates in non-Caucasian patients with SSc, especially among African-American females, possibly due to an increase in

frequency, severity and an earlier disease onset (Laing, Gillespie et al. 1997; Reveille, Fischbach et al. 2001). The incidence of SSc in African-American women is 20 cases/million/year, more the twice the incidence of their Caucasian female counterparts (8 cases/million/year). In addition, the peak age-specific incidence for dcSSc in African-American women, which is 35-44 years, occurs about 10 years earlier compared to Caucasian women. Differences are also reported in the disease characteristics between various ethnic groups in the United States. In a comparative study, non-Caucasian subjects, especially African-Americans, were more likely to have diffuse skin involvement, skin pigmentary changes, digital ulcers, pulmonary arterial hypertension, anti-U1-ribonucleoprotein and anti-U3-RNP (fibrillarin) autoantibodies (Reveille, Fischbach et al. 2001). In contrast, Caucasians were more likely to have facial telangiectasias, hypothyroidism, and anti-centromere autoantibodies (Arnett, Reveille et al. 1996; Reveille, Fischbach et al. 2001). An interesting observation regarding the ethnic influence of SSc is the clustering of the disease among Choctaw Native Americans, with a prevalence of 469 cases/100,000 in “full-bloods,” that is well above that of the general U.S. population, as well as non-full-blood Choctaw Native Americans (Arnett, Howard et al. 1996; Tan, Tercero et al. 2003). Poor prognostic factors for SSc survival has been reported to include organ involvement, dcSSc disease, Black race, male sex, and older age of onset (Valentini and Black 2002).

1.3 COMPLICATIONS OF SYSTEMIC SCLEROSIS

Multiple organ systems are affected by the destructive processes involved in the pathogenesis of SSc, and organ involvement correlates with poor prognosis. Visceral organ

complications may involve the kidneys, lungs, heart, and the gastrointestinal tract and can ultimately lead to organ failure.

1.3.1 SSc renal disease

Renal disease in SSc results from fibrosis of the capillaries and the glomeruli, within the renal capsule, which compromises the filtration of blood and excretory capacity of the kidneys. SSc renal disease results in major complications, with the most severe being a sudden hypertensive crisis, associated with oliguria (decreased urine output), headache, dyspnea, edema, and rapidly rising serum creatinine levels, with acute renal failure leading to dependence on dialysis, and possible death (Bar, Ehrenfeld et al. 2001; Adams 2003). Renal complications were the leading cause of death in patients with SSc, until the development of ACE-inhibitors as anti-hypertensive therapy (Adams 2003). Nevertheless, scleroderma renal crisis, if left untreated, is the most acute and life-threatening complication of scleroderma, occurring in 10-20% of patients, with about half occurring early in the first 2.5 years of SSc disease onset (Bar, Ehrenfeld et al. 2001).

1.3.2 SSc lung disease

Involvement of the lungs in SSc disease can result in fibrosis of the lung parenchyma manifesting as restrictive lung disease, or as pulmonary arterial hypertension (Kane, Varga et al 1996). Currently, pulmonary involvement, specifically pulmonary arterial hypertension has replaced SSc renal disease and hypertensive crisis as the leading cause of mortality and a principal cause of morbidity in patients with SSc (Bar, Ehrenfeld et al. 2001). Severe restrictive lung disease, found in 10-15% of patients, has a mortality rate of 40-45% at 10 years. However, the most rapid decline of lung function occurs early in the course of the

disease, within the first 4 years of SSc (Clements 2000). Treatments for lung complications in SSc are currently insufficient to counter the underlying cause of the disease.

1.3.3 SSc cardiac disease

Cardiac involvement occurs early in the course of the disease, within the first 5 years (Clements 2000). Myocardial fibrosis is the chief cardiac manifestation, resulting in the degeneration of healthy myocardial fibers, diastolic dysfunction, and cardiac conduction defects. In addition, the coronary vessels become sclerotic compromising blood flow to the heart tissue, and resulting in angina and possibly myocardial infarction (Beranek 2001).

1.3.4 SSc gastrointestinal (G.I) disease

Involvement of the gastrointestinal tract is the most common visceral organ complication of SSc, resulting in smooth muscle hypomotility in the esophagus, stomach and bowel early in the course of the disease, within the first 3 years (Marie, Levesque et al. 2001; Denton and Black 2004; Sallam, McNearney et al. 2006). “Watermelon stomach” (also known as antral vascular ectasia) is a classic presentation in patients with SSc G.I disease, associated with high morbidity (Elkayam, Oumanski et al. 2000). Hypomotility in the large intestine occurs later in the course of the disease, and results in bacterial migration into the small intestine from the colon, leading to bacteria overgrowth, inactivation of bile acids and malabsorption of fat and fat-soluble nutrients (Marie, Levesque et al. 2001; Sallam, McNearney et al. 2006). Primary biliary cirrhosis of the liver also occurs in less than 5% of patients with SSc, especially those with lcSSc of long duration (Denton and Black 2004).

1.3.5 Other SSc associated disease

Sclerodermal myopathy results in muscle weakness and is a common complication of SSc, noted within the first 2 years (Clements 2000). SSc-associated neuropathy has also been

reported in patients, evidenced by abnormal blink reflex and painful trigeminal neuropathy (Fischoff and Sirois 2000; Casale, Frazzitta et al. 2004). Pregnancy is associated with disease progression in 50% of patients with SSc, complicated by severe pre-eclampsia and thrombocytopenia (D'Angelo and Miller 1997). Female patients with SSc have been shown to have higher rates of abortions, and lower fertility than normal females and have a higher risk for premature births (Steen 1999; Steen and Medsger 1999).

1.4 PATHOGENESIS OF SYSTEMIC SCLEROSIS

The etiology of SSc is unknown although multiple published reports suggest that this complex disease may be multifactorial influenced by genetic, immunologic, and environmental factors. The main characteristic features of SSc involve three main cell types, specifically immune cell dysfunction, endothelial cell injury (including fibrointimal proliferation and obliterative vasculopathy) leading to functional and structural microvascular destruction, and an imbalance in fibroblast activity resulting in fibrosis, the pathological hallmark of the SSc disease.

1.4.1 *Endothelial cells*

In the microvasculature, endothelial cell injury greatly augments vascular permeability, permitting increased filtration of plasma proteins into the interstitial space, and results in edema, fibrointimal proliferation and interstitial fibrosis (Prescott, Freemont et al. 1992; Black 1996; LeRoy 1996). Dermal thickening due to fibrosis in the skin results in flexion contractures, ischemic ulcerations, and resorption of distal phalanges (Black 1996). In addition, capillary abnormalities can be seen in more than 90% of patients with SSc (Grassi, Medico et al. 2001). However, defective vasculogenesis, evidenced by low circulating

endothelial precursors (CEP) in patients with SSc, is also evident (Kuwana, Okazaki et al. 2004). This suggests insufficient formation of blood vessels to replace the damaged microvasculature in SSc disease. There is also activation of endothelial cells, evidenced by increased expression of E-selectin, and endothelin-1 reported in multiple studies (Vancheeswaran, Azam et al. 1994; Vancheeswaran, Magoulas et al. 1994; Hebbar, Lassalle et al. 1995). The role of this activation of endothelial cells in SSc disease is still being elucidated.

1.4.2 Fibroblasts

Fibrosis is the pathological hallmark of SSc disease due to an aberrant activation, and overactivity of fibroblasts. Activation of fibroblasts results in excess production of collagen, fibronectin and other extracellular matrix proteins (ECM), a process tightly regulated by enzyme balance, specifically matrix metalloproteinases (MMPs), which degrade ECM proteins and tissue inhibitors of matrix metalloproteinases (TIMPs), that facilitate accumulation of ECM proteins. There is reported evidence of an ECM deposition and degradation imbalance, with increased TIMPs and a decrease in different MMPs in patients with SSc (Kikuchi, Kubo et al. 1995; Yazawa, Kikuchi et al. 2000). In addition, there is evidence of autoantibodies against fibroblasts (AFAs) in patients with SSc that have been shown to bind normal fibroblasts and induce a proadhesive and proinflammatory fibroblast phenotype, similar to fibroblasts from patients with SSc (Chizzolini, Raschi et al. 2002). Genetic analyses of fibroblasts from patients with SSc have revealed a specific genetic signature, with selective overexpression of multiple autoantigens, compared to control fibroblasts from normal donors (Zhou, Tan et al. 2001). Overexpression of TGF- β , a potent profibrotic cytokine, has also been reported in fibroblasts from skin biopsies of patients with SSc (Kubo, Ihn et al. 2001).

1.4.3 Immune cells

There is mononuclear cell infiltration, predominantly comprised of T-cells and monocytes, in early skin lesions of patients with SSc, and the degree of infiltration positively correlates with both the degree and the progression of skin thickening (Prescott, Freemont et al. 1992). Autoantibody production, an effector function of activated B-cells that requires T-cell help, is also evident in patients with SSc. In addition, multiple cytokines are overexpressed in peripheral blood and lesions from patients with SSc, some of which are profibrotic cytokines. Other immune cells, including monocytes, have been shown to be aberrantly activated in SSc, with overexpression of cytokines and chemokines that foster a chronic inflammatory environment (Sambo, Jannino et al. 1999).

1.5 T-CELLS IN SYSTEMIC PATHOGENESIS

The pathological characteristics of SSc disease, namely immune cell dysfunction (autoantibody production), endothelial cell injury, and fibrosis may involve activated T-cells. Several lines of evidence support the role of T-cells in the pathogenesis of SSc.

1.5.1 *T-cell infiltrates present in the skin biopsies from patients with SSc.*

T-cells and monocytes are the most prominent inflammatory cells infiltrating SSc lesions and are seen in the perivascular areas, at sites of active connective tissue deposition, and within the subcutaneous tissue, and dermis, where they surround small vessels, nerves and skin appendages (Prescott, Freemont et al. 1992). The T-cells infiltrating skin lesions from patients with SSc, include both the conventional $\alpha\beta$ TCR+ T-cells and the more enigmatic $\gamma\delta$ TCR+ T-cell subsets (Giacomelli, Matucci-Cerinic et al. 1998; Kalogerou,

Gelou et al. 2005). These T-cells express activation antigens, including IL-2R (CD25), HLA-DR and CD29 (Abraham and Varga 2005; Kalegerou, Gelou et al. 2005).

1.5.2 Clonally expanded T-cells in SSc lesions

In addition to the expression of activation antigens by T-cells infiltrating lesions from patients with SSc, the presence of clonally-expanded T-cells provides further evidence of an antigen-driven activation of these T-cells. Different studies report that the lesional skin biopsies from patients with SSc of recent onset contain a restricted repertoire, i.e. varying degrees of oligoclonal populations of both $\alpha\beta$ TCR+ T-cells and $\gamma\delta$ TCR+ T-cells, analyzed by TCR transcript sequencing and/or CDR3 spectratyping (Yurovsky, Sutton et al. 1994; White and Yurovsky 1995; Giacomelli, Matucci-Cerinic et al. 1998; Sakka, Xu et al. 2002). These clonally expanded $\alpha\beta$ TCR+ and $\gamma\delta$ TCR+ T-cells may have undergone specific antigen-driven proliferation and clonal expansion, in response to as yet unidentified antigens, and may play a significant role in the pathogenesis of SSc. CD8+ T-cells present in the bronchoalveolar lavage from patients with SSc express restricted TCR junctional region length diversity, also suggestive of oligoclonal expansion (Yurovsky, Wigley et al. 1996). Further evidence of antigen-driven clonal expansion of T-cells playing a role in SSc disease include, 1) usage of a restricted repertoire of V β TCR genes present in CD4-CD8- T-cells from the peripheral blood from patients with SSc (Sakamoto, Sumida et al. 1992); 2) usage of a restricted repertoire of V β TCR gene segments in T-cells infiltrating the skin of tight-skin (Tsk-2) mice, an experimental animal model of systemic sclerosis, (Wooley, Sud et al. 1998); 3) usage of a restricted repertoire of α - and β -chain TCR segments in autoreactive T-cell clones generated from patients with SSc that were specific for DNA topoisomerase-I, a putative SSc antigen (Kuwana, Medsger et al. 1997).

1.5.3 Putative antigens in SSc.

Several putative SSc antigens have emerged from multiple studies into SSc pathogenesis, including autoantigens with circulating autoantibodies that are present in SSc disease; autoantigens exposed on endothelial cells (which are destroyed in the context of the disease); and viral antigens that may result in molecular mimicry and cross reactivity with self antigens. A lack of autoantibody to a putative antigen in a patient with SSc does not necessarily preclude a cell-mediated (T-cell) immune response against that antigen in the context of SSc disease pathogenesis, but only suggests a lack of humoral (B-cell) response.

DNA topoisomerase I (DNA topo I) is a potential antigen that may be recognized by T-cells in the context of SSc pathogenesis. Autoantibodies against DNA topo I are present in SSc disease and characterize the more severe diffuse form of the disease, dcSSc (Hu, Fertig et al. 2004). The tight-skin (Tsk-2) mice model of SSc also have anti-DNA topo I autoantibodies that may recognize similar epitopes on DNA topo I as the anti-DNA topo I autoantibodies from patients with SSc (Muryoi, Kasturi et al. 1992). A study also reported peripheral blood mononuclear cells (PBMC) from 96% of patients with SSc, who had anti-DNA topo I antibody, exhibited a substantial T-cell proliferative response to human recombinant DNA topo I, evident only after 3 days in culture with DNA topo I, compared to 40% of patients with SSc without the autoantibody and 62% of the healthy controls, both of which required 7 days of incubation (Kuwana, Medsger et al. 1995).

Centromere proteins (CENP), especially CENPA and CENPB, have also been suggested to be putative antigens in SSc disease. Although anti-centromere antibodies are not specific to SSc disease, and are present in other autoimmune diseases, they characterize a subset of SSc disease, specific the limited form of SSc (Denton and Black 2004). In addition,

heat shock proteins have been reported to be overexpressed in different autoimmune diseases, including SSc (Dhillon, MacCallum et al. 1993).

Endothelial cell surface antigens may be putative antigens in the pathogenesis of SSc. There are anti-endothelial cell autoantibodies in the serum of patients with SSc, and they have been reported to bind CENPB (Servettaz, Tamby et al. 2006). Endothelial cell injury is a major pathological characteristic of SSc disease, and recognition of, and cytotoxicity against endothelial cells by $\gamma\delta$ TCR+ T-cells may occur. Studies have reported $\gamma\delta$ TCR+ T-cell recognition and cytotoxicity against endothelial cells in other autoimmune diseases. For example, the $\gamma\delta$ TCR+ T-cells from patients with Takayasu arteritis, an autoimmune vascular disease characterized by injury to the aorta and its large branches, were reported to recognize hsp60. These hsp 60-responsive $\gamma\delta$ TCR+ T-cells showed an increased cytotoxicity against the human aortic endothelial cell line, compared to the cytotoxicity observed in the total $\gamma\delta$ TCR+ T-cell population (Chauhan, Singh et al. 2007). In SSc disease, $\gamma\delta$ TCR+ T-cells from SSc patients show enhanced interaction with vascular endothelial cells *in vitro*, compared to those from normal donors (Kahaleh, Fan et al. 1999). In addition, anti-endothelial autoantibodies, present in sera of patients with SSc, bind and result in apoptosis of human dermal endothelial cells (Ahmed, Tan et al. 2006).

Human cytomegalovirus (hCMV) has also been implicated in SSc pathogenesis, through a possible molecular mimicry with endothelial cell surface antigens that may result in endothelial cell injury. Molecular mimicry is the cross-reactivity of a T-cell specific for a microorganism with a self-antigen, which may result in the development of autoimmune disease (Sakkas and Platsoucas 2004). The vasculopathy due to acute hCMV infection has extensive similarities to the microvascular injury characteristic of SSc pathogenesis, and

hCMV shows a tropism for endothelial cells during latency (Jarvis and Nelson 2007). In addition, hCMV infection may enhance vasculopathy in SSc because hCMV promoters have been reported to turn on the gene for the profibrotic cytokine, TGF- β (Michelson, Alcamì et al. 1994). Increased levels of anti-hCMV antibodies have been identified in the serum of SSc patients, compared to normal controls (Neidhart, Kuchen et al. 1999; Lunardi, Dolcino et al. 2006). These anti-hCMV antibodies may bind endothelial cells, possibly through molecular mimicry mechanisms, resulting in endothelial cell apoptosis (Lunardi, Dolcino et al. 2007). A subset of anti-hCMV antibodies specifically interacts with a normally-expressed endothelial cell surface receptor, novel antigen-2 (NAG-2), which shares similarities with the hCMV UL94 protein (Lunardi, Bason et al. 2000). In addition, similarities between the hCMV UL70 protein and DNA topoisomerase I residues 122-126 have been identified (Lunardi, Bason et al. 2005), suggestive of possible molecular mimicry. Interestingly, a study reported patients with acute hCMV infection, developing sclerodermal disease, further implicating a role for the virus in the pathogenesis of SSc (Magro, Crowson et al. 2007).

1.5.4 Autoantibody production.

Autoantibodies towards selected cellular proteins are present in the sera of over 90% of patients with SSc and are not only characteristic, but specific for clinical subsets (Harvey and McHugh 1999). For example, anti-centromere antibodies are present in patients with lcSSc, while antibodies against RNA polymerases are in dcSSc patients with risk of renal involvement (Hirakata, Okano et al. 1993; Harvey, Butts et al. 1999), and anti-DNA topoisomerase I are in patients with pulmonary involvement (Kuwana, Medsger et al. 1995; Hayakawa, Hasegawa et al. 2004; Fertig et al. 2004). Anti-DNA topoisomerase I autoantibodies from patients with SSc and the tight-skin mice model interact with similar

epitopes on DNA topoisomerase I (Muryoi, Kasturi et al. 1992). Other autoantibodies, including anti-fibrillin-1 and anti-PDGFR are present in patients with SSc and may be stimulatory to normal fibroblasts, possibly contributing to the increased activation and fibrosis (Gabrielli, Svegliati et al. 2007; Baroni, Santillo et al. 2006; Zhou, Tan et al. 2005; Tan, Arnett et al. 2000). CD40-CD40L interactions, important in the generation of the humoral response and antibody production, are involved in the mechanism of graft-versus-host disease (GVHD) and can possibly contribute similarly to the pathogenesis of SSc. There is increased expression of CD40L on the surface of activated T-cells from SSc patients (Valentini, Romano et al. 2000). CD40-CD40L interactions also mediate the upregulation of adhesion molecules and chemokines in CD40+ endothelial cells and COX-2 expression in CD40+ fibroblasts thereby regulating inflammation. This interaction between T-cells and B-cells has been shown to be essential in the autoantibody response in SSc (Valentini, Romano et al. 2000).

1.5.5 Cytokines and chemokines.

T-cells produce cytokines and chemokines in response to antigenic stimulation, and in the course chronic inflammation, these factors recruit and activate other inflammatory cells, contributing to the disease progression and the onset of complications. T-cells expressing activation markers as well as showing evidence of a possible antigen-driven clonal expansion are present in lesions and peripheral blood of patients with SSc, as previously discussed. High numbers of CD30+ CD4+ T-cells and soluble CD30, a key feature of T-cell activation, are also present in skin samples and sera of patients with SSc, particularly the lcSSc subset (Mavalia, Scaletti et al. 1997; Ihn, Yazawa et al. 2000). It is therefore not very surprising that increased levels of cytokines and chemokines have been described in SSc

patients. Various studies report an increased expression of different profibrotic cytokines, including IL-4, IL-17, IL-6, TGF- β and other fibroblast mitogens that may contribute to the pathogenesis of SSc.

IL-4 is a potent pro-fibrotic cytokine that enhances the production of extracellular matrix proteins *in vitro*, induces the production of other profibrotic cytokines, and enhances the expression of vascular cell adhesion molecule (VCAM-1) in endothelial cells (Kodera, McGaha et al. 2002; Banning, Krutmann et al. 2006). An increase IL-4 and alternatively-spliced IL-4 transcripts, (IL-4 δ), were detected in PBMCs from patients with SSc (Sakkas, Tourtellotte et al. 1999). In addition, double positive T-cells (CD4+ CD8+), with high IL-4 production potential were identified in skin lesions from patients with SSc (Parel, Aurrand-Lions et al. 2007). IL-17 is another potently profibrotic cytokine produced by specific T-cell subsets, and is overexpressed in the peripheral blood and skin of patients with SSc (Kurasawa, Hirose et al. 2000). TGF- β 1, an isoform of TGF- β , causes fibroblast activation, proliferation and increased synthesis of collagen through upregulating profibrotic cytokines (Sato, Nagaoka et al. 2000; Shi-wen, Pennington et al. 2000). SSc skin fibroblasts have higher expression of TGF- β 1 and TGF- β 2 receptors than normal controls (Gabielli, Di Loreto et al. 1993; Kubo, Ihn et al. 2001). IL-6 is a fibrogenic cytokine that induces collagen production by fibroblasts. IL-6 and IL-10 are elevated in serum from patients with SSc compared to healthy controls, and this increase correlates with disease progression (Sato, Hasegawa et al. 2001).

Various fibroblast mitogens are also elevated in SSc, including connective tissue growth factor (CTGF), and basic fibroblast growth factor (bFGF) (Sato, Nagaoka et al. 2000; Lawrence, Khanna et al. 2006). Allograft inflammatory growth factor 1 (AIF-1),

initially identified in cardiac allografts in chronic rejection, is elevated in lesions and PBMC from patients with SSc, and may be involved in the fibroproliferative vascular destruction that is characteristic of the disease (Del Galdo, Maul et al. 2006; Otieno, Lopez et al. 2007). Chemokines are key factors that regulate the recruitment of specific leukocyte subsets to the site of inflammation. In patients with SSc, serum levels of chemokines, including monocyte chemoattractant protein-1 (MCP-1), IL-8, and macrophage inflammatory protein (MIP-1 α and MIP-1 β) are elevated, and correlate with organ involvement (Hasegawa, Sato et al. 1999; Fujii, Shimada et al. 2004; Yanaba, Komura et al. 2006).

1.5.6 Apoptosis-related T-cell homeostasis

Multiple reports suggest a deregulation of apoptosis-related lymphocyte homeostasis in SSc disease. On one hand, there is increased apoptosis of CD8⁺ T-cells, suggesting a compromise in cell-mediated immunity in patients with SSc. A decrease in the CD8⁺ T-cell subpopulation in the peripheral blood of patients with SSc has been reported that may be due to low levels of the NF κ B transcription factor (Kessel, Rosner et al. 2004). In addition, high levels of soluble Fas/APO1 and CD95⁺ lymphocytes are detected in patients with SSc (Stummvoll, Aringer et al. 2000). However, a resistance to apoptosis has been reported in $\alpha\beta$ TCR⁺ and $\gamma\delta$ TCR⁺ T-cells in the peripheral blood of patients with SSc (Cipriani, Fulminis et al. 2006), suggesting a persistence of activated T-cells that may result in autoimmunity and chronic inflammation. The persistence of clonally-expanded T-cells in peripheral blood of SSc patients (White and Yurovsky 1995; Sakkas, Xu et al. 2002) further supports this notion.

1.5.7 HLA association with SSc.

Association of particular HLA alleles with various characteristics of SSc disease supports the concept of an antigen-driven T-cell response in the pathogenesis of SSc. The

strongest HLA associations in SSc are with the autoantibody subsets. HLA-DRB1*11 shows a strong correlation with the anti-topoisomerase I antibody response and HLA-DRB1*01, DRB1*04 and DQB1*0501 with anticentromere antibodies (Arnett 1995; Fanning, Welsh et al. 1998; Rands, Whyte et al. 2000; Reveille 2006). HLA-DQB1*0301 is significantly associated with SSc in all three major US ethnic groups (Blacks, Whites and Hispanics) in addition to Choctaw Native Americans (Reveille, Fischbach et al. 2001; Reveille 2006).

1.5.8 Potential therapies for SSc.

Treatment protocols for SSc disease, targeting activated T-cells, including cyclosporin A, tacrolimus, plasmapheresis and T-cell depletion, seem to have an ameliorating effect on skin tightness in patients with SSc (Dau and Callahan 1994; Morton and Powell 2000). In addition, a novel therapy may be the redirection of T-cell cytokine production to the Th1 subset, because increased expression and production of Th2 cytokines including IL-4, and IL-6 may be contributing to fibrosis in SSc disease. This approach has been tried in experimental systems with some success (Polisson, Gilkeson et al. 1996; Giacomelli, Cipriani et al. 2001; McGaha, Saito et al. 2001).

All the characteristics of SSc implicate a role for activated T-cells and their cytokines in the pathology of SSc, however the antigen(s) that induces the activation of these T-cells in SSc pathogenesis remains unknown.

1.6 GAMMA-DELTA TCR+ T-CELL BIOLOGY

1.6.1 Introduction to gamma-delta ($\gamma\delta$) TCR+ T-cells

There are two subsets of T-cells in the human body, characterized by the polypeptide T-cell receptor (TCR) present on their cell surface. The conventional T-cells, representing

approximately 95% of T-cells in the peripheral blood, are the $\alpha\beta$ TCR+ T-cells, bearing a disulfide-linked TCR consisting of the α - and β -chain. The $\gamma\delta$ TCR heterodimer, which may or may not be disulfide-linked, is expressed on a small proportion (less than 5%) of functionally mature T-cells in the peripheral blood, but are more frequent (up to 40%) in epithelial-lined mucosa and organs, including the skin, reproductive tract, lungs, and the GI tract (Ioannides, Itoh et al. 1987; Seki, Nanno et al. 1989; Ferrarini, Ferrero et al. 2002). The $\gamma\delta$ TCR+ T-cell subset has not been as well characterized as the $\alpha\beta$ TCR+ T-cells, and there still remains much to be learned about their biology and function in the immune system. Although both $\alpha\beta$ TCR+ and $\gamma\delta$ TCR+ T-cells play some similar roles in regulation of the immune response and control of infections, $\gamma\delta$ TCR+ T-cells may have unique features.

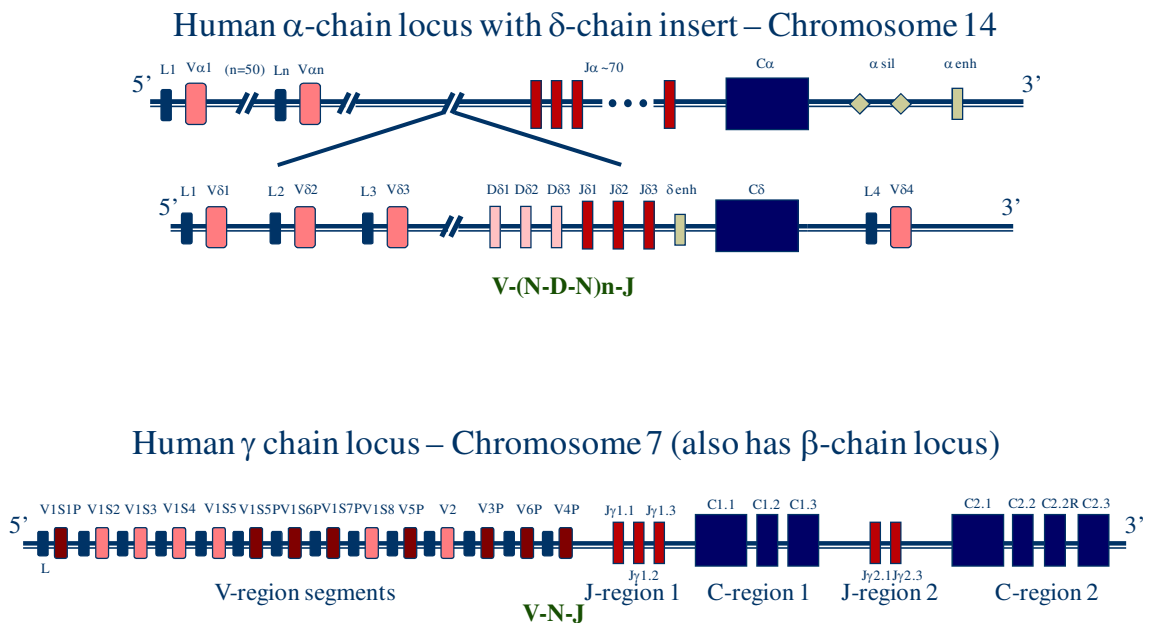


Figure 1 – Genetic locus of the human γ -chain and δ -chain showing the multiple gene segments

The $\alpha\beta$ TCR+ and $\gamma\delta$ TCR+ T-cell populations are comprised of large numbers of different T-cell clones, each expressing a distinct T-cell receptor that acts as a unique

molecular fingerprint of each T-cell clone. The TCR-dependent recognition of an antigen by a specific T-cell clone results in proliferation and clonal expansion, to facilitate an immune response against the inciting antigen. The chains of the TCR are composed of multiple regions, each coded for by multiple different gene segments in the germline DNA; the variable (V) region that contains a hypervariable (CDR3) region to facilitate antigen-specific binding, the junctional (J) region, the diversity (D) region unique to the β - and δ -chains, and the constant (C) region that contains the transmembrane domain (see figure 1) (Dariavach and Lefranc 1989; Lefranc, Chuchana et al. 1989; Lefranc and Rabbitts 1989). Interestingly, the δ -chain locus is within the α -chain locus, therefore, rearrangement of the α -chain results in a loss of the δ -chain genetic information within that T-cell. Hence, the rearrangement and expression of the $\alpha\beta$ TCR and $\gamma\delta$ TCR on T-cells is mutually exclusive, and no T-cell can express a functional $\gamma\delta$ TCR and $\alpha\beta$ TCR simultaneously (Joachims, Chain et al. 2006; Jones, Salio et al. 2007).

A major mechanism for the generation of diversity is the random recombination events occurring in the germline DNA of these gene segments, mediated by the lymphocyte-specific recombinase machinery, i.e. recombination activating proteins (RAG1/2), to form a functional chain that is expressed on the cell surface of each T-cell (Murray, O'Neill et al. 2006). In the context of the recombination process, random nucleotides are also inserted in the junction between the V-J or V-D-J segments of the hypervariable (CDR3) region, forming non-template dependent (N) regions to further increase the uniqueness of the TCR, a process catalyzed by the enzyme terminal deoxynucleotide transferase (tdt). The maximum theoretical number of $\alpha\beta$ TCR+ T-cells has been estimated to be approximately 10^{18} , while that of $\gamma\delta$ TCR+ T-cells has been estimated to be up to 10^{19} (Sakkas and Platsoucas 2004).

The actual size of the T-cell repertoire is significantly reduced due to elimination of the vast majority of precursor T-cells in the thymus during the selection processes. Nonetheless, the number of T-cell clones in the body is very large and sufficient to recognize practically most antigenic epitopes. Because of the significant size of the $\alpha\beta$ TCR and $\gamma\delta$ TCR repertoire, the probability of finding multiple identical copies of a TCR chain transcript within an independent sample of T-cells, by chance, is negligible. Therefore, the appearance of multiple identical copies may be the result of antigen-driven proliferation and clonal expansion of a T-cell clone in response to specific antigenic stimulation (see figure 2).

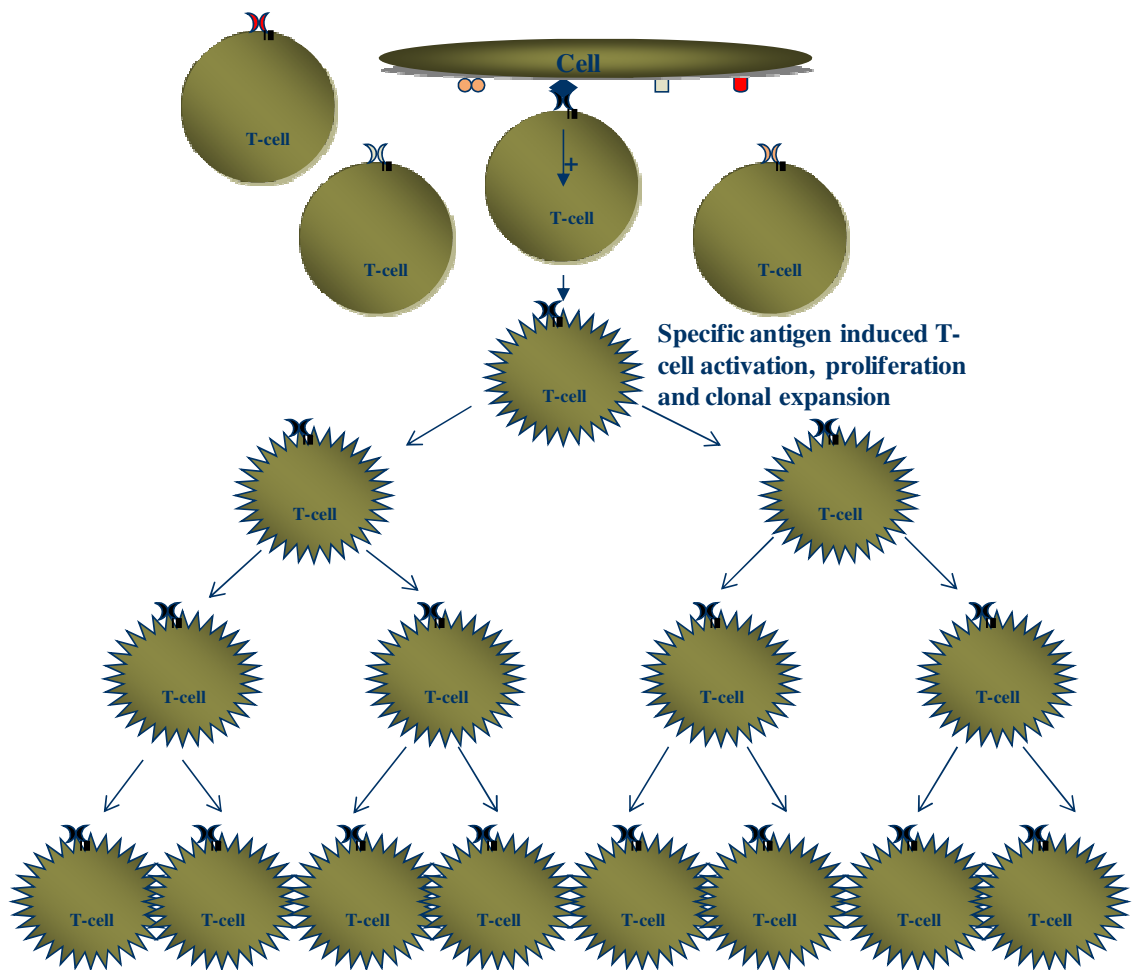


Figure 2 – Concept of antigen-driven clonal expansion of T-cells

Most of the $\alpha\beta$ TCR+ T-cells are self-MHC restricted during maturation in the thymus, and only recognize antigens as processed peptides presented in the context of MHC complexes on antigen-presenting cells (APCs). However, certain $\alpha\beta$ TCR+ T-cells as well as $\gamma\delta$ TCR+ T-cells can recognize lipids, carbohydrates, other small molecules, as well as peptides presented by CD1 in an MHC-unrestricted manner (Tanaka, Sano et al. 1994; Sieling, Chatterjee et al. 1995; Tanaka, Morita et al. 1995; Spada, Grant et al. 2000; Shin, El-Diwany et al. 2005). Recent evidence suggests that $\gamma\delta$ TCR+ T-cells do not require antigen processing by APCs, and may recognize epitopes on whole proteins without MHC-presentation, in a similar fashion to the immunoglobulin (Shin, El-Diwany et al. 2005).

The $\gamma\delta$ TCR+ T-cells are in relatively higher proportions in the reproductive tract, GI mucosa, skin, red pulp of the spleen, and during pregnancy, where they appear to have important immunologic functions. Emerging roles of $\gamma\delta$ TCR+ T-cells is also evidenced by ability to produce a variety of cytokines including IL-2, IL-3, IL-4, IL-5, GM-CSF, TNF- α/β , and IFN- γ (Aljurf, Ezzat et al. 2002). In addition $\gamma\delta$ TCR+ T-cells may function as professional antigen presenting cells, facilitating a direct interaction with $\alpha\beta$ TCR+ T-cells, to modulate their activity at sites of inflammation (Brandes, Willmann et al. 2005). $\gamma\delta$ TCR+ T-cells may function in wound repair and in surveillance of stressed and/or tumorigenic epithelial cells. $\gamma\delta$ TCR+ T-cells have direct cytotoxic activity and produce cytokines somewhat distinct from $\alpha\beta$ TCR+ T-cells, including connective tissue growth factor (CTGF), which may play an important physiologic role in maintenance of the epithelial surface integrity, but may also play a pathogenic role in a fibrotic disease such as systemic sclerosis (Varga and Bashey 1995; Sato, Nagaoka et al. 2000; Workalemahu, Foerster et al. 2003).

1.6.2 Function of $\gamma\delta$ TCR+ T-cells in disease pathogenesis

Although there are many published reports on the physiologic immune function of the still enigmatic $\gamma\delta$ TCR+ T-cells in maintaining health, emerging studies have implicated a role for this T-cell subset in the pathogenesis of different disease processes. Indirect evidence of the role of $\gamma\delta$ TCR+ T-cells in disease pathogenesis have been inferred by, 1) the presence of $\gamma\delta$ TCR clonally expansions in tissue lesions, peripheral blood or other bodily fluids (including bronchoalveolar lavage fluid from lungs, synovial fluid from rheumatoid arthritis) from patients, and 2) the presence of activated $\gamma\delta$ TCR+ T-cells infiltrates in the diseased lesion, bearing activation markers. Various investigators have reported the presence of clonal expansion of $\gamma\delta$ TCR+ T-cells in different disease processes. Sequence analysis of TCR transcripts revealed evidence of restricted V δ TCR chains in the peripheral blood from patients with systemic lupus erythematosus (SLE), while peripheral blood from healthy individuals showed a lack of restricted V δ TCR (Olive, Gatenby et al. 1994). Investigators also reported the involvement of $\gamma\delta$ TCR+ T-cells in the progression of IgA nephropathy to renal failure, evidenced by restricted diversity in $\gamma\delta$ TCR chains, after CDR3 spectratyping and sequencing, in the kidney biopsy lesions and peripheral blood lymphocytes from patients with IgA nephropathy, suggestive of antigen-driven oligoclonal expansion (Wu, Clarkson et al. 2001). In experimental mice, $\gamma\delta$ TCR+ T-cells were shown to be present in lesions from acute graft-versus-host disease (aGVHD), and the presence of $\gamma\delta$ TCR+ T-cells in the experimental animals correlated with disease-related mortality. Specifically, depletion of $\gamma\delta$ TCR+ T-cells resulted in increased survival in the mice, which was reversed with reconstitution of $\gamma\delta$ TCR+ T-cells in the animals (Maeda, Reddy et al. 2005).

Dornmair et al. (2004) first reported a molecularly defined $\gamma\delta$ TCR directly derived from an autoimmune tissue lesion, specifically a polymyositis lesion, as more direct evidence of $\gamma\delta$ TCR+ T-cell involvement in a disease process. This autoreactive monoclonal $\gamma\delta$ TCR was identified in destructive infiltrates of a lesion from a rare form of polymyositis, an autoimmune disease against skeletal muscle fibers (O'Hanlon, Messersmith et al. 1995). T-cells expressing the monoclonal $\gamma\delta$ TCR from the polymyositis lesion were shown to be cytotoxic against human skeletal muscle cell lines, including the human myoblast cell line and the TE671 rhabdomyosarcoma cell line (O'Hanlon, Messersmith et al. 1995; Dornmair, Schneider et al. 2004). Subsequent studies identified a cytosolic skeletal muscle antigen and the recognition of this autoantigen by the $\gamma\delta$ TCR was in the absence of antigen-presenting cells, suggesting an MHC-unrestricted, but CDR3-dependent recognition (Wiendl, Malotka et al. 2002; Dornmair, Schneider et al. 2004). Using a soluble form of this monoclonal $\gamma\delta$ TCR isolated from the polymyositis lesion, the investigators later identified cross-reactivity with an antigen from *E. coli* cells (Wiendl, Malotka et al. 2002; Dornmair, Schneider et al. 2004).

Chauhan et al. (2007), demonstrated reactivity of $\gamma\delta$ TCR+ T-cells isolated from peripheral blood of patients with Takayasu arteritis, with the 60kDa heat shock protein (hsp 60), which was not seen in $\gamma\delta$ TCR+ T-cells from normal or disease (SLE) controls (Chauhan, Singh et al. 2007). Takayasu's arteritis (TA) is a chronic inflammatory autoimmune disease of unknown etiology, characterized by intimal thickening, fibrosis, stenosis and aneurysm formation in the large elastic arteries, especially the aorta and its major branches. Studies have implicated a role for $\gamma\delta$ TCR+ T-cells in this disease, evidenced by; 1) increased numbers of circulating activated $\gamma\delta$ TCR+ T-cells, 2) a restricted

$\gamma\delta$ TCR repertoire in arterial lesions and peripheral blood of patients with TA, suggestive of an antigen-driven response, and 3) colocalization of $\gamma\delta$ TCR+ T-cells with hsp 60/65 (which are highly expressed in TA lesions compared to normal controls) in arterial lesions from TA patients (Seko, Minota et al. 1994; Seko, Takahashi et al. 2000). The $\gamma\delta$ TCR+ T-cells from peripheral blood of TA patients proliferated after stimulation with hsp 60. These hsp 60-responsive $\gamma\delta$ TCR+ T-cells showed increased cytotoxicity to the human aortic endothelial cell line compared to the total $\gamma\delta$ TCR+ T-cells population (Chauhan, Singh et al. 2007).

The $\gamma\delta$ TCR+ T-cell subset have also been implicated in SSc disease. There is evidence of $\gamma\delta$ TCR+ T-cells clonal expansion in peripheral blood of patients with SSc (Yurovsky, Sutton et al. 1994). These cells have been shown to be activated and accumulate in skin of SSc patients (Giacomelli, Matucci-Cerinic et al. 1998), as well as display a Th1 polarization (Giacomelli, Cipriani et al. 2001). In addition, $\gamma\delta$ TCR+ T-cells from SSc patients have increased adhesion to vascular endothelial cells *in vitro* (Kahaleh, Fan et al. 1999).

CHAPTER 2

HYPOTHESIS AND SPECIFIC AIMS

2.1 HYPOTHESIS

The $\gamma\delta$ TCR+ T-cell subset in patients with SSc may be involved in the initiation and/or propagation of SSc disease, possibly by a specific antigen-driven T-cell response directed against self or non-self antigens. The presence of clonal expansion of $\gamma\delta$ TCR+ T-cells is highly suggestive of such an antigen-driven activation, and proliferation of specific T-cell clones, and very likely is of pathological significance. These activated $\gamma\delta$ TCR+ T-cells may have effector functions of direct cytotoxicity and/or cytokines and chemokines production responsible for the recruitment of other inflammatory cells such as monocytes, to the SSc lesions. Therefore, the hypothesis tested in this study is that clonally-expanded $\gamma\delta$ TCR+ T-cells, in response to specific antigen(s), are present in skin biopsy and peripheral blood of SSc patients, and may play an important role in the initiation and/or propagation the SSc disease. Identification of the antigen(s) recognized by the $\gamma\delta$ TCR+ T-cells in lesions from SSc patients would provide important insights into the possible role $\gamma\delta$ TCR+ T-cells in the initiation and/or propagation of SSc disease.

2.2 SPECIFIC AIMS

The specific aims of the study are;

- 1) To expand on previous studies in our laboratory investigating the presence of clonally-expanded $\gamma\delta$ TCR+ T-cells in skin biopsies from patients with SSc of recent onset.
- 2) To expand on previous studies in our laboratory investigating the presence of clonally-expanded $\gamma\delta$ TCR+ T-cells in peripheral blood from patients with SSc of recent onset.
- 3) To develop an approach for the identification of the antigens recognized by the clonally-expanded $\gamma\delta$ TCR transcripts present in skin biopsies and peripheral blood of patients with SSc.
- 4) To determine whether clonally-expanded $\gamma\delta$ TCR transcripts in skin biopsies and peripheral blood of patients with SSc recognize the following putative SSc antigens (DNA topoisomerase I, centromere proteins, human cytomegalovirus, and human endothelial cell antigens)

CHAPTER 3 MATERIALS AND METHODS

3.1 CLONAL EXPANSION STUDY

3.1.1 *Samples*

Skin biopsies (5mm) from the leading edge of affected skin in the forearms of eight patients with SSc of recent onset (defined as less than 24 months from the appearance of clinically detectable skin pathology), who were followed up at the department of Internal Medicine at Temple University School of Medicine, by Dr Allen Myers, were used in this study. The removal of these skin biopsies from patients with SSc and their use in subsequent molecular biology studies were approved by the Institutional Review Board of Temple University School of Medicine. All patients and normal controls provided informed consent. Each patient sample was designated “SS-#” (for example, SS-1), and all fulfilled the criteria for classification of SSc described by the subcommittee for scleroderma criteria of the American Rheumatism Association (currently the American College of Rheumatology) Diagnostic and Therapeutic Criteria Committee. The specimens were snap-frozen and stored in liquid nitrogen and were stored until experimental use. Peripheral blood (20 ml) were also collected from the patients with SSc predominantly at the same time as the collection of the skin biopsy, in heparinized tubes and PBMC were isolated (as described below), and kept at -80 °C until experimental use.

3.1.2 *PBMC isolation*

Peripheral blood mononuclear cells (PBMC) were obtained from the peripheral blood samples using the Ficoll-Paque density cushion (following the manufacturer’s instructions). Briefly, blood samples were diluted in equal volume 1X phosphate buffered saline (PBS) without calcium or magnesium, with 2% heat inactivated fetal bovine serum

(FBS). The diluted blood samples were gently layered on 10 mL of Ficoll-Paque density cushion in a 50 mL centrifuge tube and centrifuged at 1350 rpm for 30 min at 4 °C to separate the peripheral blood into layers, specifically, a top layer of blood plasma, interface of mononuclear cells and platelets, i.e. the buffy coat, a 3rd layer of Ficoll-Paque density cushion, and bottom layer of erythrocytes. The interface was carefully transferred to a fresh 50 mL tube and washed three times with 1X DPBS with 2% FBS, by centrifuging at 1250 rpm for 5 min at 4 °C. The PBMC was quantified on a hemacytometer using Trypan Blue to exclude dead cells in the cell count.

3.1.3 Total RNA isolation

SSc samples were homogenized in Trizol® reagent (a monophasic solution of phenol and guanidine isothiocyanate) to facilitate total RNA isolation and maintenance of mRNA integrity. Specifically, 1×10^6 cells, for each SSc PBMC sample, or 5 mm of SSc skin biopsy were homogenized in 1 mL of Trizol® reagent, in RNase-free tubes, on ice. Two-phase separation was facilitated by addition of 200 µL of chloroform to the homogenate, followed by vigorous mixing by inversion. The samples were centrifuged at 13,000 rpm for 20 min at 4 °C, for separation of RNA into the aqueous phase, DNA and cellular debris at the interface and proteins in the bottom organic phase. 150 µL of the top aqueous phase, containing the total RNA, was transferred into a new RNase-free tube, being careful not to approach the interface, to avoid contamination. 600 µL of isopropanol was added to the RNA samples followed by mixing by multiple inversions, and incubation at -70 °C for at least 30 min to facilitate RNA precipitation. The samples were centrifuged at 13,000 rpm for 20 min at 4 °C to pellet the RNA, followed by washing with 500 µL of cold 75% ethanol and

centrifuged for 5 min at 13,000 rpm at 4 °C. The pellet was air-dried briefly and resuspended in DEPC-treated water.

Commercially-available total RNA, isolated from normal skin samples, including a 63 year old female donor, designated “NS-1” and a 20-week fetal female donor, designated “FNS-2” (Stratagene), were also analyzed for γ - and δ -chain clonal expansions.

3.1.4 cDNA synthesis and gene-specific PCR

One-step cDNA synthesis and TCR-chain gene-specific polymerase chain reaction (PCR) amplification was accomplished with the Invitrogen Superscript III® One step RT-PCR with a Platinum high fidelity Taq polymerase kit, in a single tube using gene-specific primers and target RNA. The kit used an enzyme mixture of Superscript III® reverse transcriptase (RT), a version of the Moloney murine leukemia virus (M-MLV) RT that had been engineered to reduce RNaseH activity and provide increased thermal stability, and high fidelity (low error) platinum Taq polymerase. The platinum Taq polymerase itself was an enzyme mixture of recombinant Taq polymerase, Pyrococcus GB-D polymerase (a proofreading enzyme with a 3' to 5' exonuclease activity to increase the fidelity of Taq polymerase by 6-fold and allow amplification of longer targets), and platinum Taq antibodies that block the activity of Taq polymerase at ambient temperatures. The Taq antibodies were designed to get denatured at 94 °C and restore Taq polymerase activity, thereby providing an automatic “hot-start” for enhanced PCR yields, and fidelity. The 2X reaction mix of the kit was a proprietary buffer system optimized for reverse transcription and PCR amplification with Mg²⁺ (2.4 mM MgSO₄), dNTPs (0.4 mM each) and stabilizers.

The RT-PCR cocktail was made in a nuclease-free tube on ice, containing nuclease-free water (18.5 μ L), RNaseOUT™ inhibitor (0.5 μ L), 2X reaction mix (25 μ L), forward and

reverse TCR chain gene-specific primers (5 μ M each), RNA sample (2.5 μ L), and the enzyme mix (5 U/ μ L).

TABLE 2 – PRIMERS USED IN RT-PCR PROTOCOL FOR CLONAL EXPANSION STUDY

IDENTITY	PRIMER SEQUENCE
<i>Sense</i>	
V γ I (V γ 1.5)	GGTGGGCCCTACTGGTGC
V γ I (V γ 1.2)	ATGCAGTGGGCCCTAGCG
V γ II (V γ 9)	CTGTCACTGCTCCACACATC
V δ 1	GTGTGGCCCAGAAAGGTTACT
V δ 2	CATTGAGTTGGTGCCTGAA
<i>Antisense</i>	
C γ	GTGAGCTGCAGCAGTAGAGTA
C δ	CTTGGATGACACGAGATTTATT

The thermal cycler conditions was initiated by one cycle at 50 °C for 30 min for cDNA synthesis followed by gene-specific PCR amplification, specifically, pre-denaturing at 94 °C for 2 min and 30 cycles of 94 °C for 1 min for denaturing, 55 °C (for V δ chains) or 59 °C (for V γ chains) for 2 min for annealing, and 72 °C for 3 min for extension. After 30 cycles, a final extension at 72 °C for 10 min completed the amplification.

The PCR products were separated by size on a 1% agarose gel in 1X TAE with 5 μ L ethidium bromide to visualize the DNA under ultraviolet light. The appropriate bands were excised from the gel and the PCR products were purified from the gel using the Q-biogene GeneClean® kit, following the manufacturer’s instructions. The gel piece containing the PCR products of interest was transferred to a spin column, and melted in 500 μ L of sodium iodide (NaI)/glassmilk solution at 55 °C for 5 min, followed by centrifugation at 13,000 rpm for 10 sec at room temperature to pellet the glassmilk bound to the DNA onto the column. After three washes using the kit’s New wash solution reconstituted in 100% ethanol, and air-drying the pellet, the cDNA was eluted from the glassmilk pellet by the addition of nuclease-

free water (10 μ L) and centrifugation at 13,000 rpm for 1 min into pre-labeled collection tubes.

3.1.5 TOPO® TA cloning of PCR products and sequencing

The Invitrogen TOPO® TA cloning kit facilitated the quick cloning of Taq polymerase-amplified PCR products, for direct insertion of Taq polymerase-amplified PCR products into the TOPO® cloning vector. The cloning vector (pCR2.1 TOPO®) was a 3.9 kB linear vector, with a single 3' thymidine base overhang (on each end) as well as a covalently attached topoisomerase enzyme bound to the vector. Since Taq polymerase had a non-template dependent terminal transferase activity that added a single deoxyadenosine base, during PCR amplification, to the 3' ends of PCR products, the linearized TOPO® vector, with its 3' deoxythymidine residues facilitated efficient ligation of the PCR product to the vector, without the need for a ligase enzyme. The topoisomerase I, isolated from the Vaccinia virus, subsequently bound to duplex DNA at specific sites and cleaved the phosphodiester backbone after 5' CCCTT 3' in one strand (of the vector), releasing energy that was conserved by formation of a covalent bond between the 3' phosphate of the cleaved strand and a tyrosine residue (Tyr 274) of topoisomerase I. The phosphor-tyrosyl bond between the DNA and the enzyme was subsequently attacked by the 5'-OH of the original cleaved strand, reversing the reaction and releasing topoisomerase I.

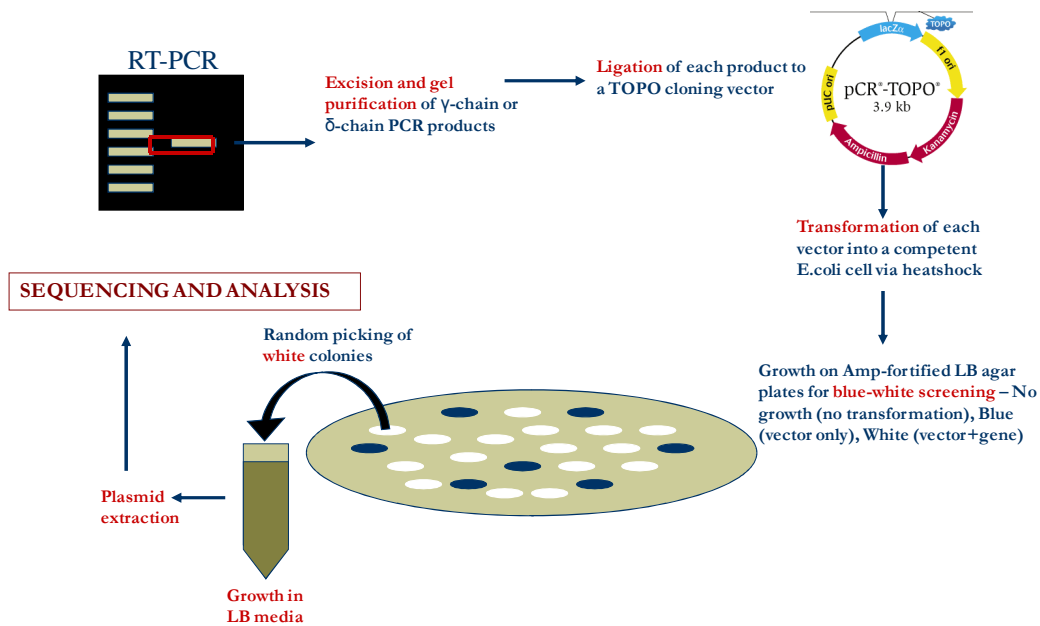


Figure 3 – Summary diagram of cloning procedure of the cDNA copies of isolated TCR transcripts

After ligation of the transcripts to the TOPO® vector, the vector was used to transform chemically-competent One-shot® TOP10 *E. coli* cells (Invitrogen). A salt solution was added to the cloning reaction to increase the number of transformants by 2-3 fold. Specifically, the salt solution allowed for longer incubation times by preventing DNA topoisomerase I from re-binding and potentially nicking the DNA after ligating the PCR products and dissociating from the DNA. Transformation of the One-shot® TOP 10 *E. coli* cells was accomplished by addition of 2 μ L of the TOPO® cloning vector containing the gene insert, to the cells, and incubation on ice for 20 min. This was followed by a heat-shock of the *E. coli* cells at 42 °C for 30 sec and a shaking incubation in 250 μ L of SOC medium (GibcoBRL) on a shaker at 200 rpm for 1 h at 37 °C.

The TOPO® cloning vector contains components of the *lac* operon, which codes for the β -galactosidase enzyme that cleaves its substrate, lactose into glucose and galactose, providing an energy source for the bacteria. The *pLac* (*Lac* promoter) gene is located at the

start site, followed by the *lacZa* gene (encodes β -galactosidase), and the ampicillin antibiotic-resistance gene. The *lacZa* gene contains the insertion site for the PCR products, therefore effective insertion results in a lack of expression of functional *lacZa* and ultimately no production of the β -galactosidase enzyme. Transformation of the One-shot® Top10 *E. coli* cells facilitated cloning of the PCR transcripts and selection of transformed *E. coli* cells by “blue/white” screening. Specifically, the transformed *E. coli* cells, after incubation in SOC medium, were spread onto Luria-Bertani (LB) agar plates, fortified with the antibiotic ampicillin (100 μ g/mL), and a non-inducing chromogenic substrate, X-gal (100 μ g/mL), similar to lactose, (and inducing β -galactosidase substrate), which yielded a blue precipitate upon cleavage by β -galactosidase.

An 18 h incubation at 37 °C allowed for growth of the *E. coli* colonies that were successfully transformed with the TOPO® vector on the fortified LB agar to facilitate blue-white screening. Colonies that produced the blue precipitate product of X-gal, showed the presence of a functional *lacZa* gene in the cloning vector and a lack of presence of the PCR product insert in the vector. Therefore white colonies, which lacked a functional *lacZa* gene due to interruption by the PCR product insert, were picked and used to inoculate LB media (5 mL), for bacterial growth and plasmid amplification, after an 18 h incubation in a shaker at 200 rpm at 37 °C (see figure 3).

In some rare instances, a “heat-shock artifact” may occur during transformation of the chemically-competent Top 10 *E. coli* cells with the plasmid containing the TCR chain insert. Specifically, there is a possibility that a cloned *E. coli* cell may have a shorter lag period after heat shock and replicate prior to spreading of the cells on LB agar plates. This results in

the presence of two identical transcripts in the sampled TCR-chain gene population, which is not a reflection of a physiologic clonal expansion.

3.1.6 Sequencing and sequence analysis

Plasmids with each TCR chain transcript clone were extracted using the Eppendorf Fastplasmid® mini prep kit, following manufacturer's instructions. A sequencing PCR reaction was performed, using the Applied Biosystems Premix solution that contained the fluorescently-labeled terminal dideoxynucleotides, polymerase enzyme, and buffer. The sequencing products were purified using the Amersham Microspin columns, vacuum-dried and resuspended in Hi Di formamide (Applied Biosystems). The resuspended products were incubated in a denaturing 90 °C water bath for 6 min, followed by an immediate ice-chill to prevent renaturing of the strands. The products were loaded onto 96-well plates and the sequence of the transcript products read on the automated 3130 Applied Biosystems 4-capillary genetic analyzer, with separation of the nucleotides facilitated by the Applied Biosystems POP 7™ polymer. The sequence raw data were obtained from the manufacturer software. Predicted amino acid sequences of the transcripts were analyzed using the ExPASy proteomics website, and the CDR3 regions (end of the V-region, N-region, and beginning of J-region) of the different transcripts were quantified.

Statistical analysis was done by Dr. John Gaughan, a statistician at Temple University School of Medicine, using binomial distribution. The binomial distribution, which is the basis for the binomial test of statistical significance, is a discrete probability distribution that facilitates the determination of the probability, p , that the number of observed multiple identical transcripts were significant, within the independent sampling of transcripts. This was tested against two potential outcomes (alternative hypotheses) that were

generated based on results obtained by using the same methodology of cloning and sequencing of V γ - and V δ -chain TCR transcripts for PBMC samples from normal donors which showed the presence of unique transcripts in most cases, with some “doublets” (2 identical transcripts) and even rarer “triplets” (3 identical transcripts). In other words, statistical significance by binominal distribution analysis was determined by comparing the observed transcript frequency (x/n) from samples of patients with SSc to non-statistically significant outcomes of polyclonal expansion, i.e. 1/n and 2/n, where n is the number of total productively-rearranged TCR-chain transcripts analyzed and x is the number of multiple identical transcripts. This was based on the hypothesis that 1/n and 2/n were polyclonal expansions as seen in normal PBMC control studies and therefore not statistically significant in an antigen-driven T-cell-mediated disease process. The presence of multiple identical transcripts and a p<0.05 value was considered to be statistically significant.

3.2 T-CELL RECEPTOR EXPRESSION

3.2.1 Full length T-cell receptor construction

Full-length transcripts for the V γ 9, and V δ 2 TCR isolated and sequenced from a systemic sclerosis patient (SS-16) and a commercially available normal skin total RNA sample, designated NS-1 were constructed for subsequent expression studies. The clonally-expanded transcripts in these patients were the V γ 9, and V δ 2 TCR subset and not the V γ I, and V δ 1 TCR subset, and therefore the V γ I, and V δ 1 TCR transcripts were not included in subsequent experiments.

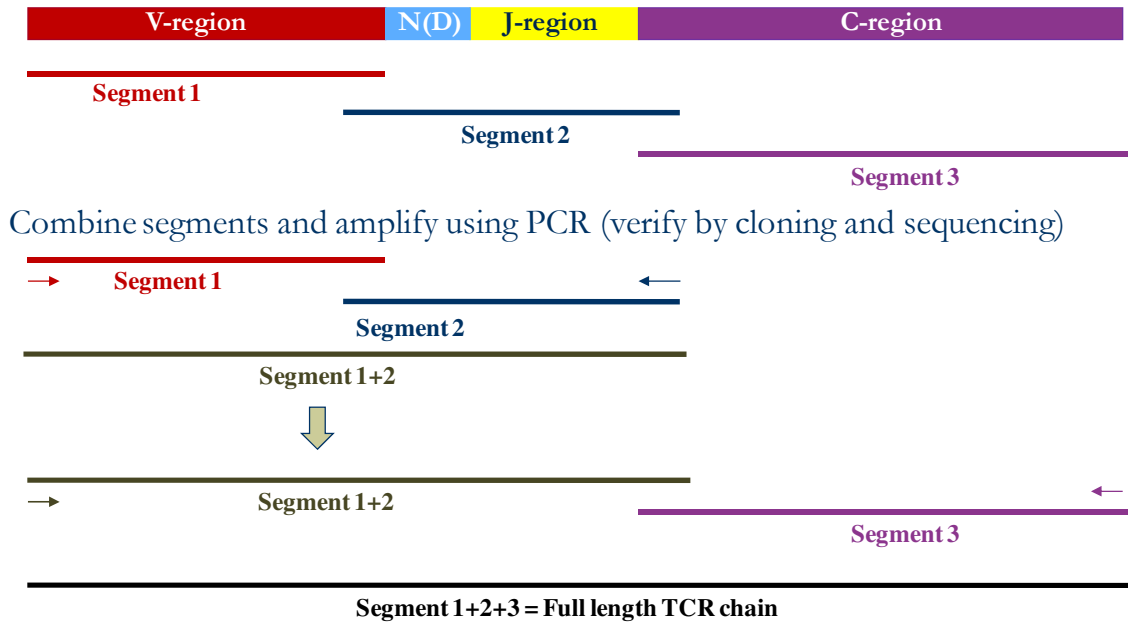


Figure 4 – Summary of full length construction of the TCR V γ - or V δ -chain

The full length copies of the transcripts were constructed by designing primers to amplify three overlapping segments spanning the entire full-length transcript followed by an overlapping PCR to combine the segments (see figure 4). The first segment i.e. segment 1, began from the initiating methionine (ATG) of the V-region to the end of the V-region and was cloned from normal PBMC. The second segment, (segment 2), contained the CDR3 region of the TCR of interest i.e. it extended from the end of the V-region (overlapping with segment 1 by approximately 6 amino acid residues), included the N-region (D-region in the case of δ -chain) and J-region, and contained the first 8 amino acids of the C-region. This segment was cloned from plasmids already containing inserts of the TCR-CDR3 region of interest for each of the SSc patient samples. The third segment, (segment 3) began from the beginning of the C-region to its stop codon (TAA), without including the poly-A tail of the transcript (a requisite of subsequent expression protocols) and was cloned from normal PBMC. The three segments of the full-length transcript overlapped sufficiently with each

other to facilitate a subsequent overlapping PCR. Each segment was verified qualitatively by size on a 1% agarose in 1X TAE gel and quantitatively by gel purification, cloning and sequencing. Then, a subsequent two-step overlapping PCR was performed to construct the final full-length transcript. Specifically, segment 1 was ligated with segment 2 and the PCR product was verified qualitatively and quantitatively as before, followed by an overlapping PCR of the segment 1+2 product with segment 3 (see figure 4). The generated full length copies of the γ - and δ -chain TCR transcripts were verified by cloning and sequencing.

3.2.2 Generation of the expression vectors

The full length copies of TCR transcripts, isolated from the patient with SSc (SS-16) and the normal skin sample (NS-1), were expressed in the β -chain negative J.RT3-T3.5 mutant Jurkat T-cell line, using the murine stem cell virus (MSCV) system, specifically, the Clontech retroviral expression system (see figure 5).

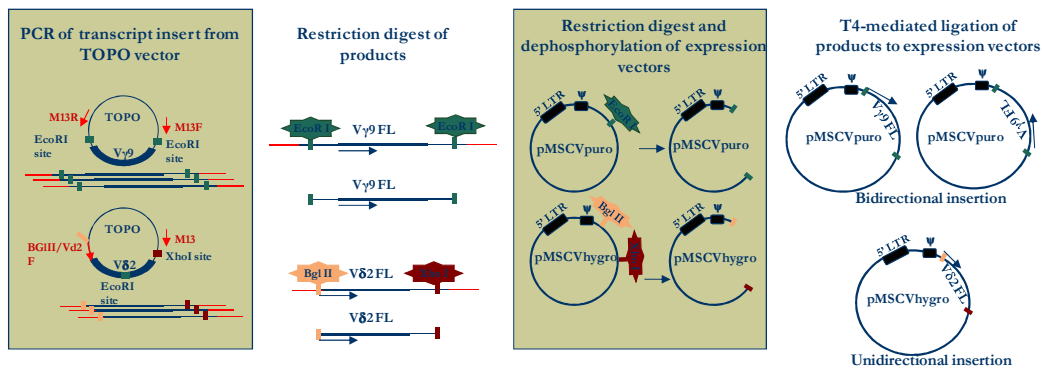


Figure 5 – Summary of insertion of transcripts to expression vector plasmid

Prior to expression, a restriction site check of the full length copies, using the online program NEBcutter, was performed to determine the choice of restriction enzymes. Specifically the restriction site for the enzyme had a unique location only in the multiple cloning site of the vector plasmid and was not present in the transcript. The MSCV vectors were derived from the Murine Embryonic Stem Cell Virus (MESV) and the LN retroviral

vectors. Upon transfection into a packaging cell line, the pMSCV vector would transiently express, or integrate and stably express a transcript containing the extended viral packaging signal Ψ , the antibiotic-resistance gene, and the TCR chain gene of interest. The 5' long terminal repeat (LTR) was specifically designed to facilitate stable, high-level gene expression in hematopoietic and embryonic stem cells. The LTR was from the murine stem cell PCMV virus, and was modified with several point mutations and a deletion that were shown to enhance transcriptional activation and prevent transcriptional suppression, resulting in high-level constitutive expression of a target gene the cell lines. The murine phosphoglycerate kinase (PGK) promoter (P_{PGK}) controlled the expression of the antibiotic resistance gene for antibiotic selection in eukaryotic cells. The expression vector plasmid also contains the pUC origin of replication and *E. coli* ampicillin-resistance gene for propagation and antibiotic selection in bacteria.

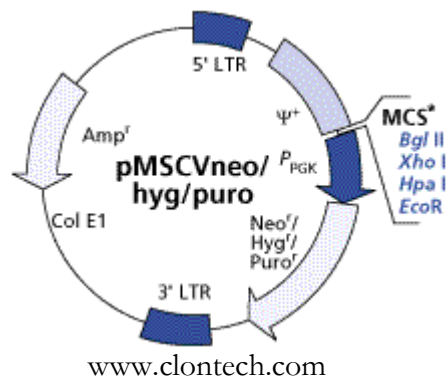
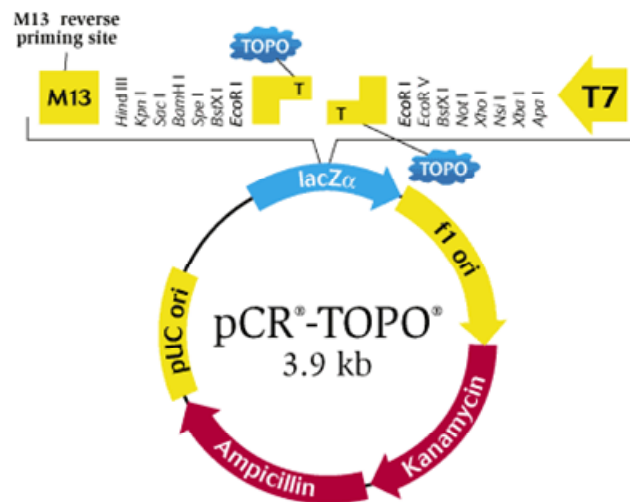


Figure 6 – MSCV expression vector plasmid

PCR amplification of transcripts from the TOPO® cloning vector (see figure 6), with products containing determined restriction site, was performed. The restriction enzyme site had to be unique and present only once in the multiple cloning site of the expression vector as well as present once in the TOPO® vector near the transcript insertion site. This limited the choices to *Bgl II*, *EcoR I*, and *Xho I* restriction enzymes. For the V γ 9 chain, M13

forward and M13 reverse primers were used in the PCR to facilitate amplification of both *EcoR I* restriction sites (see figure 7) flanking the multiple cloning site of the TOPO® cloning vector, containing the TCR chain. The Vδ2 chain required some modification because it contained an *EcoR I* restriction site within the gene. Hence, a novel *Bgl II* site had to be introduced into the PCR product, preceding the gene. Specifically, the forward primer was designed to contain a *Bgl II* site juxtaposed to the beginning nucleotides of the Vδ2 gene. The reverse primer was the M13 forward primer with its priming site after the multiple cloning site of the TOPO® vector, facilitating the use of *Bgl II* and *Xho I* for the unidirectional insertion of the Vδ2 chain transcript into the expression vector.



www.catalog.invitrogen.com

Figure 7 – pCR2.1 TOPO TA cloning vector showing restriction enzyme sites flanking gene insertion position

Restriction digest of transcripts from PCR products facilitated ligation to the Clontech expression vectors, specifically the hygromycin vector and puromycin vector. The digestion protocol involved a cocktail of nuclease free water, 2 µL digest buffer, 0.2 µL bovine serum albumin (BSA), Template DNA (7-10 µL), 1µL enzyme (or 0.5 µL of double digest enzymes) followed by incubation at 37 °C for 1-3 h, and inactivation 65 °C for 15 min.

The expression vectors were digested at the multiple cloning sites with the same enzymes as the PCR products, as described above, followed by dephosphorylation of the nicked ends of the expression vectors to prevent relegation of the plasmid. The dephosphorylation protocol involved the cocktail of nuclease free water, 10X buffer, 10 μ L vector, 1 μ L shrimp alkaline phosphatase enzyme and incubation at 37 °C for 1 h, followed by enzyme inactivation 65 °C for 15 min. The digested PCR products were ligated to the digested expression vectors, mediated by T4 ligase. The ligation protocol involved a cocktail of nuclease free water, 5 μ L Clonables® reaction solution containing T4 ligase, the digested PCR product of the TCR chain gene insert, and 1 μ L of dephosphorylated vector, with incubation at room temperature for 10 min.

For the V γ 9 chain, the *EcoR I* restriction enzyme was used, because there was only a single site in the expression vector, which was located within the MCS, while there were two *EcoR I* sites that flanked the transcript within multiple cloning site of the TOPO® vector, and was not present within the gene of interest. However, the insertion was bi-directional in the expression vector, with some transcripts in the correct forward orientation to produce a functional gene product and others in the reverse direction, resulting in a prematurely terminated false product. The V γ 9 chain gene inserts were ligated to the puromycin expression vector, containing the puromycin resistance gene.

For the V δ 2 chain, the *EcoR I* restriction enzyme could not be used because the δ -chain sequence contained an *EcoR I* site, as determined by NEBcutter program, in the junctional region. Therefore, to facilitate ligation, a novel *Bgl II* site, which was not present in the TOPO® vector, was inserted by PCR to the beginning of the V δ 2 gene during PCR amplification of the full length transcripts. The *Xho I* site was located downstream of the

TOPO® multiple cloning site, therefore a double digest of the PCR products, with *Bgl II* and *Xho I* restriction enzymes facilitated a unidirectional T4-ligase mediated ligation and insertion into the similarly digested hygromycin expression vector, with the hygromycin resistance gene.

Cloning of the ligated products into One-shot® TOP10 *E. coli*, from the TOPO® TA cloning kit, was performed as previously described followed by sequencing and sequence analysis. This enabled selection of clones with complete copies the appropriate γ - or δ -chain TCR genes, in the correct orientation and without any aberrant bases or premature termination.

3.2.3 Transfection of the packaging cell line

The RetroPack™ PT67 cell line is an NIH-3T3 immortalized mouse fibroblast cell line, and was provided in the Clontech retroviral expression kit. This packaging cell line had been modified to have stable integration of the critical retroviral structural genes, *gag*, *pol*, and *env* necessary for particle formation and replication, into its genome. Introduction of the expression vector plasmids into the packaging cell line resulted in production of high-titer, replication-incompetent infectious virus particles to infect target cells and transmit the gene of interest. The process of separately introducing and integrating the structural genes into the packaging cell line minimized the chances of producing replication-competent virus due to recombination events during cell proliferation. RetroPack™ PT67 cells packaged retrovirus particles with a dualtropic envelope, 10A1, that recognized receptors on most mammalian cells, specifically, the amphotropic retrovirus receptor, RAM1 (Pit2), or the GALV (Pit1) receptor.

Transfection of the packaging cell line, RetroPack™ PT67, with the plasmid vectors containing the full length TCR chain genes of interest (either Hygromycin+ δ 2 or Puromycin+ γ 9) was accomplished using the calcium phosphate-mediated transfection of eukaryotic cells protocol; specifically the Promega Profection® kit. It has been established that cellular uptake of DNA is markedly enhanced when DNA is presented as a co-precipitate with calcium phosphate. Specifically the insoluble precipitate attaches to the cell surface and is endocytosed. In preparation for transfection, the packaging cells were grown to about 60% confluency in 100mm² tissue culture plates in Dulbecco's Modified Eagle Medium (DMEM) fortified with L-glutamine, 10% FBS, penicillin/streptomycin, and sodium pyruvate. A mock transfection was done to generate a negative control using the hygromycin and puromycin vectors only, without any γ - or δ -chain TCR genes. Three hours before transfection, fresh DMEM complete media was added to the cells and all transfection reagents were brought to room temperature. Under a sterile hood, the plasmid vector (15 μ g) was diluted with nuclease-free water then mixed in 62 μ L of calcium chloride. The mixture was added drop-wise into 500 μ L of 2X HBS while slowly vortexing, and left to incubate at room temperature for 30 min, to facilitate precipitation. The precipitate mixture was then added drop-wise unto the packaging cells, swirling to mix with each drop. The cells were incubated overnight at 37 °C with 5% CO₂, and the next day, a DMSO shock was performed to increase transfection efficiency. Briefly, the media was removed from the cells and replaced with 10% DMSO in 1X PBS (DMSO shock solution) followed by an incubation at room temperature for 3 min. The shock solution was then removed and replaced with fresh DMEM complete media and the cells were allowed to grow for 1-2 days. The cells containing the expression vectors were selected using DMEM selection media (DMEM

complete media fortified with 450 µg/mL of hygromycin B or 3 µg/mL of puromycin) to produce stable cell lines that produce retrovirus containing TCR chain gene of interest. After 2 weeks of selection, and re-growth of the cells, the media was replaced with complete media for harvesting the retrovirus in preparation for transduction.

3.2.4 Retroviral transduction of the mutant Jurkat T-cell line

The retrovirus-containing supernatants produced by the transfected packaging cell lines were filtered in 0.45 µM CA syringe filters (Millipore) and used in transducing the β-chain negative J.RT3-T3.5 mutant Jurkat T-cell leukemia cell line. Different combinations of Vγ9 and Vδ2 were used, including a combination of the most clonally expanded Vγ9 and Vδ2 chain from SS-16 skin biopsy. The mutant Jurkat T-cells were grown in a cell suspension in 45 mm² filter-cap flasks in Roswell Park Memorial Institute (RPMI) culture media fortified with penicillin/streptomycin, 10% FBS, sodium bicarbonate, sodium pyruvate, for three days because transduction efficiency is increased in actively dividing cells. On day of transduction, the cells were resuspended and counted. 1x10⁶ cells/mL was used for each transduction experiment, including the different Vγ9/Vδ2 experimental combinations, an untransduced control and the mock vector only (hygromycin+puromycin) control. The cells were pelleted by centrifugation at 250 g, 4 °C, for 5 min. Equal proportions of puromycin+γ9 and hygromycin+δ2 retrovirus supernatant (or the vector only control) were combined and used to resuspend the mutant Jurkat T-cells pellet to a volume of 1 mL, with 8 ug/mL polybrene to increase transduction efficiency. The cell suspension was transferred to 24-well fibronectin-coated plates (Retronectin™), in appropriately labeled wells. The plate was centrifuged at 1800 rpm, 32 °C, for 10 min to facilitate interaction between the cells, retroviral vectors and fibronectin fragments.

Following 5 h incubation at 37 °C, 5% CO₂ in the retroviral supernatants, 1 mL of fresh RPMI culture media was added and the cells were incubated overnight. The next day, the process was repeated after cells were harvested using cell dissociation buffer (GIBCO). The cells were grown for three days, followed by selection of cells transduced with both puromycin-V γ 9 and hygromycin-V δ 2, using both hygromycin (400 μ g/mL) and puromycin (0.5 μ g/mL) in RPMI culture media for three weeks. Dead cell debris was removed using the Ficoll-Paque density cushion, as previously described.

Hygromycin B is an aminoglycoside antibiotic produced by *Streptomyces hygroscopicus* that inhibits protein synthesis by interfering with translocation and causing mistranslation at the 70S ribosome. The gene that confers resistance to hygromycin is the hygromycin phosphotransferase (*hpt*, *hph* or *aphIV*) gene, originally derived from *Escherichia coli*, and codes for the kinase, hygromycin phosphotransferase (HPT), which inactivates hygromycin B by phosphorylation. Puromycin dihydrochloride is an aminonucleoside antibiotic produced by *Streptomyces alboniger*, that specifically inhibits peptidyl transfer on both prokaryotic and eukaryotic ribosomes causing premature chain termination during translation. In addition, part of the puromycin resembles the 3' end of the aminoacylated tRNAs and therefore it enters the A site and transfers to the growing chain, causing premature chain release. Puromycin also inhibits protein import into mitochondria by interfering with an intramitochondrial ATP-dependent reaction. Resistance to puromycin is conferred by the *pac* gene, encoding a puromycin N-acetyl-transferase (PAC) that is found in a *Streptomyces* strain.

3.2.5 Verification of expression of TCR chain in transduced cells

Expression of the transduced V γ 9/V δ 2 genes was analyzed, as before, using the Invitrogen Superscript III® One step RT-PCR with high fidelity platinum Taq polymerase.

In addition, surface expression of the transduced V γ 9V δ 2 TCR was verified by flow cytometric analysis. Specifically, the cells were washed twice in FACs buffer (2% FBS in 1X DPBS without Ca²⁺ or Mg²⁺) in 5 mL snap-cap Falcon tubes, and centrifuged at 1250 rpm for 5 min at 4 °C. After decanting the final wash buffer from the cells, 2 μ L of the appropriate antibodies were added to the cells (including single color compensation, and isotype controls), followed by incubation on ice and in the dark for 30 min. The cells were washed twice in wash buffer, with centrifugation, as previously described. After decanting the final wash supernatant, the cells were resuspended on 350 μ L of wash buffer and the surface expression analyzed using the Cyan flow cytometer at the Wistar institute in Philadelphia. Positive cells for the surface $\gamma\delta$ TCR were selected by flow cytometry and re-cultured in preparation for the antigen recognition study, also at the Wistar institute. Analysis of the fluorescence data from surface TCR expression was performed on the Flow-Jo software.

3.3 ANTIGEN RECOGNITION STUDY

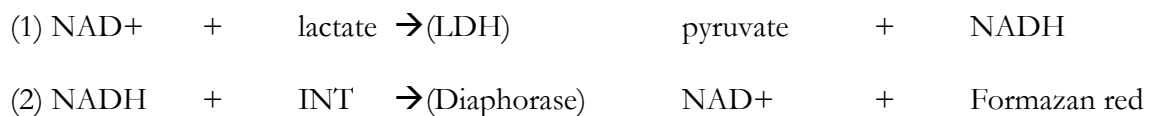
3.3.1 Introduction to $\gamma\delta$ TCR+ T-cell antigen recognition and rationale

The different transduced Jurkat T-cell lines were stimulated with putative SSc antigens and analyzed for recognition. The different antigens studied were soluble antigens i.e. recombinant whole proteins of DNA topoisomerase I, centromere proteins A and B, heat shock proteins (hsp 90 and the estrogen-regulated hsp 27), the viral lysate of human cytomegalovirus (hCMV) and human endothelial cell surface antigens, specifically, the human umbilical vein endothelial cell (HUVECs) and human lung microvascular endothelial cell lines (HLMVECs). Possible recognition of the human endothelial cell surface antigens

was analyzed using a cytotoxicity assay, with the transduced Jurkat T-cells as effectors and the human endothelial cells as the target cell lines, and cytotoxicity was assessed by quantification of lactate dehydrogenase release. Recognition of the soluble antigens was assessed using an intracellular fluorescent calcium flux assay. The positive control stimulations for the calcium flux assay were soluble anti-CD3 antibody (OKT3), and isopentenyl pyrophosphate (IPP) stimulation of the V γ 9V δ 2 transduced TCR+ T-cells.

3.3.2 Assessment for endothelial cell cytotoxicity

Endothelial cell cytotoxicity was analyzed using the Promega CytoTox 96® assay kit. This assay is a colorimetric alternative to chromium release cytotoxicity assays and it quantitatively measures lactate dehydrogenase (LDH) release, a stable cytosolic enzyme that is released upon cell lysis (similar to chromium release in radioactive assays). Released LDH in the culture supernatant is measured with a 30 min, coupled enzymatic assay, which results in the conversion of a tetrazolium salt (INT) into a red formazan product. The amount of red color formed is proportional to the number of lysed cells. The general chemical reactions of the assay are



Visible wavelength absorbance data are collected using a standard 96-well microplate reader.

To setup the assay (see figure 8), the cells were counted to generate the different effector:target ratios, (0.5:1, 5:1, 20:1) used in the study. The controls for the cytotoxicity assay were i) the effector cell alone to correct for effector cell spontaneous release of LDH, ii) target cell alone to correct for target cell spontaneous LDH release, iii) maximum (100%) target cell LDH release, using a lysis solution, iv) volume correction control, after addition of

the lysis solution to the maximum LDH release control, v) culture medium background control, because two factors in culture media contribute to background of this assay, including phenol red and LDH from the serum. To decrease the culture medium background without affecting cell viability, the serum concentration in the culture media was decreased from 10% to 5% decrease serum. The effector cells at each cell concentration for the ratios, in a final volume of 50 μ L, were added to the appropriate experimental wells and control wells (see figure), in triplicate. 50 μ L of media alone were added to the appropriate control wells (specifically the target only wells, and medium only wells). Then 50 μ L of target cells (20,000 endothelial cells i.e. HLMVEC or HUVECs) or media alone were added to the appropriate experiment and control wells, bringing the volume in the wells to 100 μ L.

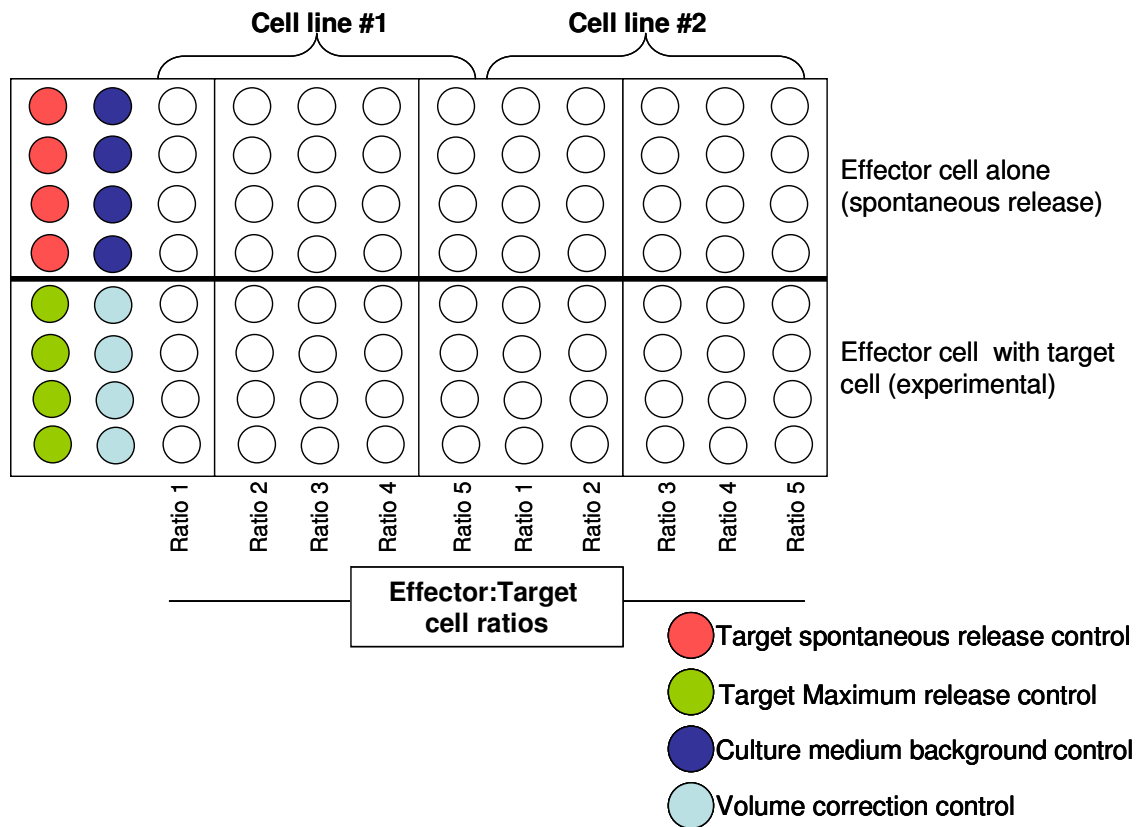


Figure 8 – Set-up of cytotoxicity assay

The target cell number was determined in a preliminary experiment testing the maximum LDH release using the lysis solution of a serial dilution of target cell numbers to determine the optimal number for the cell-mediated cytotoxicity experiment. The plate was centrifuged at 250 g for 4 min (to ensure contact of the cells).

The plate was incubated for 5 h at 37 °C, in 5% CO₂. Approximately 45 min before the end of the incubation, 10 µL of 10X lysis solution was added to the volume control and the maximum lysis control wells. After incubation, the plates were centrifuged at 250 g for 5 min to pellet cells and 50 µL of the supernatant was carefully and immediately harvested and transferred to a fresh 96-well ELISA plate using a multichannel pipette. The light-sensitive assay buffer (12 mL) was added to the substrate solution in the dark. After thorough shaking to mix, 50 µL of the reconstituted substrate solution was added to the wells using a multichannel pipette and the plate was incubated in the dark for 20-30 min at room temperature. The stop solution (50 µL) was then added to terminate the reaction and the plate was read at an absorbance of 490 nm. To correct for the culture medium background, the average culture medium background value was subtracted from all absorbance values of the experimental wells, target cell spontaneous release, and effector cell spontaneous release. Also, the average volume correction control value was subtracted from the target cell maximum LDH release control. Then, the percent cytotoxicity was calculated using the following formula;

$$\% \text{ cytotoxicity} = \frac{(\text{Experimental} - \text{Effector spontaneous} - \text{Target spontaneous})}{(\text{Target maximum} - \text{Target spontaneous})} \times 100$$

3.3.3 Assessment for antigen recognition via measuring intracellular calcium

Release of calcium from intracellular stores is one of the earliest events that occur in response to T-cell stimulation with antigens, facilitating activation of calcium-sensitive enzymes in the T-cell cytoplasm, required in the activation of the T-cell.

The transduced cell line expressing the most clonally-expanded $\gamma\delta$ TCR from the SS_c patient (SS-16) were used in the study. Control cell lines were the transduced cell lines expressing the mock (vector only) control, or the non clonally-expanded V γ 9V δ 2 TCRs isolated from SS-16. The intracellular calcium protocol used was the Invitrogen Fluo-4 NW (No wash) assay kit (based on Fluo-4 AM dye), modified for flow cytometry. Fluo-4 AM, is a fluorescent calcium indicator widely used for intracellular measurement of agonist or antagonist-mediated calcium signaling. The advantages of this fluorescent dye include, high sensitivity, large fluorescence increase upon binding to calcium and visible wavelength excitation. The Fluo-4 NW kit required neither a wash (therefore lower variability due to potential loss of cells) nor a quencher dye (shown to interact negatively with some receptor systems), and therefore achieved larger increases in fluorescence intensity than Fluo-3 and conventional Fluo-4. The Fluo-4 NW indicator was non-fluorescent and stable in the buffer (pH 7-7.5) for several hours, so spontaneous conversion to the calcium sensitive form, in the absence of experimental stimulation, was not a significant source of background fluorescence. In addition, contributions to baseline fluorescence by the growth media (esterase activity, serum proteins, phenol red) were eliminated by removing the media prior to adding the indicator dye to the wells. Probenecid, an inhibitor of anion transporters on the cell surface, was added to prevent extrusion of the indicator from cells thereby potentially contributing to transfer of fluorescence outside the cells by organic anion

transporters, further contributing to increased background. Probenecid was in a water soluble form provided in the kit, for dissolution in the kit buffer, as opposed to the free acid form that required caustic 1M NaOH to dissolve.

The cells were counted and placed in labeled experimental or control 5 mL tubes. 500,000 cells were centrifuged at 250 g for 5 min to remove the culture media, resuspended in 250 μ L of assay buffer, and incubated at 37 $^{\circ}$ C, 5% CO₂ for 1 h to allow cells to acclimate to the buffer. Meanwhile, the calcium binding dye solution was prepared by adding 5 mL of assay buffer and 100 μ L of 250 mM stock solution of probenecid, to the calcium dye stock solution. The mixture was vortexed to ensure complete dye dissolution. After the 1 h incubation, 250 μ L of the prepared 2X calcium-binding dye solution was added to the cells, which were allowed to incubate at 37 $^{\circ}$ C, 5% CO₂ for another 30 min. The cells were incubated for 30 min at room temperature, while being taken to the Wistar institute, with the control and experimental TCR agonists (on ice). In these experiments, the antigens analyzed were; positive controls, i.e. OKT3 (10 μ g/mL) for anti-CD3 stimulation, and IPP (50 uM) for V γ 9V δ 2-specific TCR stimulation; experimental SSc putative antigens (all at 15 μ g/mL) as soluble human recombinant proteins including DNA topoisomerase I, centromere protein A, centromere protein B, hsp 27, hsp 90, as well as viral lysate of hCMV (AD619 strain). The antigen concentration was determined after literature review of use of soluble whole proteins for TCR-dependent stimulation of $\gamma\delta$ TCR+ T-cells, although none were exactly similar to the study undertaken. For example, proliferation of PBMC in patients with SSc was observed in response to one-week stimulation with soluble DNA topoisomerase I at concentrations ranging from 5 μ g/mL and 20 μ g/mL (Rands, Whyte et al. 2000).

The kinetics of intracellular calcium fluorescence over time, in gated live T-cells, was measured for 250 sec using flow cytometry. Initial background fluorescence was obtained for approximately 15 sec prior to addition the antigens, and change in fluorescence over time was acquired in response to subsequent stimulation with the antigens. Fluo-4 fluorescence was measured using same green laser channel as the FITC fluorochrome, because both share similar excitation and emission wavelengths (494 nm and 516 nm respectively). At 200 sec, the acquisition was paused and the calcium ionophore, ionomycin, was added to each experiment as a normalization control, with subsequent completion of the acquisition. Ionomycin facilitated an influx of extracellular calcium into the cells to obtain a quantification of the maximum loaded calcium dye, as a normalization control, to account for differential calcium dye loading into cells in the different experimental tubes. The FlowJo software kinetics platform allowed for analysis of the raw dot plot data, by obtaining the mean fluorescent line over time. The results were expressed as $(F-F_0)/F_{\max}$ where F is peak fluorescence after stimulation, F_0 is the background fluorescence before stimulation, and F_{\max} is the maximum fluorescence after ionomycin stimulation.

CHAPTER 4 RESULTS

4.1 PREVIOUS STUDY

Previous study on normal human peripheral blood mononuclear cells (PBMC) in our laboratory showed evidence of polyclonal $\gamma\delta$ TCR expansion, based on the presence of unique $V\gamma 1$, $V\gamma 9$ and $V\delta 2$ chain transcripts in 3 of 3 patients analyzed. However, there were multiple identical $V\delta 1$ transcripts suggestive of an oligoclonal expansion in 2 of 3 patients analyzed, a finding also reported by other investigators (Shen J et al., 1998). The significance of this $V\delta 1$ expansion unknown, however, the pairing of the $V\delta$ to unique $V\gamma$ transcripts, still facilitates the generation of unique $\gamma\delta$ T-cell receptor specificities. In addition, previous studies began in our laboratory, showed evidence of multiple identical $V\gamma$ and $V\delta$ transcripts in skin biopsies and peripheral blood of different patients with SSc, suggestive of an antigen-driven clonal expansion (see table 35). These findings were expanded upon and completed in the current study.

4.2 CLONAL EXPANSION STUDY

To determine if fresh, not expanded in culture, $\gamma\delta$ TCR⁺ T-cells present in skin lesions and peripheral blood from patients with SSc (see table 3) contained clonally-expanded populations, transcripts of all the $V\gamma$ and $V\delta$ -chain TCR chain subsets, specifically the $V\gamma 1$, $V\gamma 9$, $V\delta 1$, and $V\delta 2$ chains, were amplified, cloned and sequenced, using standard molecular biology techniques. The predicted amino acid sequence revealing productively-rearranged transcripts were used in quantification for clonal expansion analysis. Of the

results obtained, less than 4% of predicted amino acid sequences of V γ or V δ chain transcripts were non productively-rearranged and these were not included in the analysis.

TABLE 3 - CLINICAL DEMOGRAPHICS OF THE STUDY SSc PATIENTS

SSc patient	Age	Ethnicity	Sex	Clinical information	SSc disease characteristics
SSc-1	51 y	African-American	F	Diagnosed in 2002 Diffuse SSc ANA(+) 1:640 homogenous pattern anti-Scl70 (+)	RP, sclerodactyly, telangiectasias, skin thickening, GERD, digital ulcers, oral aperture (3cm), DIP erosion, esophageal dysmotility, interstitial lung disease
SSc-2	28 y	African-American	F	Diagnosed in 2002 ANA(-), anti-cytoplasmic antigens (+)	RP, severe GERD, pulmonary fibrosis, arthritis, peripheral neuropathy, perioral facial numbness, dysphagia, pericardial and bilateral pleural effusions, bilateral sclerodactyly
SSc-3	47 y	NA	F	Diagnosed in 1984, Limited SSc Biopsy taken 2003	RP, telangiectasias, calcinosis, sclerodactyly, digital atrophy without ulceration, tight facial skin over nose and forehead.
SSc-5	75 y	NA	F	Diagnosed in 1977 Limited SSc	RP, GERD, telangiectasias, skin thickening, dry mucosa (eyes, mouth, skin), thickened stomach wall
SSc-6	60 y	African-American	F	Diffuse SSc	RP, Skin hardening (chest, hands, forearm, thigh, abdomen), arthritis
SSc-11	67 y	African-American	F	Diagnosed in 2004 Limited SSc ANA(+) >1:1280 (nucleolar pattern) Biopsy taken 2005	Severe RP, telangiectasias (GI, face), sclerodactyly, skin thickening (chest, abdomen and back), skin tightness (hands and forearms), GERD, pulmonary HTN, serositis of pleura and pericardium (with cardiac tamponade)

TABLE 3 - CLINICAL DEMOGRAPHICS OF PATIENTS WITH SSc continued

SSc patient	Age	Ethnicity	Sex	Clinical information	SSc disease characteristics
SSc-16	57 y	African-American	F	Diffuse SSc ANA(+) 1:640 nucleolar anti-Scl-70 (-), anti-centromere (-)	RP, mild distal and proximal SSc skin thickening, digital ulcers in 4 th fingertip, synovitis of PIP bilaterally, finger thickening, oral aperture (3.5cm), GERD, arthralgias, pulmonary HTN, diffuse hyperpigmentation, hypopigmentation (face, arms)
SSc-17	49 y	Caucasian		Anti-cardiolipin antibody +	RP, GERD, arthritis (hands), sclerodactyly, telangiectasias of face, no lung involvement, synovitis (2 nd and 3 rd bilateral MCP joint), visible nail fold dilatation, skin thickening

Abbreviations – NA (information not available), SSc (Systemic sclerosis), F- female, RP – Raynaud’s phenomenon, ANA – anti-nuclear antibody, anti-Scl-70 (anti-topoisomerase I antibody), GERD (gastro-esophageal reflux disease), GI – gastrointestinal, HTN – hypertension, DIP – Distal interphalangeal joint, PIP – proximal interphalangeal joint

4.2.1 Sequencing results of V γ I-chain TCR Transcripts

In this study, V γ I TCR chain transcripts from the skin biopsy of 2 patients, and peripheral blood mononuclear cells (PBMC) of 3 patients, were amplified, cloned and sequenced. The predicted amino acid sequence of the transcripts was generated using the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB). Results revealed the presence of statistically significant proportions of multiple identical V γ I TCR chain transcripts in the SSc skin biopsy of 1 out of 2 patients and the PBMC of 1 out of 3 patients, suggesting the presence of oligoclonal populations of T-cells. No conserved sequences were identified in the γ -chain transcripts, nor were there any homologies after comparison to γ -chain transcripts in the GENBANK database using BLAST searches, suggesting that these clones were previously unidentified and were novel.

TABLE 4 – PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ I SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-3)

Clone	Variable region	N-region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
GS3P1.2	C A T W D TGTGCCACCTGGGAC	S R K N AGTCGAAAAAAT	Y K K L F TATAAGAAACTCTTT	Vγ5;Jγ2.3;Cγ2 5/16-31%	0.002	0.0307
GS3P1.21	C A T W D R TGTGCCACCTGGGACAGG	P P CCCCCT	S D W I K T F AGTGATTGGATCAAGACGTTT	V γ 5;J γ 2.1;C γ 2 3/16-19%	0.0591	0.1928
GS3P1.4	C A T W TGTGCCACCTGG	G D GGGGAT	Y K K L F TATAAGAAACTCTTT	V γ 5;J γ 2.3;C γ 2 2/16-13%	0.1899	0.2891
GS3P1.38	C A T W D R TGTGCCACCTGGGACAGG	P CCT	S D W I K T F AGTGATTGGATCAAGACGTTT	V γ 5;J γ 2.1;C γ 2 2/16-13%	0.1899	0.2891
GS3P1.23	C A T W D R TGTGCCACCTGGGACAGG	Y R R M TACCGACGGATG	K L F AAACTCTTT	V γ 5;J γ 2.3;C γ 2 1/16-6%	0.3798	NS
GS3P1.41	C A T TGTGCCACC	C TGT	N Y Y K K L F AATTATTATAAGAAACTCTTT	V γ 5;J γ 2.3;C γ 2 1/16-6%	0.3798	NS
GS3P1.45	C A T W D R TGTGCCACCTGGGACAGG	L W CTCTGG	N Y Y K K L F AATTATTATAAGAAACTCTTT	V γ 5;J γ 2.3;C γ 2 1/16-6%	0.3798	NS
GS3P1.33	E S G L S P GAATCAGGACTCAGTCCA		D K Q L D GATAAACAACCTTGAT	V γ 5;C γ 2 1/16-6%	0.3798	NS

TABLE 5 – PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ I SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-4)

Clone	Variable region	N-region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
GS4P1.35	C A T W TGTGCCACCTGG	I ATT	G T T L V GGAACAACACTTGTT	V γ 5;J γ 2.3;C γ 2 3/25-12%	0.0600	0.1881
GS4P1.7	E S G L S P GAATCAGGACTCAGTCCA		D K Q L D GATAAACAACCTTGAT	V γ 5;C γ 2 2/25-8%	0.1877	NS
GS4P1.23	C A T W D R TGTGCCACCTGGGACAGA		R N S L A AGAAACTCTTTGGCA	V γ 5;J γ 2.3int;C γ 2 2/25-8%	0.1877	NS
GS4P1.38	C A T W D TGTGCCACCTGGGAC	G R P A S S A GGAAGACCCGCTCATCAGCC	G T T L V GGAACAACACTTGTT	V γ 5;J γ 2.3;C γ 2 2/25-8%	0.1877	NS
GS4P1.3	C A T W D R TGTGCCACCTGGGACAGG	L E CTTGAG	K L F AAACTCTTT	V γ 5;J γ 2.3;C γ 2 1/25-4%	0.3754	NS
GS4P1.9	C A T TGTGCCACT	H P A Y CATCCAGCCTAT	W I K T F TGGATCAAGACGTTT	V γ 5;J γ 2.1;C γ 2 1/25-4%	0.3754	NS
GS4P1.16	C A T W D R TGTGCCACCTGGGACAGG	R G CGAGGT	I R N S L A ATAAGAACTCTTTGGCA	V γ 5;J γ 2.3int;C γ 2 1/25-4%	0.3754	NS
GS4P1.18	C A T W D TGTGCCACCTGGGAC	S Y AGCTAT	Y Y K K L F TATTATAAGAACTCTTT	V γ 5;J γ 2.3;C γ 2 1/25-4%	0.3754	NS
GS4P1.20	C A T W D R TGTGCCACCTGGGACAGG	L E R D CTTGAAAGGGAT	Y K K L F TATAAGAACTCTTT	V γ 5;J γ 2.3;C γ 2 1/25-4%	0.3754	NS
GS4P1.22	C A T W D R TGTGCCACCTGGGACAGG	A GCC	Y Y K K L F TATTATAAGAACTCTTT	V γ 5;J γ 2.3;C γ 2 1/25-4%	0.3754	NS
GS4P1.24	C A T W D R TGTGCCACCTGGGACAGG	R D CGTGAT	Y K K L F TATAAGAACTCTTT	V γ 5;J γ 2.3;C γ 2 1/25-4%	0.3754	NS
GS4P1.26	C A T W D TGTGCCACCTGGGAC	G K V GGGAAAGTT	Y K K L F TATAAGAACTCTTT	V γ 5;J γ 2.3;C γ 2 1/25-4%	0.3754	NS

TABLE 5 – PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ I SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-4) continued

Clone	Variable region	N-region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
GS4P1.27	C A T W D TGTGCCACCTGGGAC	T I ACAATC	Y Y K K L F TATTATAAGAAACTCTTT	V γ 5;J γ 2.3;C γ 2 1/25-4%	0.3754	NS
GS4P1.28	C A T W D R TGTGCCACCTGGGACAGG	P P D P CCGCCTGATCCG	Y K K L F TATAAGAAACTCTTT	V γ 5;J γ 2.3;C γ 2 1/25-4%	0.3754	NS
GS4P1.30	C A T W D TGTGCCACCTGGGAC	S I AGTATT	N Y Y K K L F AATTATTATAAGAAACTCTTT	V γ 5;J γ 2.3;C γ 2 1/25-4%	0.3754	NS
GS4P1.34	C A T W D TGTGCCACCTGGGAC	S P I Y F AGCCCGATCTACTTT	Y K K L F TATAAGAAACTCTTT	V γ 5;J γ 2.3;C γ 2 1/25-4%	0.3754	NS
GS4P1.36	C A T TGTGCCACC	R CGA	N Y Y K K L F AATTATTATAAGAAACTCTTT	V γ 5;J γ 2.3;C γ 2 1/25-4%	0.3754	NS
GS4P1.40	C A T W D R TGTGCCACCTGGGACAGG	R CGA	Y K K L F TATAAGAAACTCTTT	V γ 5;J γ 2.3;C γ 2 1/25-4%73	0.3754	NS
GS4P1.42	C A T W D R TGTGCCACCTGGGACAGG	R R CGTCGT	Y K K L F TATAAGAAACTCTTT	V γ 5;J γ 2.3;C γ 2 1/25-4%	0.3754	NS
GS4P1.45	C A T W D TGTGCCACCTGGGAC	L D TTGGAT	Y K K L F TATAAGAAACTCTTT	V γ 5;J γ 2.3;C γ 2 1/25-4%	0.3754	NS

TABLE 6 – PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ I SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE SKIN BIOPSY TISSUE FROM A SYSTEMIC SCLEROSIS PATIENT (SS-6)

Clone	Variable region	N-region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
GS6T1.2	C A T W D R TGTGCCACCTGGGACAGG	P N CCGAAT	Y K K L F TATAAGAAACTCTTT	V γ 5; J γ 2.3; C γ 2 13/35–37.2%	<0.0001	<0.0001
GS6T1.1	C A T W D R TGTGCCACCTGGGACAGG	P CCT	Y Y K K L F TATTATAAGAAACTCTTT	V γ 5; J γ 2.3; C γ 2 9/35–25.7%	<0.0001	<0.0001
GS6T1.12	E S G L S P GAATCAGGACTCAGTCCA		D K Q L D GATAACAACCTTGAT	V γ 5; C γ 2 8/35–22.8%	<0.0001	0.0005
GS6T1.8	C A T W D TGTGCCACCTGGGAC	G GGA	Y K K L F TATAAGAAACTCTTT	V γ 5; J γ 2.3; C γ 2 5/35–14.3%	0.0026	0.0339

TABLE 7 – PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ I SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE SKIN BIOPSY TISSUE FROM A SYSTEMIC SCLEROSIS PATIENT (SS-16)

Clone	Variable region	N-region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
GS16T1.1	C A T W TGTGCCACCTGG	F L E A C TTCCTGGAAGCTTGT	Y K K L F G S G TATAAGAAACTCTTTGGCAGTGGA	V γ 3;J γ 2.3;C γ 2.3 2/15-13%	NS	NS
GS16T1.3	C A T W D R TGTGCCACCTGGGACAGG		N Y Y K K L F G S G AATTATATAAGAAACTCTTTGGCAGTGGA	V γ 5;J γ 2.3;C γ 2 2/15-13%	NS	NS
GS16T1.8	C A T W TGTGCCACCTGG	F L E A C TTCCTGGAAGCTTGT	Y K K L F G S G TATAAGAAACTCTTTGGCAGTGGA	V γ 3;J γ 2.3;C γ 2.2 2/15-13%	NS	NS
GS16T1.17	C A T W D G TGTGCCACCTGGGACGGG	L Q CTTCAG	K L F G S G AAACTCTTTGGCAGTGGA	V γ 2;J γ 2.3;C γ 2.3 2/15-13%	NS	NS
GS16T1.3b	C A T W TGTGCCACCTGG	A P W Y GCCCCGTGGTAT	W I K T F A K TGGATCAAGACGTTTGCAAAA	V γ 2;J γ 2.1;C γ 2.2 1/15-6.7%	NS	NS
GS16T1.13	C A T W TGTGCCACCTGG	G R F GGCCGGTTT	G S G GGCAGTGGA	V γ 5;J γ 2.3;C γ 1 1/15-6.7%	NS	NS
GS16T1.14	C A T W D G TGTGCCACCTGGGATGGA	Y TAC	Y Y K K L F G S G TATTATAAGAAACTCTTTGGCAGTGGA	V γ 4;J γ 2.3;C γ 2.3 1/15-6.7%	NS	NS
GS16T1.20	C A T W D G TGTGCCACCTGGGATGGG	L P G S G CTCCCCGGATCGGGG	K K L F G S G AAGAAACTCTTTGGCAGTGGA	V γ 4;J γ 2.3;C γ 2.3 1/15-6.7%	NS	NS
GS16T1.22	C A T W TGTGCCACCTGG	T ACG	Y Y K K L F G S G TATTATAAGAAACTCTTTGGCAGTGGA	V γ 4;J γ 2.3;C γ 1 1/15-6.7%	NS	NS
GS16T1.29	C A T W TGTGCCACCTGG	T ACG	Y Y K K L F G S G TATTATAAGAAACTCTTTGGCAGTGGA	V γ 4;J γ 2.3;C γ 2.2 1/15-6.7%	NS	NS
GS16T1.30	C A T W D R TGTGCCACCTGGGACAGG	R A CGAGCG	K L F G S G AAACTCTTTGGCAGTGGA	V γ 5;J γ 2.3;C γ 2.2 1/15-6.7%	NS	NS

TABLE 8 – PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ I SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-16)

Clone	Variable region	N-region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
GS16P1.5	C A T W D R TGTGCCACCTGGGACAGG	Q R CAACGG	S S D W I K T AGTAGTGATTGGATCAAGACG	V γ 5;J γ 2.1;C γ 2 2/17 - 11.7%	NS	NS
GS16P1.3	C A T W TGTGCCACCTGG	V Y GTTTAT	N Y Y K K L F AATTATTATAAGAAACTCTTT	V γ 4;J γ 2.3;C γ 2 1/17 - 5.9%	NS	NS
GS16P1.4	C A T W TGTGCCACCTGG	E H GAACAT	Y Y K K L F TATTATAAGAAACTCTTT	V γ 2;J γ 2.3;C γ 2 1/17 - 5.9%	NS	NS
GS16P1.6	C A TGTGCC	A A K E S R L GCCGCGAAGGAGAGCCGGTTG	N Y Y K K L I AATTATTATAAGAAACTCATT	V γ 3;J γ 2.3;C γ 2 1/17 - 5.9%	NS	NS
GS16P1.7	C A T W TGTGCCACCTGG	E N H GAGAATCAT	Y K K L F TATAAGAAACTCTTT	V γ 4;J γ 2.3;C γ 2 1/17 - 5.9%	NS	NS
GS16P1.9	C A T W D G TGTGCCACCTGGGACGGG	L P CTCCCG	K L F AAACTCTTT	V γ 2;J γ 2.3;C γ 2 1/17 - 5.9%	NS	NS
GS16P1.10	C A T W TGTGCCACCTGG	G GGT	S D W I K T AGTGATTGGATCAAGACG	V γ 4;J γ 2.1;C γ 2 1/17 - 5.9%	NS	NS
GS16P1.10b	C A T W D R TGTGCCACCTGGGACAGG	P G CCGGGT	Y Y K K L F TATTATAAGAAACTCTTT	V γ 5;J γ 2.3;C γ 2 1/17 - 5.9%	NS	NS
GS16P1.11	C A T W D G TGTGCCACCTGGGATGGG	L CTT	Y K K L F TATAAGAAACTCTTT	V γ 4;J γ 2.3;C γ 2 1/17 - 5.9%	NS	NS
GS16P1.13	C A T W D R TGTGCCACCTGGGACAGG	P G CCGGGG	Y K K L F TATAAGAAACTCTTT	V γ 3;J γ 2.3;C γ 2 1/17 - 5.9%	NS	NS
GS16P1.16	C A T W D TGTGCCACCTGGGAT	V Q GTTTACG	Y Y K K L F TATTATAAGAAACTCTTT	V γ 4;J γ 2.3;C γ 2 1/17 - 5.9%	NS	NS
GS16P1.18	C A T W TGTGCCACCTGG	A P A D GCCCCGGCGGAT	Y Y K K L F TATTATAAGAAACTCTTT	V γ 4;J γ 2.3;C γ 2 1/17 - 5.9%	NS	NS

TABLE 8 – PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ I SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-16) continued

Clone	Variable region	N-region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
GS16P1.20	C A T W TGTGCCACCTGG	V H GTCCAT	Y K K L F TATAAGAAACTCTTT	V γ 2;J γ 2.3;C γ 2 1/17 – 5.9%	NS	NS
GS16P1.22	C A T W D G TGTGCCACCTGGGACGGG	P V N CCGGTTAAT	Y K K L F TATAAGAAACTCTTT	V γ 2;J γ 2.3;C γ 2 1/17 – 5.9%	NS	NS
GS16P1.23	C A T W TGTGCCACCTGG	G V GGTGTT	Y Y K K L F TATTATAAGAAACTCTTT	V γ 4;J γ 2.3;C γ 2 1/17 – 5.9%	NS	NS
GS16P1.24	C A T W D R TGTGCCACCTGGGACAGG	P P CCCCCT	G S G T T GGCAGTGAACAACA	V γ 3;J γ 2.3;C γ 2 1/17 – 5.9%	NS	NS
GS16P1.15	C A T W TGTGCCACCTGG	T K ACTAAG	N Y Y K K L F AATTATTATAAGAAACTCTTT	V γ 5;J γ 1.3;C γ 1 1/17 – 5.9%	NS	NS

TABLE 9 – SUMMARY TABLE OF CLONAL EXPANSION OF V γ I-CHAIN TRANSCRIPTS IN SSc PATIENT SAMPLES

Tissue	Patient	Clone	CDR3 region	Identity	Frequency	Statistics	Clonal Expansion (Yes/No)
PBMC	SS3P γ I	clone 2	CATWD <u>SRKN</u> YKKLF	V γ 5;J γ 2.3;C γ 2	5/16-31%	p=0.002	Yes
PBMC	SS4P γ I	clone 35	CATW <u>I</u> GTTLV	V γ 5;J γ 2.3;C γ 2	3/25-12%	p=0.0600	No
PBMC	SS16P γ I	clone 5	CATWDR <u>QR</u> SSDWIKT	V γ 5;J γ 2.1;C γ 2	2/17-11.7%	p>0/05	No
Biopsy	SS6T γ I	clone 2	CATWDR <u>PN</u> YKKLF	V γ 5;J γ 2.3;C γ 2	13/35-37.2%	p<0.0001	Yes
		clone 1	CATWDR <u>P</u> YYKKLF	V γ 5;J γ 2.3;C γ 2	9/35-25.7%	p<0.0001	Yes
		clone 12	ESGLSP <u>DKQLD</u>	V γ 5;C γ 2	8/35-22.8%	p<0.0001	Yes
		clone 8	CATWD <u>G</u> YKKLF	V γ 5;J γ 2.3;C γ 2	5/35-14.3%	p=0.0026	Yes
Biopsy	SS16T γ I	clone 1	CATW <u>FLEAC</u> YKKLF	V γ 3;J γ 2.3;C γ 2	2/15-13%	p>0.05	No

Statistical analysis by binomial distribution (p<0.05 is statistically significant)

In the PBMC from patient SS-3 (see table 4), V γ I chain analysis revealed 5 out of 16 (31%) transcripts had identical CDR3 regions (transcript #2), representing a statistically significant clonal expansion; CATWD SRKN YKKLF, V γ 5;J γ 2.3;C γ 2, $p=0.002$. There were three clones, each with 3 out of 16 (19%), or 2 out of 16 (13%) identical transcripts, but these represented a statistical insignificant clonal expansion ($p>0.05$). In addition, there were four clones, each with unique CDR3 regions (1 out of 16). For patient SS-4 (see table 5), no statistically significant clonal expansion was observed in the V γ I chain transcripts present in the PBMC. There were four clones, each with 3 out of 25 (12%), or 2 out of 25 (8%) identical transcripts, but these represented a statistical insignificant clonal expansion ($p>0.05$). The remaining sixteen clones each had unique CDR3 regions (1 out of 25), indicative of a polyclonal population. In the PBMC from patient SS-16 (see table 8), 2 out of 17 (17%) transcripts were identical, representing a statistically insignificant expansion ($p>0.05$). The remaining sixteen transcripts had unique CDR3 regions (1 out of 17).

In the skin biopsy sample of patient SS-6 (see table 6), V γ I chain analysis revealed 13 out of 35 (37.2%) identical transcripts (transcript #2), CATWDR PN YKKLF, V γ 5;J γ 2.3;C γ 2, $p<0.0001$; 9 out of 35 (25.7%) identical transcripts (transcript #1), CATWDR P YYKKLF, V γ 5;J γ 2.3;C γ 2, $p<0.0001$; 8 out of 35 (22.8%) identical transcripts (transcript #12), ESGLSP DKQLD, V γ 5;C γ 2, $p<0.0001$; 5 out of 35 (14.3%) identical transcripts (transcripts #8), CATWD G YKKLF, V γ 5;J γ 2.3;C γ 2, $p=0.0026$. All these transcripts showed evidence of statistically significant clonal expansions. There were no unique V γ I chain transcripts in the clones analyzed in this patient sample. The skin biopsy of patient SS-16 (see table 7), revealed the presence of four clones with 2 out of 15 (13%) identical transcripts, each representing a statistically insignificant clonal expansion ($p>0.05$).

There were seven transcripts each with unique CDR3 regions (1 out of 15), indicative of a polyclonal population. The deduced amino acid sequence of all V γ I transcripts analyzed revealed neither amino acid sequence homology nor the presence of conserved motifs, when compared using BLAST searches to transcripts in the GENBANK database, suggesting these were unique, previously unidentified, clones.

4.2.2 Sequencing results of V γ 9-chain TCR Transcripts

In the current study, V γ 9 TCR chain transcripts from skin biopsy of 1 patient with SSc, and peripheral blood mononuclear cells (PBMC) of 3 patients, including a patient with SSc (SS-16) from which both PBMC and skin biopsy were obtained, were amplified, cloned, sequenced. The predicted amino acid sequence of the transcripts was generated using the ExPASy proteomics server. Results revealed the presence of statistically significant proportions of multiple identical V γ 9-chain TCR+ T-cell transcripts in the SSc skin biopsy of 1 out of 1 patients and the peripheral blood of 2 out of 3 patients, suggesting the presence of oligoclonal populations of $\gamma\delta$ TCR+ T-cells infiltrating the SSc lesions. No conserved sequences were identified in the γ -chain transcripts, nor were there any homologies after comparison to γ -chain transcripts in the GENBANK database using BLAST searches, suggesting that these clones were previously unidentified and were therefore novel.

TABLE 10 – PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ II (V γ 9) SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-4)

Clone	Variable region	N-region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
GS4P2.1	C A L W TGTGCCTTGTGG	D K GACAAA	Q E L G K K CAAGAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1 1/20-5%	NS	NS
GS4P2.2	C A L W E V TGTGCCTTGTGGGAGGTG	R CGC	G Q E L G K K GGCCAAGAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1/20-5%	NS	NS
GS4P2.3	C A L W E V TGTGCCTTGTGGGAGGTG	G GGG	E L G K K GAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1/20-5%	NS	NS
GS4P2.4	C A L W E TGTGCCTTGTGGGAG	E GAG	Q E L G K K CAAGAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1/20-5%	NS	NS
GS4P2.5	C A L W E TGTGCCTTGTGGGAG	A S GCCTCT	Q E L G K K CAAGAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1 1/20-5%	NS	NS
GS4P2.6	C A L W E V TGTGCCTTGTGGGAGGTT	K AAG	E L G K K GAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 2 1/20-5%	NS	NS
GS4P2.7	C A L W E TGTGCCTTGTGGGAG	N H R AACCACCGA	E L G K K GAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1/20-5%	NS	NS
GS4P2.8	C A L W E V TGTGCCTTGTGGGAGGTG	H K CATAAA	K K L F AAGAACTCTTT	V γ 9;J γ 2.3;C γ 1 1/20-5%	NS	NS
GS4P2.9	C A L W E TGTGCCTTGTGGGAG	A GCG	Q E L G K K CAAGAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1 1/20-5%	NS	NS
GS4P2.10	C A L W E V TGTGCCTTGTGGGAGGTC	P P P R V K CCTCCGCCGAGAGTTAAG	L G K K TTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1 1/20-5%	NS	NS
GS4P2.11	C A L W TGTGCCTTGTGG	A I R GCCATACGG	E L G K K GAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1 1/20-5%	NS	NS
GS4P2.12	C A TGTGCC	P CCA	Y Y K K L F TATTATAAGAAACTCTTT	V γ 9;J γ 2.3;C γ 2 1/20-5%	NS	NS

TABLE 10 – PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ II (V γ 9) SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-4) continued

Clone	Variable region	N-region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
GS4P2.13	C A L W TGTGCCTTGTGG	V V S GTGGTGCC	Q E L G K K CAAGAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1/20-5%	NS	NS
GS4P2.14	C A L W E V TGTGCCTTGTGGGAGGTG	L K CTCAAG	L G K K TTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1/20-5%	NS	NS
GS4P2.15	C A L W E TGTGCCTTGTGGGAG	E V GAGGTG	L G K K TTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 2 1/20-5%	NS	NS
GS4P2.16	C A L W E V TGTGCCTTGTGGGAGGTC	S A TCCGCG	L G K K TTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1 1/20-5%	NS	NS
GS4P2.17	C A L W E TGTGCCTTGTGGGAG	A R G L N GCGAGAGGACTGAAT	K K L F AAAAAACTCTTT	V γ 9;J γ 2.3;C γ 1/20-5%	NS	NS
GS4P2.18	C A L W E V TGTGCCTTGTGGGAGGTG		Y Y K K L F TACTATAAGAAACTCTTT	V γ 9;J γ 2.3;C γ 1 1/20-5%	NS	NS
GS4P2.19	C A L W E V TGTGCCTTGTGGGAGGTT	G M GGGATG	G K K GGCAAAAAA	V γ 9;J γ 1.2;C γ 1/20-5%	NS	NS
GS4P2.20	C A L W E V TGTGCCTTGTGGGAGGTG	R CGG	K K L F AAGAAACTCTTT	V γ 9;J γ 2.3;C γ 2 1/20-5%	NS	NS

TABLE 11 – PRODUCTIVELY-REARRANGED CDR3 & CONSTANT REGION SEQUENCES OF V γ II (V γ 9) SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-7)

Clone	Variable region	N- region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
GS7P2.13	C A L W E V TGTGCCTTGTGGGAGGTG	W TGG	Y Y K K L F TATTATAAGAAACTCTTT	Vγ9; Jγ2.3; Cγ2 5/21–24%	0.0023	0.0321
GS7P2.1	C A L W E TGTGCCTTGTGGGAG	F TTC	N Y Y K K L F AATTATTATAAGAAACTCTTT	V γ 9; J γ 2.3; C γ 3/21–14%	0.0597	0.1896
GS7P2.6	C A L TGTGCCTTG	C TGT	Y Y K K L F TATTATAAGAAACTCTTT	V γ 9; J γ 2.3; C γ 2 2/21–9.5%	0.1884	0.2844
GS7P2.3	C A L W E V TGTGCCTTGTGGGAGGTG	H S P H G CATTGCCACACGGG	L G K K I K TTGGGCAAAAAATCAAG	V γ 9; J γ 1.2; C γ 2 1/21–4.8%	0.3769	NS
GS7P2.4	C A L W E V TGTGCCTTGTGGGAGGTG	P R V G CCACGTGTGGGT	Y Y K K L F TATTATAAGAAACTCTTT	V γ 9; J γ 2.3; C γ 1/21–4.8%	0.3769	NS
GS7P2.7	C A L W E V TGTGCCTTGTGGGAGGTG	P T CCCACG	N Y Y K K L F AATTATTATAAGAAACTCTTT	V γ 9; J γ 2.3; C γ 1 1/21–4.8%	0.3769	NS
GS7P2.10	C A L W E TGTGCCTTGTGGGAG	D R GACCGA	N Y Y K K L F AATTATTATAAGAAACTCTTT	V γ 9; J γ 2.3; C γ 2 1/21–4.8%	0.3769	NS
GS7P2.12	C A L W E V TGTGCCTTGTGGGAGGTC	L CTA	E L G K K I K GAGTTGGGCAAAAAATCAAG	V γ 9; J γ 1.2; C γ 1 1/21–4.8%	0.3769	NS
GS7P2.16	C A L W E TGTGCCTTGTGGGAG	L T D CTTACGGAT	Y Y K K L F TATTATAAGAAACTCTTT	V γ 9; J γ 2.3; C γ 1/21–4.8%	0.3769	NS
GS7P2.17	C A L W E V TGTGCCTTGTGGGAGGTG	P CCA	E L G K K I K GAGTTGGGCAAAAAATCAAG	V γ 9; J γ 1.2; C γ 1/21–4.8%	0.3769	NS
GS7P2.18	C A L W E TGTGCCTTGTGGGAG	A E GCAGAA	E L G K K I K GAGTTGGGCAAAAAATCAAG	V γ 9; J γ 1.2; C γ 2 1/21–4.8%	0.3769	NS
GS7P2.21	C A L TGTGCCTTG	S V S K T TCGGTATCCAAGACT	Y Y K K L F TATTATAAGAAACTCTTT	V γ 9; J γ 2.3; C γ 1/21–4.8%	0.3769	NS

TABLE 11 – PRODUCTIVELY-REARRANGED CDR3 & CONSTANT REGION SEQUENCES OF V γ II (V γ 9) SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-7) continued

Clone	Variable region	N- region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
GS7P2.23	C A L W E TGTGCCTTGTGGGAG	T K ACGAAA	E L G K K I K GAGTTGGCAGAAAAATCAAG	V γ 9;J γ 1.2;C γ 1/21-4.8%	0.3769	NS
GS7P2.11	Mid-V-region I S A T S V ATTTCTGCAACATCTGTA		Mid-C-region Y L C L L E TACCTTTGCTTCTTGAG	V γ 9;C γ 1 1/21-4.8%	0.3769	NS

TABLE 12 – PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ II (V γ 9) SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE SKIN BIOPSY TISSUE FROM A SYSTEMIC SCLEROSIS PATIENT (SS-16)

Clone	Variable region	N-region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
GS16T2.1	C A L W E V TGTGCCTTGTGGGAGGTG	L A A G CTGGCGGCGGGG	L G K K TTGGGCAAAAAA	V γ 9; J γ 1.2; C γ 1 20/24–83.3%	<0.0001	<0.0001
GS16T2.7	C A L W E V TGTGCCTTGTGGGAGGTG	H CAT	Q E L G K K CAAGAGTTGGGCAAAAAA	V γ 9; J γ 1.2; C γ 1 2/24–8.3%	0.1879	0.2826
GS16T2.2	C A L W E V TGTGCCTTGTGGGAGGTG	P A CCGGCG	L G K K TTGGGCAAAAAA	V γ 9; J γ 1.2; C γ 1 1/24–4.2%	0.3757	0.2703
GS16T2.23	C A L W TGTGCCTTGTGG	D Y G GATTACGGC	T G W F K ACTGGTTGGTTCAAG	V γ 9; J γ 1.2; C γ 1 1/24–4.2%	0.3757	0.2703

TABLE 13 – PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ II (V γ 9) SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-16)

Clone	Variable region	N-region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
GS16P2.2	C A L W E V TGTGCCTTGTGGGAGGTG	L A A G CTGGCGGCGGGG	L G K K TTGGGCAAAAAA	Vγ9;Jγ1.2;Cγ1 27/34 – 79%	<0.0001	<0.0001
GS16P2.1	C A L W E V TGTGCCTTGTGGGAGGTG	L P CTTCCA	E L G K K GAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1 1/34 – 3%	NS	NS
GS16P2.15	C A L W E TGTGCCTTGTGGGAG	G I GGGATT	Q E L G K K CAAGAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1 1/34 – 3%	NS	NS
GS16P2.18	C A L W E TGTGCCTTGTGGGAG	G A K GGAGCTAAA	E L G K K GAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1 1/34 – 3%	NS	NS
GS16P2.22	C A L W TGTGCCTTGTGG		Q E L G K K CAAGAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1 1/34 – 3%	NS	NS
GS16P2.31	C A L W E V TGTGCCTTGTGGGAGGTG	S TCA	E L G K K GAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1 1/34 – 3%	NS	NS
GS16P2.33	C A L W E V TGTGCCTTGTGGGAGGTG	R CGG	E L G K K GAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1 1/34 – 3%	NS	NS
GS16P2.40	C A L W E V TGTGCCTTGTGGGAGGTG		E L G K K GAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1 1/34 – 3%	NS	NS

TABLE 14 – SUMMARY TABLE OF CLONAL EXPANSION OF V γ II (V γ 9)-CHAIN TRANSCRIPTS IN SSc PATIENT SAMPLES

Tissue	Patient	Clone	CDR3 region	Identity	Frequency	Statistics	Clonal Expansion (Yes/No)
PBMC	SS4P γ II	clone 1	CALW <u>DK</u> QELGKK	V γ 9;J γ 1.2;C γ 1	1/20-5%	p>0.05	No
PBMC	SS7P γ II	clone 13	CALWEV <u>W</u> YYKKLF	V γ 9;J γ 2.3;C γ 2	5/21-24%	p=0.0023	Yes
PBMC	SS16P γ II	clone 2	CALWEV <u>LAAG</u> LGKK	V γ 9;J γ 1.2;C γ 1	27/34-79%	p<0.0001	Yes
Biopsy	SS6T γ II	clone 1	CALWEV <u>LAAG</u> LGKK	V γ 9;J γ 1.2;C γ 1	20/24-83.3%	p<0.0001	Yes

Statistical analysis by binomial distribution (p<0.05 is statistically significant)

In the PBMC from patient SS-4 (see table 10), V γ 9 transcript analysis revealed that all transcripts were unique clones (1 out of 20), indicative of a polyclonal population. Patient SS-7 (see table 11) had 5 out of 21 (24%) transcripts with identical CDR3 regions (transcript #13) in the PBMC, representing a statistically significant clonal expansion; CALWEV WYYKKLF, V γ 9;J γ 2.3;C γ 2, $p=0.0023$. There were two clones, each with 3 out of 21 (14%), or 2 out of 21 (9.5%) identical transcripts, but these represented a statistical insignificant clonal expansion ($p>0.05$). Eleven clones each had unique CDR3 regions (1 out of 21). For patient SS-16, an interesting finding was the presence of a transcript that was expanded in both the SSc lesional skin biopsy and the PBMC (see table 12 and 13 respectively). There were 20 out of 24 (83.3%) transcripts with identical CDR3 regions (transcripts #1) in the skin biopsy, and 27 out of 34 (79%) transcripts with identical CDR3 regions (transcripts #2) in the PBMC, representing a statistically significant clonal expansion; CALWEV LAAG LGKK, V γ 9;J γ 1.2;C γ 1, $p<0.0001$ in both cases. In the PBMC of SS-16, there were seven other clones that each had unique CDR3 regions (1 out of 34), whereas in the skin biopsy of the patient, there were 2 out of 24 identical transcripts that represented statistically insignificant clonal expansion ($p>0.05$), and two clones each with unique CDR3 regions (1 out of 24). The deduced amino acid sequence of all V γ 9 transcripts analyzed revealed neither amino acid sequence homology nor the presence of conserved motifs, when compared using BLAST searches to transcripts in the GENBANK database, suggesting these were unique, previously unidentified, clones.

4.2.3 Sequencing results of V δ 1-chain TCR Transcripts

In the current study, V δ 1 TCR chain transcripts from skin biopsy of 3 patients with SSc, and peripheral blood mononuclear cells (PBMC) of 6 patients, including 2 patients with

SSc (SS-7 and SS-16) from which both PBMC and skin biopsies were obtained, were amplified, cloned, sequenced. The predicted amino acid sequence of the transcripts was generated using the ExPASy proteomics server. Results revealed the presence of statistically significant proportions of multiple identical V δ 1-chain TCR+ T-cell transcripts in the SSc skin biopsy of 2 out of 3 patients, and peripheral blood of 4 out of 6 patients, suggesting the presence of oligoclonal populations of $\gamma\delta$ TCR+ T-cells infiltrating the SSc lesions. No conserved sequences were identified in the δ -chain transcripts, nor were there any homologies after comparison to δ -chain transcripts in the GENBANK database using BLAST searches, suggesting that these clones were previously unidentified and were therefore novel.

TABLE 15 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-1)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS1P1.8	C A L G TGTGCTCTTGGG	N K P H W G Y A S F T Y AATAAACCCAC TGGGGA TACGCGTCCTTCACCTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 8/24–33.3%	<0.0001	0.0004
DS1P1.2	C A L G E TGTGCTCTTGGGGAG	L T I D W G I H CTAACAAATC GACTGGGGGATA CAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 2/24–8.3%	0.1879	0.2826
DS1P1.3	C A L G TGTGCTCTTGGG	S F L G D W G L TCCTTCCTAGGA GACTGGGGG TTG	S S W D T R AGCTCCTGGGACACCCGA	V δ 1;D δ 2F3;J δ 3 2/24–8.3%	0.1879	0.2826
DS1P1.5	C A L G E TGTGCTCTTGGGGAA	L K T G G P Y CTGAAA ACTGGGGGC CCCTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F1;J δ 1 2/24–8.3%	0.1879	0.2826
DS1P1.6	C A L G E TGTGCTCTTGGGGAA	W G G Q Q TGGGGA GGG CAGCAA	D K L I F GATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 2/24–8.3%	0.1879	0.2826
DS1P1.7	C A L G TGCCTCTTGGG	L G G L R G I P Y TTA GGGGGT CTACGG GGGATA CCTTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F1;D δ 2F3;J δ 1 2/24–8.3%	0.1879	0.2826
DS1P1.1	C A L G TGTGCTCTTGGC	L W G G E H CTCTGG GGGGGA GAACAC	D K V I F GATAAAGTCATCTTT	V δ 1;D δ 2F1;J δ 1 1/24–4.1%	0.3757	NS
DS1P1.11	C A L G E TGTGCTCTTGGGGAA	G L P I P D W G F Y GGA CTCCT ATACCG GACTGGGGA TTTTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 1F2;D δ 2F3;J δ 1 1/24–4.1%	0.3757	NS
DS1P1.13	C A L G E TGTGCTCTTGGGGAA	P P R T G D CCACCCGTA CTACT GGGGAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F2;J δ 1 1/24–4.1%	0.3757	NS
DS1P1.17	C A L G E TGTGCTCTTGGGGAA	P N Y V T R Y W G I R W CCGAAC TACGTTACTCGCTAC TGGGGGATACGT TGG	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2.3;J δ 1 1/24–4.1%	0.3757	NS
DS1P1.19	C A L G E TGTGCTCTTGGGGAA	L V F R L P T F G D T N CTTGTATTCCGC CTTTCT ACGTTC GGGGACACCAAT	K L I F AAACTCATCTTT	V δ 1;D δ 1F2;J δ 1 1/24–4.1%	0.3757	NS

TABLE 16 – PRODUCTIVELY REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-2)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS2P1.6	C A L TGTGCTCTT	E L H P F Y W G I L Y GAATTGCACCCATTCTAC TGGGGGATT CTGTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 5/20–25%	0.0022	0.0319
DS2P1.9	C A L G TGTGCTCTTGGG	G T Y Y GGTACTTATTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;J δ 1 3/20–15%	0.0596	0.1901
DS2P1.4	C A L G TGTGCTCTTGGG	P P F H W G I R G K F G CCCCCATTCAC TGGGGGATACGC GGAAGTTTGGG	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 2/20–10%	0.1887	0.2852
DS2P1.17	C A L G TGTGCTCTTGGG	V H L G W G Y GTCCACCTAGGC TGGGGG TAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 2/20–10%	0.1887	0.2852
DS2P1.20	C A L G E TGTGCTCTTGGGGAA	L P P V L T G G I R N CTACCCCCGTTCTAACC GGGGGG ATACGG AAC	D K L I F GATAAACTCATCTTT	V δ 1;D δ 2F1;D δ 2F2;J δ 1 2/20–10%	0.1887	0.2852
DS2P1.1	C A L G E TGTGCTCTTGGGGAA	L P F L L W G I CTCCCC TTCCTA CTC TGGGGGATC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 1F3;D δ 2F3;J δ 1 1/20–5%	0.3774	NS
DS2P1.2	C A L G TGTGCTCTTGGG	D L T W Y W G I E G GATCTGACCTGGTAC TGGGGGATA GAGGGC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 1/20–5%	0.3774	NS
DS2P1.5	C A L G TGTGCTCTTGGG	D Q R A T W G I L K GATCAAAGGGCTACC TGGGGGATA CTAAAG	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 1/20–5%	0.3774	NS
DS2P1.19	C A L G E TGTGCTCTTGGGGAA	L E T G G L P H P W G N CTCGAAACCGGTGGC CTTCCC CACCCC TGGGGA AAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 1/20–5%	0.3774	NS
DS2P1.11	C A L G P L S L L L T E Y TGTGCTCTTGGG CCCCTTTCACTTCTCTCACCGAGTAC TGGGGGATACGG TCGTAC	W G I R S Y	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;D δ 1F1;J δ 1 1/20–5%	0.3774	NS
DS2P1.18	C A L G L P S F L N V D W W I L T G G S E S Y T D K L I F TGTGCTCTTGGGCTCCCGTCCTTCTGAATGTGGATTGGTGGATTTTAACTGGGGGATCAGAAAAGTTACACCGATAAACTCATCTTT			V δ 1;D δ 1;D δ 2;J δ 1 1/20–5%	0.3774	NS

TABLE 17 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-3)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS3P1.6	C A L G E N H M A F L R G T G G Y P L Q Y TGTGCTCTGGGGAAAATCATATGGCCTTCTACGAGGTACTGGGGGATACCCCTTCAATAC		T D K L I ACCGATAAACTCATC	V δ 1;D δ 1F3;D δ 2F1;J δ 1 2/20 – 10%	0.1884	0.2852
DS3P1.1	C A L G E TGTGCTCTGGGGAA	R T L P S Y R L G K Y CGGACATTG CCTTCCTAC CGG CTGGGG AAATAC	T D K L I ACCGATAAACTCATC	V δ 1;D δ 1F1;D δ 2F2;J δ 1 1/20 – 5%	0.3774	NS
DS3P1.2	C A L G E TGTGCTCTGGGGAA	G G P I P T L Y W G K S Y GGGGGG CCGATTCTACCTGTAC TGGGGG AAA TCGTAC	T D K L I ACCGATAAACTCATC	V δ ;D δ 1.1;D δ 2F1;D δ 2F3;J δ 1 1/20 – 5%	0.3774	NS
DS3P1.3	C A L G E TGTGCTCTGGGGAA	V P H H Y Y R G R N GTTCCGCACCATACTACCGGGGTCGGAAC	T D K L I ACCGATAAACTCATC	V δ 1;J δ 1 1/20 – 5%	0.3774	NS
DS3P1.4	C A L G E TGTGCTCTGGGGAA	L G P T G G D T P N CTAGGT CCT ACAGGAGGG GATACC CCAAAC	T D K L I ACCGATAAACTCATC	V δ 1;D δ 2F2;D δ 2F1;J δ 1 1/20 – 5%	0.3774	NS
DS3P1.5	C A L G TGTGCTCTTGGG	G T Y W G I M G GGGACCTAC TGGGGGATA ATGGGC	T D K L I ACCGATAAACTCATC	V δ 1;D δ 2F3;J δ 1 1/20 – 5%	0.3774	NS
DS3P1.7	C A L G E TGTGCTCTGGGGAA	S F T S L Y W G I R G V L Y TCTTTTACGTCCCTTTAC TGGGGGATACGC GGGGTTTTGTAC	T D K L I ACCGATAAACTCATC	V δ 1;D δ 2F3;J δ 1 1/20 – 5%	0.3774	NS
DS3P1.8	C A L G TGTGCTCTTGGG	G P P F L R Y W G M P L GGACCCCC TTCCTA CGGTAC TGGGGG ATGCCCTC	D K L I GATAAACTCATC	V δ 1;D δ 1F3;D δ 2F3;J δ 1 1/20 – 5%	0.3774	NS
DS3P1.9	C A L G TGTGCTCTTGGG	D I S R N H GATATTTCCCGTAACCAC	T D K L I ACCGATAAACTCATC	V δ 1;J δ 1 1/20 – 5%	0.3774	NS
DS3P1.10	C A L G TGTGCTCTTGGG	D E F R G I S S T L G Y GACGAATTCCGG GGGATA TCATCTACT CTCGGG TAC	T D K L I ACCGATAAACTCATC	V δ 1;D δ 2F3;D δ 2F2;J δ 1 1/20 – 5%	0.3774	NS
DS3P1.11	C A L G TGTGCTCTTGGG	D L P P S L S G V GAC CTCCCC CCGTCC CTATCTGGGGTT	T D K L I ACCGATAAACTCATC	V δ 1;D δ 1F2;D δ 1F1;J δ 1 1/20 – 5%	0.3774	NS
DS3P1.12	C A L G E TGTGCTCTGGGGAA	Q S F L R V K Y W G I Y CAGAGC TTCCTA CGGGTCAAGTAC TGGGGGATA TAC	T D K L I ACCGATAAACTCATC	V δ 1;D δ 1F3;D δ 2F3;J δ 1 1/20 – 5%	0.3774	NS

TABLE 17 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-3) continued

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS3P1.13	C A L G TGTGCTCTTGGG	D G L P L Q Y GACGGC CTTCCC CTACAATAC	T D K L I ACCGATAAACTCATC	V δ 1;D δ 1F.2;J δ 1 1/20 - 5%	0.3774	NS
DS3P1.14	C A L G E TGTGCTCTTGGGGAA	L G P S S S Y W G I R S H P H CTAGGACCTTCTCTTCATACTGGGGGATACGCTCCCACCCGCAC	T D K L I ACCGATAAACTCATC	V δ 1D δ 2F2D δ 1F1D δ 2F3J δ 1 1/20 - 5%	0.3774	NS
DS3P1.17	C A L G E TGTGCTCTTGGGGAA	V I P I L H W G S P L G Y GTAATTCCTATCCTCCAC TGGGGG TCTCCC CTGGG TAC	T D K L I ACCGATAAACTCATC	V δ 1;D δ 2F3;D δ 2F2;J δ 1 1/20 - 5%	0.3774	NS
DS3P1.18	C A L G TGTGCTCTTGGG	V A F S I L G D T G A GTCGCCTTCTCCATT CTGGGGGATACG GGAGCT	L T A Q L TTGACAGCACAACTC	V δ 1;D δ 2F2;J δ 2 1/20 - 5%	0.3774	NS
DS3P1.20	C A L G E TGTGCTCTTGGGGAA	L V T C Y P T R W G S P A CTAGTGACATGCTATCCTACGAGA TGGGGA TCCCCGGCC	D K L I GATAAACTCATC	V δ 1;D δ 2F3;J δ 1 1/20 - 5%	0.3774	NS
DS3P1.22	C A L G TGTGCTCTTGGG	D P L L G GACCCTTTG TTGGGG	D K L I GATAAACTCATC	V δ 1;D δ 2F2;J δ 1 1/20 - 5%	0.3774	NS
DS3P1.23	C A L G E TGTGCTCTTGGGGAA	P L G P CCC TTGGGG CCT	D K L I GATAAACTCATC	V δ 1;D δ 2F2;J δ 1 1/20 - 5%	0.3774	NS

TABLE 18 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-4)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS4P1.5	C A L G TGTGCTCTTGGG	D L P R G F L R L G I P S GACCTCCCGCGGGC TTCCTA CGT TTGGGG ATACCATCG	D K L I F GATAAACTCATCTTT	Vδ1;Dδ1F3;Dδ2F2;Jδ1 5/20-25%	0.0022	0.0319
DS4P1.9	C A L G TGTGCTCTTGGG	G T Y Y GGTACTTATTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;J δ 1 2/20-10%	0.1887	0.2852
DS4P1.14	C A L G E TGTGCTCTTGGGGAA	L S L H L G I R G D Y CTGAGCCTTCACCTG GGGATACGG GGAGATTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 2/20-10%	0.1887	0.2852
DS4P1.3	C A L G E TGTGCTCTTGGGGAA	L S F D W G I P R R CTTTCCTTT GACTGGGGGATC CCTCGTAGG	K L I F AAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 1/20-5%	0.3774	NS
DS4P1.4	C A L G E TGTGCTCTTGGGGAA	L G R Y W G I A CTAGGA AGGTAC TGGGGGATA GCC	D K L I F GATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 1/20-5%	0.3774	NS
DS4P1.10	C A L G TGTGCTCTTGGG	P P F H W G I R G K F G CCCCATTCCAC TGGGGGATACGC GGAAGTTTGGG	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 1/20-5%	0.3774	NS
DS4P1.11	C A L G E TGTGCTCTTGGGGAA	L P L L G D K G G CTGCCGTTA CTGGGGGAT AAAGCGGC	D K L I F GATAAACTCATCTTT	V δ 1;D δ 2F2;J δ 1 1/20-5%	0.3774	NS
DS4P1.16	C A L G TGTGCTCTTGGG	D P F P T P T G G Q I R GATCCCTTTCTACGCCT ACTGGGGG CAA ATACGG	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F1;D δ 2F3;J δ 1 1/20-5%	0.3774	NS
DS4P1.17	C A L G E TGTGCTCTTGGGGAA	P R S W G G N CCCCGAGCTGG GGGGA AAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F1;J δ 1 1/20-5%	0.3774	NS
DS4P1.18	C A L G TGTGCTCTTGGG	D P T W Y W G I E G GATCCGACCTGGTAC TGGGGGATA GAGGGC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 1/20-5%	0.3774	NS
DS4P1.21	C A L G E TGTGCTCTTGGGGAA	I S L T G G Y P ATTTCCTT ACTGGGGGATAC CCC	D K L I F GATAAACTCATCTTT	V δ 1;D δ 2F1;J δ 1 1/20-5%	0.3774	NS
DS4P1.22	C A L G TGTGCTCTTGGG	T L P T G W G P Y ACC CTCCT ACTGGC TGGGG CTTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 1F2;D δ 2F3;J δ 1 1/20-5%	0.3774	NS

TABLE 18 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-4) continued

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS4P1.23	C A L G E L Y G R L P Y I P P Y TGTGCTCTTGGGGAA	C T T A T G G T C G T C T G C C A T A T A T C C C C C C T A C	T D K L I F ACCGATAAACTCATCTTT	V δ 1;J δ 1 1/20-5%	0.3774	NS
DS4P1.24	C A L G A S F V A R S Q Y W G I N E P W G T R Q M F TGTGCTCTTGGGGCTTCATTTGTTGCTCGAAGTCAGTACTGGGGGATAAACGAGCCCTGGGGCACCCGACAGATGTTT			V δ 1;D δ 2F2;J δ 3 1/20-5%	0.3774	NS

TABLE 19 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE SKIN BIOPSY TISSUE FROM A SYSTEMIC SCLEROSIS PATIENT (SS-7)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS7T1.4	C A L G TGTGCTCTTGGG	G T Y Y GGTACTTATTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;J δ 1 8/22-36.4%	<0.0001	0.0004
DS7T1.5	C A L G E TGTGCTCTTGGGGAA	L P F L L W G I CTCCCC TTCCTA CTC TGGGGGATC	T D K L I F ACCGATAAGCTCATCTTT	V δ 1;D δ 1;D δ 2;J δ 1 6/22-27.3%	0.0003	0.0092
DS7T1.1	C A L G TGTGCTCTTGGG	D Q A Q G I W W G I R Q W W A L A GATCAGGCCCAAGGTATTTGG TGGGGGATACGC CAGTGGTGGGCGTTAGCC	D K L I F GATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 3/22-13.6%	0.0598	0.1892
DS7T1.2	C A L G TGTGCTCTTGGG	D L S P S A G G G G A GACCTATCTCCCTCG GCGGGT GGGGGG GGAGCT	L T A Q L F TTGACAGCACAACTCTTC	V δ 1;D δ 2F1;J δ 2 1/22-4.5%	0.3765	NS
DS7T1.10	C A TGTGCT	S P P S L R P G P TCCCCA CCTTCC TTGAGACCCGGGCC	D K L I F GATAAACTCATCTTT	V δ 1;D δ 1F1;J δ 1 1/22-4.5%	0.3765	NS
DS7T1.21	C A L G TGTGCTCTTGGG	D L F L P L P Y W G I Q GACCTC TTCCTA CCG CTTCCC TAC TGGGGGATA CAG	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 1;D δ 2;J δ 1 1/22/4.5%	0.3765	NS
DS7T1.3	C A L G E TGTGCTCTTGGGGAA	C V F L R F F G G P V A R L L G S P R L L Y TGTGCTCTTGGGGAAATGTGTCTTCCTACGATTCTTTGGGGGACCCGTCGCCAGACTTCTGGGCTCACCAAGACTGCTGTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 1;D δ 2;J δ 1 1/22/4.5%	0.3765	NS
DS7T1.17	C A L G E TGTGCTCTTGGGGAA	C A F L L W G I G H E L I F V K G T R V M V E A TGTGCTCTTGGGGAAATGCGCCTTCCTACTATGGGGGATCGGACATGAACTCATATTTGTGAAAGGAACAAGAGTGATGGTGAAGCT	K I L F AAAATCCTCTTT	V δ 1;D δ 1;D δ 2;J δ 1 1/22/4.5%	0.3765	NS

TABLE 20 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-7)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS7P1.1	C A L G E TGTGCTCTTGGGGAA	L P F L L W G I CTCCCC TTCCTA CTC TGGGGGATC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 1F3;D δ 2F3;J δ 1 10/20–50%	<0.0001	<0.0001
DS7P1.3	C A L G TGTGCTCTTGGG	D P T W Y W G I E G GATCCGACCTGGTAC TGGGGGATA GAGGGC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 3/20–15%	0.0596	0.1901
DS7P1.9	C A L G D Q A Q G I W W G I R Q W W A L A D K L I F TGTGCTCTTGGGGATCAGGCCCAAGGTATTTGGTGGGGGATACGCCAGTGGTGGGCGTTAGCCGATAAACTCATCTTT			V δ 1;D δ 2F3;J δ 1 2/20–10%	0.1887	0.2852
DS7P1.4	C A L G TGTGCTCTTGGG	D L S P S A G G G G A GACCTATCTCCCTCGGCGGGT GGGGGG GGAGCT	L T A Q L F TTGACAGCACAACCTCTTC	V δ 1;D δ 2F1;J δ 2 1/20–5%	0.3774	NS
DS7P1.13	C A L G E TGTGCTCTTGGGGAA	L P S L P W G I CTCCCCTCCCTACCC TGGGGGATC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 1/20–5%	0.3774	NS
DS7P1.21	C A L G TGTGCTCTTGGG	D L F L P L P Y W G I Q GACCTC TTCCTA CCG CTTCCC TAC TGGGGGATA CAG	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 1F3;D δ 1F2;D δ 2F3;J δ 1 1/20–5%	0.3774	NS
DS7P1.19	C A L G E C V F L Q F F G G A D A R L V G S P T L M Y T D K L TGTGCTCTTGGGGAATGTGTCTTCCTACAATTCTTTGGGGGAGCCGACGCCAGACTTGTGGGCTCACCAACTGATGTACACCGATAAACTC			V δ 1;D δ 1;D δ 2;J δ 1	0.3774	
DS7P1.24	C A L G E C V F L R F F G G P V A R L L G S P R L L Y T D K L TGTGCTCTTGGGGAATGTGTCTTCCTACGATTCTTTGGGGGACCCGTCGCCAGACTTCTGGGCTCACCAAGACTGCTGTACACCGATAAACTC			V δ 1;D δ 1;D δ 2;J δ 1	0.3774	

TABLE 21 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE SKIN BIOPSY TISSUE FROM A SYSTEMIC SCLEROSIS PATIENT (SS-11)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS11T1.2	C A L G TGTGCTCTTGGG	N K P H W G Y A S F T Y AATAAACCCAC TGGGGA TACGCGTCCTTCACCTAC	T D K L I F ACCGATAAACTCATCTTT	Vδ1;Dδ2F3;Jδ1 14/22–63.6%	<0.0001	<0.0001
DS11T1.1	C A L G E TGTGCTCTTGGGGAG	L T I D W G I H CTAACAATC GACTGGGGGATA CAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 1/22–4.5%	0.3765	NS
DS11T1.4	C A L G TGTGCTCTTGGG	D R L L F L T G G N GATCGCCTTCTTTTTTA ACGGGGGGA AAT	K L I F AAACTCATCTTT	V δ 1;D δ 2F1;J δ 1 1/22–4.5%	0.3765	NS
DS11T1.6	C A L G E TGTGCTCTTGGGGAA	P P R T G D I CCACCCCGTACT GGGGAC ATC	D K L I F GATAAACTCATCTTT	V δ 1;D δ 2F2;J δ 1 1/22–4.5%	0.3765	NS
DS11T1.8	C A L G TGTGCTCTTGGG	T R Q G D T R F V L Y ACCCGGCAG GGGGATACG CGTTTGTCTTTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F2;J δ 1 1/22–4.5%	0.3765	NS
DS11T1.10	C A L G E TGTGCTCTTGGGGAG	C Y P W Q F P Y W G I P G TGTTACCCCTGGCAATTTCCGTAC TGGGGGATA CCGGGG	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 1/22–4.5%	0.3765	NS
DS11T1.14	C A L G E TGTGCTCTTGGGGAA	P G S I G Y R I G P CCAGGCTCTATTGGGTACAGAATAGGCCCC	D K L I F GATAAACTCATCTTT	V δ 1;J δ 1 1/22–4.5%	0.3765	NS
DS11T1.16	C A L G TGTGCTCTTGGG	L F L H TTA TTCCTA CAC	D K L I F GATAAACTCATCTTT	V δ 1;D δ 1F3;J δ 1 1/22–4.5%	0.3765	NS
DS11T1.18	C A L G TGTGCTCTTGGG	D P T W Y W G I E G GATCCGACCTGGTAC TGGGGGATA GAGGGC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 1/22–4.5%	0.3765	NS
DS11T1.23	C A L G E TGTGCTCTTGGGGAA	L A P A P V W G I P S G D R T D E L I F CTAGCTCCCGCACCCGTC TGGGGGATA CCCAGC GGGGAC AGA ACCGATGAACTCATCTTT	T D E L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 1/22–4.5%	0.3765	NS

TABLE 22 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE SKIN BIOPSY TISSUE FROM A SYSTEMIC SCLEROSIS PATIENT (SS-16)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS16T1.4	C A L G E H S S D Y Y W G I R G G TGTGCTCTTGGGGAA	CATTCCTCCGATTACTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 3/20-15%	0.0596	0.1901
DS16T1.2	C A L G E A T L L G V T G R P TGTGCTCTTGGGGAA	GCTACTTTG CTGGGG	D K L I F GATAAACTCATCTTT	V δ 1;D δ 2F2;J δ 1 2/20-10%	0.1887	0.2852
DS16T1.12	C A L G K A L P D W G I L P TGTGCTCTTGGG	AAGGCCCTTCCG GACTGGGGGATA	D K L I F GATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 2/20-10%	0.1887	0.2852
DS16T1.14	C A L G D W G T R A P TGTGCTCTTGGG	GACTGGGGA ACCCGGGCCCCG	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 2/20-10%	0.1887	0.2852
DS16T1.16	C A L G E G P Q Y TGTGCTCTTGGGGAG	GGTCCCCAGTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;J δ 1 2/20-10%	0.1887	0.2852
DS16T1.1	C A L G E L P T F P V P Y W G I R P Y TGTGCTCTTGGGGAA	CTCCCGACCTTCCCGGTACCCTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 1/20-5%	0.3774	NS
DS16T1.6	C A L G G P Y S Y P N V L G D S Y TGTGCTCTTGGG	GGGCCCTAT TCCTAC CCGAATGTA	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 1F1,2F2;J δ 1 1/20-5%	0.3774	NS
DS16T1.10	C A L G D P I F L S G R T G G L TGTGCTCTTGGG	GACCCTATC TTCCTA TCGGGACGT	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 1F3,2F1;J δ 1 1/20-5%	0.3774	NS
DS16T1.18	C A L G A I S G V V P E P P Y W G I R R G L S W D T R TGTGCTCTTGGGGCCATATCGGGGGT	CGTTCCTGAACCCCGTACTGGGGGATACGTCGAGGGCTATCCTGGGACACCCGA		V δ 1;D δ 2F3;J δ 3 1/20-5%	0.3774	NS
DS16T1.23	C A L G E L I T G D T K K W D TGTGCTCTTGGGGAA	TTGATAACG GGGGATACG	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F2;J δ 1 1/20-5%	0.3774	NS
DS16T1.28	C A L G G P S F H R T G G Y I F L TGTGCTCTTGGG	GGACCATCCTTCCACCGT ACTGGGGTTAC	D K L I F GATAAACTCATCTTT	V δ 1;D δ 2F1,1F3;J δ 1 1/20-5%	0.3774	NS
DS16T1.29	C A L G E S S Y L G L L G D Y TGTGCTCTTGGGGAA	AGT TCCTAC TTAGGTTA	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 1F1,2F2;J δ 1 1/20-5%	0.3774	NS

TABLE 22 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE SKIN BIOPSY TISSUE FROM A SYSTEMIC SCLEROSIS PATIENT (SS-16) continued

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS16T1.33	C A L G E TGTGCTCTTGGGGAG	I A L P N W A P A L T Y ATTGCCTTGCCCAACTGGGCCCTGCCCTTACCTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;J δ 1 1/20-5%	0.3774	NS
DS16T1.44	C A L G TGTGCTCTTGGG	G L M P S Y A Y T G G Y GGTTTAATG CCTTCCTAC GCTTAT	A E Y T D K L I F ACTGGAGGATAC GCGGAATAC ACCGATAAACTCATCTTT	V δ 1;D δ 1F1,2F1;J δ 1 1/20-5%	0.3774	NS

TABLE 23 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-16)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS16P1.4	C A L G TGTGCTCTTGGC	A Q S P GCCCAGTCCCCT	F G TTTGGA	V δ 1;J δ 1 2/16-12.5%	NS	NS
DS16P1.3	C A L G TGTGCTCTTGGG	Q G W G K Y CAGGGT TGGGGG AAGTAC	T D K L I F G ACCGATAAACTCATCTTTGGA	V δ 1;D δ 2F3;J δ 1 1/16-6.25%	NS	NS
DS16P1.6	C A L G E TGTGCTCTTGGGGAA	L W G I K CTC TGGGGGATA AAG	S W D T R Q M TCCTGGGACACCCGACAGATG	V δ 1;D δ 2F3;J δ 3 1/16-6.25%	NS	NS
DS16P1.8	C A L G TGTGCTCTTGGG	V S S Y A G V R S P G GTCTCT TCCTAC GCGGGGTTGCTCCCCGGGC	T D K L I F G ACCGATAAACTCATCTTTGGA	V δ 1;D δ 1F1;J δ 1 1/16-6.25%	NS	NS
DS16P1.9	C A L G TGTGCTCTTGGG	V E A F L P F D R GTAGAGGCC TTCCTA CCTTTGATCGC	D K L I F G GATAAACTCATCTTTGGA	V δ 1;D δ 1F3;J δ 1 1/16-6.25%	NS	NS
DS16P1.10	C A L G E TGTGCTCTTGGGGAA	R V W G I R R L CGGGTC TGGGGGATAAAGG AGGCTC	W D T R Q M TGGGACACCCGACAGATG	V δ 1;D δ 2F3;J δ 3 1/16-6.25%	NS	NS
DS16P1.12	C A L G TGTGCTCTTGGG	K A S Y F P Y AAGGCCTCGTACTTTCCGTAC	T D K L I F G ACCGATAAACTCATCTTTGGA	V δ 1;J δ 1 1/16-6.25%	NS	NS
DS16P1.14	C A L G TGTGCTCTTGGG	P K E A S N N W G G L CCAAAGGAGGCTTCCAATAACTGG GGGGGC CTT	K L I F G AAACTCATCTTTGGA	V δ 1;D δ 2F1;J δ 1 1/16-6.25%	NS	NS
DS16P1.15	C A L G TGTGCTCTTGGG	V R N S GTACGGAATTCA	L T A Q L F F CTGACAGCACAACCTCTTCTTT	V δ 1;J δ 2 1/16-6.25%	NS	NS
DS16P1.16	C A L G TGTGCTCTTGGGA	G V H Y W G N P E G GGAGTCCACTAC TGGGGG AACCCCGAGGGG	T D K L I F G ACCGATAAACTCATCTTTGGA	V δ 1;D δ 2F3;J δ 1 1/16-6.25%	NS	NS
DS16P1.17	C A L G E TGTGCTCTTGGGGAA	L S S P P P I L G D A CTAAGTCCCCGCCCCCTATA CTGGGGGAT GCT	D K L I F G GATAAACTCATCTTTGGA	V δ 1;D δ 2F2;J δ 1 1/16-6.25%	NS	NS
DS16P1.20	C A L G E TGTGCTCTTGGGGAA	L F F P P R R R S Y CTATTTTTTCCCCTCGGCGGGAGTTAC	T D K L I F G ACCGATAAACTCATCTTTGGA	V δ 1;J δ 1 1/16-6.25%	NS	NS

TABLE 23 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-16) continued

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS16P1.22	C A L G E TGTGCTCTTGGGGAA	L G P T G A G S L CTCGGGCCTACCGGGGCTGGTAGTCTT	T D K L I F G ACCGATAAACTCATCTTTGGA	V δ 1;J δ 1 1/16-6.25%	NS	NS
DS16P1.23	C A L G E TGTGCTCTTGGGGAA	L G F G Y W G I H P CTAGGTTTCGGGTAC TGGGGGATA CACCCC	W D T R Q M TGGGACACCCGACAGATG	V δ 1;D δ 2F3;J δ 3 1/16-6.25%	NS	NS
DS16P1.24	C A L G TGTGCTCTTGGG	G L P T E A GGT CTCCT ACTGAGGCC	D K L I F G GATAAACTCATCTTTGGA	V δ 1;D δ 1F2;J δ 1 1/16-6.25%	NS	NS

TABLE 24 – SUMMARY TABLE OF CLONAL EXPANSION OF V δ 1-CHAIN TRANSCRIPTS IN SS_c PATIENT SAMPLES

Tissue	Patient	Clone	CDR3 region	Identity	Frequency	Statistics	Clonal Expansion (Yes/No)
PBMC	SS1P δ 1	clone 8	CALG <u>NKPH</u> WG <u>YASFTY</u> TDKLIF	V δ 1;D δ 2F3;J δ 1	8/24-33.3%	p<0.0001	Yes
PBMC	SS2P δ 1	clone 6	CAL <u>ELHPFY</u> WGI <u>LY</u> TDKLIF	V δ 1;D δ 2F3;J δ 1	5/20-25%	p=0.0022	Yes
PBMC	SS3P δ 1	clone 6	CALGE <u>NHMA</u> FL <u>RG</u> TGGY <u>PLOQY</u> TDKLIF	V δ 1;D δ 1F3;D δ 2F1;J δ 1	2/20-10%	p=0.1884	No
PBMC	SS4P δ 1	clone 5	CALG <u>DLPRG</u> FL <u>R</u> LG <u>IPS</u> DKLIF	V δ 1;D δ 1F3;D δ 2F2;J δ 1	5/20-25%	p=0.0022	Yes
PBMC	SS7P δ 1	clone 1	CALGE <u>LP</u> FL <u>L</u> WGI TDKLIF	V δ 1;D δ 1F3;D δ 2F3;J δ 1	10/20-50%	p<0.0001	Yes
PBMC	SS16P δ 1	clone 4	CALG <u>AQSP</u> FG	V δ 1;J δ 1	2/16-12.5%	p>0.05	No
Biopsy	SS7T δ 1	clone 4	CALG <u>GTYY</u> TDKLIF	V δ 1;J δ 1	8/22-36.4%	p<0.0001	Yes
		clone 5	CALGE LP FL <u>L</u> WGI TDKLIF	V δ 1;D δ 1;D δ 2;J δ 1	6/22-27.3%	p=0.0003	Yes
Biopsy	SS11T δ 1	clone 2	CALG <u>NKPH</u> WG <u>YASFTY</u> TDKLIF	V δ 1;D δ 2F3;J δ 1	14/22-63.6%	p<0.0001	Yes
Biopsy	SS16T δ 1	clone 4	CALGE <u>HSSDYY</u> WGIR GG TDKLIF	V δ 1;D δ 2F3;J δ 1	3/20-15%	p=0.0596	No

Statistical analysis by binomial distribution (p<0.05 is statistically significant)

In the PBMC from patient SS-1 (see table 15), V δ 1 chain analysis revealed 8 out of 24 (33.3%) transcripts had identical CDR3 regions (transcript #8), representing a statistically significant clonal expansion; CALG NKPH WG YASFTY TDKLIF, V δ 1;D δ 2F3;J δ 1, $p < 0.0001$. There were five clones with 2 out of 24 (8.3%) identical transcripts, but these represented a statistically insignificant clonal expansion ($p > 0.05$). In addition, there were five clones, each with unique CDR3 regions (1 out of 24). For patient SS-2 (see table 16), 5 out of 20 (25%) transcripts had identical CDR3 regions (transcript #6), representing a statistically significant clonal expansion; CAL ELHPFY WGI LY TDKLIF, V δ 1;D δ 2F3;J δ 1, $p = 0.0022$. There were four clones with 3 out of 25 (12%), or 2 out of 25 (8%) identical transcripts, but these represented a statistically insignificant clonal expansion ($p > 0.05$). The remaining six transcripts had unique CDR3 regions (1 out of 25). The PBMC from patient SS-3 (see table 17), had no statistically significant clonally-expanded transcripts, but had eighteen clones, each with a unique CDR3 region (1 out of 20), and a clone with 2 out of 20 (10%) identical transcripts that represented a statistically insignificant clonal expansion ($p > 0.05$). Hence, the V δ 1 transcripts in this patient were polyclonal. In the PBMC from patient SS-4 (see table 18), there were 5 out of 20 (25%) identical transcripts (transcript #5), representing a statistically significant clonal expansion; CALG DLPRG FL R LG IPS DKLIF, V δ 1;D δ 1F3;D δ 2F2;J δ 1, $p = 0.0022$. There were two clones with 2 out of 20 (10%) identical transcripts, but these represented a statistically insignificant clonal expansion ($p > 0.05$). Eleven clones each had transcripts with a unique CDR3 region (1 out of 20). For patient SS-7 (see table 20), V δ 1 chain analysis of the PBMC revealed that 10 out of 20 (50%) transcripts were identical (transcript #1), representing a statistically significant clonal expansion; CALGE LP FL L WGI TDKLIF, V δ 1;D δ 1F3;D δ 2F3;J δ 1, $p < 0.0001$. There

were two clones with 3 out of 20 (15%), or 2 out of 20 (10%) identical transcripts, but these represented a statistically insignificant clonal expansion ($p>0.05$). The remaining five clones had unique CDR3 regions (1 out of 20). The PBMC from patient SS-16 (see table 23), had no statistically significant clonally-expanded transcripts, but had fourteen clones, each with unique CDR3 regions (1 out of 16), and a clone with 2 out of 16 (12.5%) identical transcripts that represented a statistically insignificant clonal expansion ($p>0.05$). Hence, the V δ 1 transcripts in this patient were polyclonal.

In the skin biopsy sample of patient SS-7 (see table 19), V δ 1 chain analysis revealed 8 out of 22 (36.4%) identical transcripts (transcript #4); CALG GTYY TDKLIF, V δ 1;J δ 1, $p<0.0001$; 6 out of 22 (27.3%) identical transcripts (transcript #5); V δ 1;D δ 1;D δ 2;J δ 1, $p=0.0003$, both representing statistically significant clonal expansions. There were 3 out of 22 (13.6%) identical transcripts, which was a statistically insignificant clonal expansion ($p>0.05$). The remaining five clones each had unique CDR3 region transcripts (1 out of 22). There were transcripts that were present in both the skin biopsy of patient SS-7 and in the PBMC (see table 19 and 20). These included the clonally-expanded skin biopsy transcript #5 (6/22) and PBMC transcript #1 (10/20); CALGE LP FL L WGI TDKLIF, the skin biopsy transcript #17 and PBMC transcript #24, CALGE CV FL RFF GG PVARL LG SPRLLY TDKLIF, the skin biopsy transcript #1 and PBMC transcript #9, CALG DQAQGIW WGIR QWWALA DKLIF, the skin biopsy transcript #2 and PBMC transcript #4, CALG DLSPSAG GG GA LTAQLF; and finally, the skin biopsy transcript #21 and PBMC transcript #21 CALG DL FL P LP Y WGI Q TDKLIF. For patient SS-11 (see table 21), V δ 1 chain analysis in the skin biopsy revealed the presence of 14 out of 22 (63.6%) identical transcripts (transcript #2); CALG NKPH WG YASFTY TDKLIF, V δ 1;D δ 2F3;J δ 1,

$p < 0.0001$, which was a statistically significant clonal expansion. The rest of the nine clones each had unique CDR3 transcripts (1 out of 22). The skin biopsy of patient SS-16 (see table 22), had no statistically significant clonal expansion, but had five clones, each with 3 out of 20 (15%), or 2 out of 20 (10%) identical transcripts, representing statistically insignificant clonal expansion ($p > 0.05$). The remaining nine clones each had unique CDR3 region transcripts (1 out of 20). The deduced amino acid sequence of all V δ 1 transcripts analyzed revealed neither amino acid sequence homology nor the presence of conserved motifs, when compared using BLAST searches to transcripts in the GENBANK database, suggesting these were unique, previously unidentified, clones.

4.2.4 Sequencing results of V δ 2-chain TCR Transcripts

In the current study, V δ 2 TCR chain transcripts from skin biopsy of 2 patients with SSc, and peripheral blood mononuclear cells (PBMC) of 7 patients, including the 2 SSc patients (SS-4 and SS-16) from which both skin biopsies and PBMC were obtained, were amplified, cloned, and sequenced. The predicted amino acid sequence of the transcripts was generated using the ExPASy proteomics server. Results revealed the presence of statistically significant substantial proportions of multiple identical V δ 2-chain TCR+ T-cell transcripts in the SSc skin biopsy of 2 out of 2 patients, and peripheral blood of 3 out of 7 patients, suggesting the presence of oligoclonal populations of T-cells infiltrating the SSc lesions. No conserved sequences were identified in the δ -chain transcripts, nor were there any homologies after comparison to δ -chain transcripts in the GENBANK database using BLAST searches, suggesting that these clones were previously unidentified and were therefore novel.

TABLE 25 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-1)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS1P2.2	C A C D T TGTGCCTGTGACACC	E E T GAGGAAACA	T D K L I F ACCGATAAACTCATCTTT	V δ 2;J δ 1 4/22-18.1%	0.0135	0.0890
DS1P2.1	C A C D T TGTGCCTGTGACACC	G Y G D T R GGTTAT GGGGATACG CGT	S S W D T R AGCTCCTGGGACACCCGA	V δ 2;D δ 2F2;J δ 3 3/22-13.6%	0.0598	0.1892
DS1P2.10	C A C D T TGTGCCTGTGACACT	P S T G A L Y CCTTCT ACGGGCGCCTTATAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 1F1;J δ 1 3/22-13.6%	0.0598	0.1892
DS1P2.4	C A C D TGTGCCTGTGAT	P T G L G I R V CCT ACCGGGCTG GGGATACGA GTA	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F3;J δ 1 1/22-4.5%	0.3765	NS
DS1P2.5	C A C D T TGTGCCTGTGACACT	V G D R G GTGGGGGAT AGGGGT	K L I F AAACTCATCTTT	V δ 2;D δ 3;J δ 1 1/22-4.5%	0.3765	NS
DS1P2.6	C A C D T TGTGCCTGTGACACC	P P V L G S R G CCACCGGTA CTGGGC TCGAGGGGG	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/22-4.5%	0.3765	NS
DS1P2.7	C A C D T TGTGCCTGTGACACC	L S T T G G Y H CTTTCCACT ACTGGGGGATAT CAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F1;J δ 1 1/22-4.5%	0.3765	NS
DS1P2.8	C A C D TGTGCCTGTGAC	L L G V P Y Y CTC CTGGGG GTTCCCTACTAT	K L I F AAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/22-4.5%	0.3765	NS
DS1P2.9	C A C D T TGTGCCTGTGACACC	D Y G D T R GATTAT GGGGATACT CGT	S S W D T R AGCTCCTGGGACACCCGA	V δ 2;D δ 2F2;J δ 3 1/22-4.5%	0.3765	NS
DS1P2.11	C A C D T TGTGCCTGTGACACC	L G I L Y TTG GGGATT CTGTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F3;J δ 1 1/22-4.5%	0.3765	NS
DS1P2.14	C A C D TGTGCCTGTGAC	S R T G G Y R Y TCCCGG ACTGGGGGATAT CGGTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F1;J δ 1 1/22-4.5%	0.3765	NS
DS1P2.18	C A C D T TGTGCCTGTGACACC	G D T G G W Y GGGGATACT GGGGGC TGGTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;D δ 2F1;J δ 1 1/22-4.5%	0.3765	NS

TABLE 25 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-1) continued

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS1P2.20	C A C D TGTGCCTGTGAT	P N E T CCCAACGAAACA	T D K L I F ACCGATAAACTCATCTTT	V δ 2;J δ 1 1/22-4.5%	0.3765	NS
DS1P2.21	C A C D T TGTGCCTGTGACACC	D W G R GACTGGGGG AGA	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F3;J δ 1 1/22-4.5%	0.3765	NS
DS1P2.24	C A C D TGTGCCTGTGAC	V L G D T N GTA CTGGGGGATACG AAC	D K L I F GATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/22-4.5%	0.3765	NS

TABLE 26 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-2)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS2P2.1	C A C D T TGTGCCTGTGACACA	L G D CTGGGGGAT	S S R D T R AGTTCCCGGGACACCCGA	V δ 2;D δ 2F2;J δ 3 2/21-9.5%	0.1884	0.2844
DS2P2.10	C A C D TGTGCCTGTGAC	I L G E ATT CTGGGG GAA	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 2/21-9.5%	0.1884	0.2844
DS2P2.3	C A C D TGTGCCTGTGAC	P V L G E K Y CCGGTA CTGGGG GAAAAGTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/21-4.7%	0.3769	NS
DS2P2.4	C A C D T TGTGCCTGTGATACG	L L G D I N TTA CTGGGGGAT ATCAAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/21-4.7%	0.3769	NS
DS2P2.5	C A C D T TGTGCCTGTGACACC	A G T G G Y G GCGGGT ACTGGGGGATAC GGC	S W D T R TCCTGGGACACCCGA	V δ 2;D δ 2F1;J δ 3 1/21-4.7%	0.3769	NS
DS2P2.6	C A C D TGTGCCTGTGAC	R V G D AGA GTGGGGGAT	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 3;J δ 1 1/21-4.7%	0.3769	NS
DS2P2.7	C A C D T TGTGCCTGTGACACC	V G N P D GTGGGG AACCCGGAC	D K L I F GATAAACTCATCTTT	V δ 2;D δ 3;J δ 1 1/21-4.7%	0.3769	NS
DS2P2.9	C A C D TGTGCCTGTGAC	N V L G D T R AACGTA CTGGGGGATACG AGA	W D T R TGGGACACCCGA	V δ 2;D δ 2F2;J δ 3 1/21-4.7%	0.3769	NS
DS2P2.12	C A C D TGTGCCTGTGAC	A V G E K G G GCG GTGGGG GAAAAA GGGGGC	D K L I F GATAAACTCATCTTT	V δ 2; D δ 3;D δ 2F1;J δ 1 1/21-4.7%	0.3769	NS
DS2P2.13	C A C D T TGTGCCTGTGACACC	L G D P Y S TTGGGGGAT CCCTACTCC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/21-4.7%	0.3769	NS
DS2P2.14	C A C D T TGTGCCTGTGACACG	M V T G D T Q ATGGTTACG GGGGATACG CAG	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/21-4.7%	0.3769	NS
DS2P2.16	C A C D T TGTGCCTGTGACACC	V L G E T GTT CTGGGG GAAACC	W D T R TGGGACACCCGA	V δ 2;D δ 2F2;J δ 3 1/21-4.7%	0.3769	NS

TABLE 26 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-2) continued

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS2P2.17	C A C D TGTGCCTGTGAC	P V L G E K Y CCGGTA CTGGGG GAAAAGTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/21-4.7%	0.3769	NS
DS2P2.18	C A C D TGTGCCTGTGAC	S M G D T Q A AGTATG GGGGATACG CAAGCT	L T A Q L F TTGACAGCACAACCTCTC	V δ 2;D δ 2F2;J δ 2 1/21-4.7%	0.3769	NS
DS2P2.20	C A C D T TGTGCCTGTGACACC	L P L G D G T G TTGCC CTGGGGGAT GGT ACTGGA	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;D δ 2F1;J δ 1 1/21-4.7%	0.3769	NS
DS2P2.21	C A C D TGTGCCTGTGAC	E G D I T GAG GGGGAT ATCACC	S W D T R TCCTGGGACACCCGA	V δ 2;D δ 2F2;J δ 3 1/21-4.7%	0.3769	NS
DS2P2.22	C A C D T TGTGCCTGTGACACG	A E T G G F GCTGAG ACTGGGGC TTC	S S W D T R AGCTCCTGGGACACCCGA	V δ 2;D δ 2F1;J δ 3 1/21-4.7%	0.3769	NS
DS2P2.23	C A C D TGTGCCTGTGAC	R L G G T CGACTG GGGGGT ACC	W D T R TGGGACACCCGA	V δ 2;D δ 2F1;J δ 3 1/21-4.7%	0.3769	NS
DS2P2.24	C A C D TGTGCCTGTGAC	S V L G D T K TCCGTA CTGGGGGATACG AAG	S W D T R TCCTGGGACACCCGA	V δ 2;D δ 2F2;J δ 3 1/21-4.7%	0.3769	NS

TABLE 27 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-3)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS3P2.22	C A C D T TGTGCCTGTGACACC	P G G A T L V CCT GGGGGA GCCACCTTGGTC	W D T R Q TGGGACACCCGACAG	V δ 2;D δ 2F1;J δ 3 3/20 – 15%	0.0596	0.1901
DS3P2.4	C A C D T TGTGCCTGTGACACC	A W G I R D GCG TGGGGGATACGC GAC	S W D T R Q TCCTGGGACACCCGACAG	V δ 2;D δ 2F3;J δ 3 1/20 – 5%	0.3774	NS
DS3P2.5	C A C D T TGTGCCTGTGACACA	I T G E D V Y ATAACTGGGGAGGACGTGTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;J δ 1 1/20 – 5%	0.3774	NS
DS3P2.8	C A C D T TGTGCCTGTGACAGC	G N S G G R T Q G I P GGCAATAGC GGGGGA AGGACCCAG GGCATTCCC	W D T R Q TGGGACACCCGACAG	V δ 2;D δ 2F1;J δ 3 1/20 – 5%	0.3774	NS
DS3P2.13	C A C D T TGTGCCTGTGACACG	I S G G G I A ATATCCGGCGGG GGGATA GCC	W D T R Q TGGGACACCCGACAG	V δ 2;D δ 2F3;J δ 3 1/20 – 5%	0.3774	NS
DS3P2.15	C A C D TGTGCCTGTGAC	R V G T R D R V L G D T R CGG GTGGGC ACCCGAGACCGTGTA CTGGGGGATACG CGA	K L I F AAACTCATCTTT	V δ 2;D δ 3;D δ 2F2;J δ 1 1/20 – 5%	0.3774	NS
DS3P2.16	C A C D TGTGCCTGTGAC	R V L G D L AGGGTA CTGGGGGAC CTC	S S W D T R Q AGCTCCTGGGACACCCGACAG	V δ 2;D δ 2F2;J δ 3 1/20 – 5%	0.3774	NS
DS3P2.24	C A C TGTGCCTGT	A E L L G D T R G A D GCCGAATTA CTGGGGGATACG AGGGGGGCGGAC	D K L I F GATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/20 – 5%	0.3774	NS
DS3P2.26	C A C D T TGTGCCTGTGACACC	V G G G P Y GTTGGT GGGGG CCCTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 3;D δ 2F1;J δ 1 1/20 – 5%	0.3774	NS
DS3P2.29	C A C D TGTGCCTGTGAC	P L N T G G R D CCTTTAAAT ACTGGGGGA CGTGAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F1;J δ 1 1/20 – 5%	0.3774	NS
DS3P2.31	C A C D T TGTGCCTGTGACACA	L P V P V L G D T CTTCCT GTGCCGGTA CTGGGGGACACC	D K L I F GATAAACTCATCTTT	V δ 2;D δ 1F2;D δ 2F2;J δ 1 1/20 – 5%	0.3774	NS
DS3P2.33	C A C D TGTGCCTGTGAC	S V V T G G Q S V Y TCCGTGGTC ACTGGGGGA CAATCAGTGTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F1;J δ 1 1/20 – 5%	0.3774	NS

TABLE 27 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-3) continued

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS3P2.34	C A C D TGTGCCTGTGAT	A V L G G GCCGTACTG GGGGGA	D K L I F GATAAACTCATCTTT	V δ 2;D δ 2F1;J δ 1 1/20 - 5%	0.3774	NS
DS3P2.36	C A C D TGTGCCTGTGAC	Q P V L G D CAACCCGTA CTGGGGGAC	S S W D T R Q AGCTCCTGGGACACCCGACAG	V δ 2;D δ 2F2;J δ 3 1/20 - 5%	0.3774	NS
DS3P2.37	C A C D TGTGCCTGTGAC	A Y D W G P L R GCCTATGAT TGGGGG CCCTAAGG	S W D T R Q TCCTGGGACACCCGACAG	V δ 2;D δ 2F3;J δ 3 1/20 - 5%	0.3774	NS
DS3P2.41	C A C D T TGTGCCTGTGACACC	L Y S Y G T G F TTATAT TCCTAC GGC ACTGGT TTC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 1F1;D δ 2F1;J δ 1 1/20 - 5%	0.3774	NS
DS3P2.44	C A C D TGTGCCTGTGAC	G L G D N P G GGG CTGGGGGAT AATCCGGGG	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/20 - 5%	0.3774	NS
DS3P2.47	C A C D TGTGCCTGTGAC	V L G D D L L Y GTA CTGGGGGAC GATCTACTGTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/20 - 5%	0.3774	NS

TABLE 28 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE SKIN BIOPSY TISSUE FROM A SYSTEMIC SCLEROSIS PATIENT (SS-4)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS4T2.2	C A C D T TGTGCCTGTGACACC	V R G T A GTGAGGGGGACCGG	S S W D T R AGCTCCTGGGACACCCGA	Vδ2;Jδ3 4/22-18%	0.0135	0.0899
DS4T2.5	C A C D TGTGCCTGTGAC	G M M G D T Q A L GGGATGATG GGGGATACG CAAGCCCTC	S W D T R TCCTGGGACACCCGA	V δ 2;D δ 2F2;J δ 3 2/22-9%	0.1882	0.2838
DS4T2.6	C A C D T TGTGCCTGTGACACC	V G H T G D L G R E GTGGGG CACACGGGGGACCTCGGGCGGGAA	L I F CTCATCTTT	V δ 2;D δ 3;J δ 1 2/22-9%	0.1882	0.2838
DS4T2.11	C A C D TGTGCCTGTGAC	D V R G L G G GACGTGAGGGGA CTGGGT GGT	K L I F AAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 2/22-9%	0.1882	0.2838
DS4T2.1	C A C D T TGTGCCTGTGACACC	V G G S L G GTC GGGGGA TCTCTCGGG	D T R GACACCCGA	V δ 2;D δ 2F1;J δ 3 1/22-4.5%	0.3765	NS
DS4T2.9	C A C D TGTGCCTGTGAC	R L G G R S CGACTG GGGGGA CGATCC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F1;J δ 1 1/22-4.5%	0.3765	NS
DS4T2.10	C A C D T TGTGCCTGTGACACC	V T G G P S E G GTG ACTGGGGGA CCGTCGGAGGGT	D K L I F GACAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/22-4.5%	0.3765	NS
DS4T2.12	C A C TGTGCCTGT	E D W G R GAG GACTGGGGG CGT	S S W D T R AGCTCCTGGGACACCCGA	V δ 2;D δ 2F3;J δ 3 1/22-4.5%	0.3765	NS
DS4T2.13	C A C D TGTGCCTGTGAC	S L L G D TCCTTA CTGGGGGAT	T D K L I F ACCGACAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/22-4.5%	0.3765	NS
DS4T2.14	C A C D TGTGCCTGTGAC	R V L G I R AGGGTACTG GGGATCCGA	S S W D T R AGCTCCTGGGACACCCGA	V δ 2;D δ 2F3;J δ 3 1/22-4.5%	0.3765	NS
DS4T2.16	C A C D T TGTGCCTGTGACACC	V A G G D H G Q D Y GTGGCCGGAGGGGACCACGGACAGGACTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;J δ 1 1/22-4.5%	0.3765	NS

TABLE 28 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF Vδ2 SUBGROUP δ-CHAIN TCR TRANSCRIPTS IN THE SKIN BIOPSY TISSUE FROM A SYSTEMIC SCLEROSIS PATIENT (SS-4) continued

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS4T2.17	C A C D TGTGCCTGTGAC	G M M G D T Q A L S W G GGGATGATG GGGGATACG CAAGCCCTCTCC TGGGGC	T R ACCCGA	Vδ2;Dδ2F2;Dδ2F3;Jδ3 1/22-4.5%	0.3765	NS
DS4T2.18	C A C D T TGTGCCTGTGACACG	V P V L G A S Y GTACCCGTA CTGGGG GCGAGTTAC	T D K L I F ACCGATAAACTCATCTTT	Vδ2;Dδ2F2;Jδ1 1/22-4.5%	0.3765	NS
DS4T2.19	C A C D TGTGCCTGTGAT	G V A S G D T S GGTGTGGCTTCG GGGGATACG TCC	D K L I F GATAAACTCATCTTT	Vδ2;Dδ2F2;Jδ1 1/22-4.5%	0.3765	NS
DS4T2.24	C A C D T TGTGCCTGTGACACT	L A G G Y K S TTAGCA GGGGATAC AAATCT	T D K L I F ACCGATAAACTCATCTTT	Vδ2;Dδ2F1;Jδ1 1/22-4.5%	0.3765	NS
DS4T2.25	C A C D T TGTGCCTGTGACACT	L G D L E P D CTGGGGGAT CTTGAGCCTGAC	D K L I F GATAAACTCATCTTT	Vδ2;Dδ2F2;Jδ1 1/22-4.5%	0.3765	NS

TABLE 29 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-4)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS4P2.2	C A C D TGTGCCTGTGAC	R L G G D K A CGA CTGGGGGAT AAGGCC	D K L I F GATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/20 - 5%	0.3774	NS
DS4P2.3	C A C D TGTGCCTGTGAC	I V G V L G D T L Y ATC GTTGA GTA CTGGGGGATACG CTGTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 3;D δ 2F2;J δ 1 1/20 - 5%	0.3774	NS
DS4P2.5	C A C D TGTGCCTGTGAC	A L G D T A GCA CTGGGGGATACT GCC	D K L I F GATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/20 - 5%	0.3774	NS
DS4P2.6	C A C D T TGTGCCTGTGACACC	L L Y TTACTGTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;J δ 1 1/20 - 5%	0.3774	NS
DS4P2.7	C A C D T TGTGCCTGTGACACG	V G D F S GTGGGGGAT TTTAGC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 3;J δ 1 1/20 - 5%	0.3774	NS
DS4P2.8	C A C D TGTGCCTGTGAC	Y W G TAC TGGGGG	S S W D T R Q M F AGCTCCTGGGACACCCGACAGATGTTT	V δ 2;D δ 2F3;J δ 3 1/20 - 5%	0.3774	NS
DS4P2.9	C A C TGTGCCTGT	E N L G D T P GAGAAC CTGGGGGATACA CCC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/20 - 5%	0.3774	NS
DS4P2.10	C A C TGTGCCTGC	A V L G T Q R D GCAGTA CTGGGG ACCCAACGAGAC	D K L I F GATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/20 - 5%	0.3774	NS
DS4P2.11	C A C D T TGTGCCTGTGACACC	V L G D P R S GTC CTGGGGGAC CCGAGGAGC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/20 - 5%	0.3774	NS
DS4P2.12	C A C D TGTGCCTGTGAC	S I L G D Y TCTATA CTGGGGGAC TAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/20 - 5%	0.3774	NS
DS4P2.13	C A C D TGTGCCTGTGAC	R V G D Y A AGG GTGGGGGAT TATGCT	L T A Q L F TTGACAGCACAACCTCTTC	V δ 2;D δ 3;J δ 2 1/20 - 5%	0.3774	NS
DS4P2.15	C A C D T TGTGCCTGTGACACG	L G G D A I CTGGGG GGCAC GCTATC	S W D T R Q M F TCCTGGGACACCCGACAGATGTTT	V δ 2;D δ 2F2;J δ 3 1/20 - 5%	0.3774	NS

TABLE 29 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-4) continued

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS4P2.17	C A C TGTGCCTGT	E G L G E T Y Y GAGGGA CTGGGG GAGACTTATTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/20 – 5%	0.3774	NS
DS4P2.18	C A C D T TGTGCCTGTGACACT	L L G E K G I F L TTA CTGGGG GAAAAAGGTATC TTCCTC	F G K R T L TTTGAAAACGAACACTC	V δ 2;D δ 2F2;D δ 1F3 1/20 – 5%	0.3774	NS
DS4P2.19	C A C D T TGTGCCTGTGACACC	V G R L G G Y G G GTAGGA AGACTT GGGGGATAC GGGGGT	K L I F AAACTCATCTTT	V δ 2;D δ 3;D δ 2F1;J δ 1 1/20 – 5%	0.3774	NS
DS4P2.20	C A C D TGTGCCTGTGAC	P L K T T G G Y L CCTCTTAAAACT ACTGGGGGATAC TTA	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F1;J δ 1 1/20 – 5%	0.3774	NS
DS4P2.21	C A C D T TGTGCCTGTGACACG	L A G G Y D A TTAGCT GGGGGATAC GACGCC	W D T R Q M F TGGGACACCCGACAGATGTTT	V δ 2;D δ 2F1;J δ 3 1/20 – 5%	0.3774	NS
DS4P2.22	C A C D T TGTGCCTGTGACACC	A S T G G Y Y P GCCAGT ACTGGGGGATAC TATCCC	S W D T R Q M F TCCTGGGACACCCGACAGATGTTT	V δ 2;D δ 2F1;J δ 3 1/20 – 5%	0.3774	NS
DS4P2.23	C A C TGTGCCTGT	E T L R T G G Y G A N S GAGACCCTTAGA ACTGGGGGATAC GGCGCTAACTCC	D K L I F GATAAACTCATCTTT	V δ 2;D δ 2F1;J δ 1 1/20 – 5%	0.3774	NS
DS4P2.24	C A C D T TGTGCCTGTGACACC	V G W G I R T I GTGGGG TGGGGGATACGA ACCATC	D K L I F GATAAACTCATCTTT	V δ 2;D δ 3;D δ 2F3;J δ 1 1/20 – 5%	0.3774	NS

TABLE 30 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-6)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS6P2.3	C A C D T TGTGCCTGTGACACC	W L D W G G I P G G G TGGCTA GACTGGGGGATA CCGGGA GGGGGC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F3;D δ 2F1;J δ 1 6/26 – 23%	0.0003	0.0096
DS6P2.16	C A C D T TGTGCCTGTGACACC	V R G T A GTGAGGGGGACCGCG	S S W D T R Q AGCTCCTGGGACACCCGACAG	V δ 2;J δ 3 4/26 – 15%	0.0138	0.0900
DS6P2.24	C A C D TGTGCCTGTGAC	S L F Q M G E Y AGCCTATTTTCAGATGGGGGAATAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;J δ 1 2/26 – 8%	0.1876	0.2816
DS6P2.1	C A C D T TGTGCCTGTGACACC	P Y T G G S CCTTAT ACTGGGGT TCG	S S W D T R Q AGCTCCTGGGACACCCGACAG	V δ 2;D δ 2F1;J δ 3 1/26 – 4%	0.3751	NS
DS6P2.6	C A C D TGTGCCTGTGAT	L L L Q S G D T A TTGCTCTACAATCG GGGGATACC GCC	D K L I F GATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/26 – 4%	0.3751	NS
DS6P2.8	C A C D T TGTGCCTGTGACACC	L G V N CTGGGG GTGAAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/26 – 4%	0.3751	NS
DS6P2.9	C A C D T TGTGCCTGTGACACC	L F A P G D TTGTTGCCCCG GGGGAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;J δ 1 1/26 – 4%	0.3751	NS
DS6P2.14	C A C D TGTGCCTGTGAC	S V L G D T P TCCGTA CTGGGGGATACG CCT	S W D T R Q TCCTGGGACACCCGACAG	V δ 2;D δ 2F2;J δ 3 1/26 – 4%	0.3751	NS
DS6P2.20	C A C D TGTGCCTGTGAC	P L G D CCA CTGGGGGAT	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/26 – 4%	0.3751	NS
DS6P2.28	C A C TGTGCCTGT	V P V L G V H S Y GTCCCCGTA CTGGGG GTCCACTCGTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/26 – 4%	0.3751	NS
DS6P2.32	C A C D TGTGCCTGTGAC	A I S Y A G G S M G GCTATT TCCTAC GCC GGGGGA TCAATGGGC	D K L I F GATAAACTCATCTTT	V δ 2;D δ 1F1;D δ 2F1;J δ 1 1/26 – 4%	0.3751	NS
DS6P2.35	C A C D T TGTGCCTGTGACACC	A G S H Y F P G GCCGGGAGCCATTACTTCCCTGGC	S W D T R Q TCCTGGGACACCCGACAG	V δ 2;J δ 3 1/26 – 4%	0.3751	NS

TABLE 30 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-6) continued

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS6P2.36	C A C D T TGTGCCTGTGACACC	V G T G E T GTGGGA ACTGGG GAAACC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 3;D δ 2F1;J δ 1 1/26 - 4%	0.3751	NS
DS6P2.41	C A C D T TGTGCCTGTGACACC	V V G D T D S R T GTG GTGGGGGAT ACGGATCCAGGACC	W D T R Q TGGGACACCCGACAG	V δ 2;D δ 3;J δ 3 1/26 - 4%	0.3751	NS
DS6P2.45	C A C D TGTGCCTGTGAC	P T G T G G P T CCGACCGGG ACTGGGGGA CCAACC	W D T R Q TGGGACACCCGACAG	V δ 2;D δ 2F1;J δ 3 1/26 - 4%	0.3751	NS
DS6P2.46	C A C D TGTGCCTGTGAC	A L L G S S K Y GCTTTA CTGGGG TCATCAAATAAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/26 - 4%	0.3751	NS
DS6P2.48	C A C D T TGTGCCTGTGACACC	V G I Y P GTG GGGATT TATCCC	D K L I F GATAAACTCATCTTT	V δ 2;D δ 2F3;J δ 1 1/26 - 4%	0.3751	NS

TABLE 31 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-7)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS7P2.2	C A C D TGTGCCTGTGAC	G L R Y T G G L G D S E R GGACTACGATATACG GGGGGA CTGGGGGAT AGCGAAAGG	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F1;D δ 2F2;J δ 1 3/19-15.8%	0.0595	0.1907
DS7P2.9	C A C D T TGTGCCTGTGACACT	S Y A G G Y V V R TCCTAC GCT GGGGGATAC GTGGTCCGG	D T R Q M F GACACCCGACAGATGTTT	V δ 2;D δ 1F1;D δ 2F1;J δ 3 2/19-10.5%	0.1889	0.2860
DS7P2.5	C A C D T TGTGCCTGTGACACC	L I P I G L G D T A TTAATTCCTATAGGG CTGGGGGATACG GCT	K L I F AAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/19-5.3%	0.3779	NS
DS7P2.6	C A C D T TGTGCCTGTGACACC	V L G L W G T S R GTTCTAGGA CTC TGGGGG ACCTCGCGC	T D K L I F ACCGATAAGCTCATCTTT	V δ 2;D δ 2F3;J δ 1 1/19-5.3%	0.3779	NS
DS7P2.7	C A C D T TGTGCCTGTGACACG	L G A P CTGGGG GCCCCC	D K L I F GATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/19-5.3%	0.3779	NS
DS7P2.8	C A C D TGTGCCTGTGAT	R L G E A L Y AGA CTGGGG GAGGCACTGTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/19-5.3%	0.3779	NS
DS7P2.11	C A C D TGTGCCTGTGAC	S V L G D P P E G G AGCGTA CTGGGGGAC CCCCCGGAAGGAGGG	T A Q L F ACAGCACAACCTCTTC	V δ 2;D δ 2F2;J δ 2 1/19-5.3%	0.3779	NS
DS7P2.14	C A C D T TGTGCCTGTGACACG	I S G G G I A ATATCCGGCGGG GGGATA GCC	W D T R Q M F TGGGACACCCGACAGATGTTT	V δ 2;D δ 2F3;J δ 3 1/19-5.3%	0.3779	NS
DS7P2.17	C A C D T TGTGCCTGTGACACC	R G G CGAGGCGGT	K L I F AAACTCATCTTT	V δ 2;J δ 1 1/19-5.3%	0.3779	NS
DS7P2.18	C A C D TGTGCCTGTGAC	S L T G G P D T Y TCCCTA ACTGGGGGA CCCGACACGTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F1;J δ 1 1/19-5.3%	0.3779	NS
DS7P2.20	C A C D T TGTGCCTGTGACACC	L A P D R G T S T G G Q P TTAGCCCCTGACCGGGTACTAGT ACTGGGGGT CAACCT	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F1;J δ 1 1/19-5.3%	0.3779	NS
DS7P2.21	C A C D T TGTGCCTGTGACACC	L G L G D T G V TTAGGA CTGGGGGATACA GGCCTT	S W D T R Q M F AGCTGGGACACCCGACAGATGTTT	V δ 2;D δ 2F2;J δ 3 1/19-5.3%	0.3779	NS

TABLE 31 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD FROM A SYSTEMIC SCLEROSIS PATIENT (SS-7) continued

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS7P2.23	C A C D TGTGCCTGTGAC	S L L L G D T TCCCTTTTA CTGGGGGATACG	D K L I F GATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/19-5.3%	0.3779	NS
DS7P2.24	C A C D TGTGCCTGTGAC	S I V L G E T A TCCATCGTA CTGGGG GAAACCGCC	W D T R Q M F TGGGACACCCGACAGATGTTT	V δ 2;D δ 2F2;J δ 3 1/19-5.3%	0.3779	NS
DS7P2.25	C A C D TGTGCCTGTGAC	L L G D P G TTA CTGGGGGAC CCCGGC	S S W D T R Q M F AGTCCTGGGACACCCGACAGATGTTT	V δ 2;D δ 2F2;J δ 3 1/19-5.3%	0.3779	NS
DS7P2.26	C A C D T TGTGCCTGTGACACC	L L G K G G P Y T CTC CTGGGG AAA GGGGGA CCTTACACC	L I F CTCATCTTT	V δ 2;D δ 2F2;D δ 2;J δ 1 1/19-5.3%	0.3779	NS

TABLE 32 – PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE SKIN BIOPSY TISSUE FROM A SYSTEMIC SCLEROSIS PATIENT (SS-16)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS16T2.1	C A C D T TGTGCCTGTGACACC	I V G G Y L G ATTGTT GGGGATAC TTGGGC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F1;J δ 1 20/25-80%	<0.0001	<0.0001
DS16T2.11	C A C D T TGTGCCTGTGACACC	V Q A G G S G P GTCCAAGCG GGGGGA TCTGGTCCC	D K L I F GATAAACTCATCTTT	V δ 2;D δ 2F1;J δ 1 2/25-8%	0.3754	NS
DS16T2.3	C A C D T TGTGCCTGTGACACC	A S G G Y R R G GCCTCT GGGGATAC AGGAGAGGT	K L I F AAACTCATCTTT	V δ 2;D δ 2F1;J δ 1 1/25-4%	0.3754	NS
DS16T2.10	C A C D T TGTGCCTGTGACACC	R W G T P AGG TGGGGG ACTCCT	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F3;J δ 1 1/25-4%	0.3754	NS
DS16T2.22	C A C D TGTGCCTGTGAC	S I T G G Y A I G S A TCGATT ACTGGGGGATAC GCAATTGGGAGTGCC	D K L I F GATAAACTCATCTTT	V δ 2;D δ 2F1;J δ 1 1/25-4%	0.3754	NS

TABLE 33 – PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF Vδ2 SUBGROUP δ-CHAIN TCR TRANSCRIPTS IN THE PERIPHERAL BLOOD OF SYSTEMIC SCLEROSIS PATIENT (SS-16)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DS16P2.1	C A C D T TGTGCCTGTGACACC	I V G G Y L G ATTGTT GGGGATAC TTGGGC	T D K L I F ACCGATAAACTCATCTTT	Vδ2;Dδ2F1;Jδ1 8/33 – 24%	<0.0001	0.0005
DS16P2.4	C TGT	G L A P L G G S T Y GGACTGGCCCCCTG GGGGGT TCCACCTAC	T D K L I F ACCGATAAACTCATCTTT	Vδ2;Dδ2F1;Jδ1 4/33 – 12%	0.0141	0.0901
DS16P2.3	C A C D T TGTGCCTGTGACACC	A G G GCC GGGGGT	S S W D T R Q AGCTCCTGGGACACCCGACAG	Vδ2;Dδ2F1;Jδ3 1/33 – 3%	NS	NS
DS16P2.7	C A C D T TGTGCCTGTGACACT	L T G G T L G V CTC ACTGGGGG ACC TTGGGG GTC	T D K L I F ACCGATAAACTCATCTTT	Vδ2;Dδ2F1;Jδ1 1/33 – 3%	NS	NS
DS16P2.14	C A C D T TGTGCCTGTGACACC	V T G G L L G D F P GTC ACGGGGGC TTA CTGGGGGAT TTCCCC	D K L I F GATAAACTCATCTTT	Vδ2;Dδ2F1;Dδ2F2;Jδ1 1/33 – 3%		NS
DS16P2.16	C A C D TGTGCCTGTGAC	Q L G V S G N CAA CTGGGG GTCAGTGGGAAC	T D K L I F ACCGATAAACTCATCTTT	Vδ2;Dδ2F2;Jδ1 1/33 – 3%	NS	NS
DS16P2.17	C A C D T TGTGCCTGTGACACC	V G P T V G L G D T R G S Y GTCGGCCCTACTGTCGGT CTGGGGGATACG CGTGGGTCGTAC	T D K L I F ACCGATAAACTCATCTTT	Vδ2;Dδ2F2;Jδ1 1/33 – 3%	NS	NS
DS16P2.18	C A C D TGTGCCTGTGAC	P L G D CCA CTGGGGGAC	T D K L I F ACCGATAAACTCATCTTT	Vδ2;Dδ2F2;Jδ1 1/33 – 3%	NS	NS
DS16P2.33	C A C D TGTGCCTGTGAC	A V L G D T G D GCCGTA CTGGGGGATACG GGCGAT	S S W D T R Q AGCTCCTGGGACACCCGACAG	Vδ2;Dδ2F2;Jδ3 1/33 – 3%	NS	NS
DS16P2.37	C A C D TGTGCCTGTGAC	A V K A T L G D H GCTGTAAAGCGACA CTGGGGGAT CAC	T D K L I F ACCGATAAACTCATCTTT	Vδ2;Dδ2F2;Jδ1 1/33 – 3%	NS	NS
DS16P2.39	C A C TGTGCCTGT	E P I T R GAACCGATAACACGA	T D K L I F ACCGATAAACTCATCTTT	Vδ2;Jδ1 1/33 – 3%	NS	NS
DS16P2.41	C A C D T TGTGCCTGTGACACG	L G D CTGGGGGAT	T D K L I F ACTGATAAACTCATCTTT	Vδ2;Dδ2F2;Jδ1 1/33 – 3%	NS	NS
DS16P2.42	C A C D T TGTGCCTGTGACACC	I V L G D T R T ATCGTA CTGGGGGATACG CGTACT	W D T R Q TGGGACACCCGACAG	Vδ2;Dδ2F2;Jδ3 1/33 – 3%	NS	NS

TABLE 34 – SUMMARY TABLE OF CLONAL EXPANSION OF V δ 2-CHAIN TRANSCRIPTS IN SSc PATIENTS SAMPLES

Tissue	Patient	Clone	CDR3 region	Identity	Frequency	Statistics	Clonal Expansion (Yes/No)
PBMC	SS1P δ 2	clone 2	CACDT <u>EET</u> TDKLIF	V δ 2;J δ 1	4/22-18.1%	p=0.0135	Yes
PBMC	SS2P δ 2	clone 1	CACDT LGD SSRDTR	V δ 2;D δ 2F2;J δ 3	2/21-9.5%	p=0.1884	No
PBMC	SS3P δ 2	clone 22	CACDT <u>P</u> GG <u>ATLV</u> WDTRQ	V δ 2;D δ 2F1;J δ 3	3/20 – 15%	p=0.0596	No
PBMC	SS4P δ 2	clone 2	CACD <u>R</u> LGD <u>KA</u> DKLIF	V δ 2;D δ 2F2;J δ 1	1/20 – 5%	p= 0.3774	No
PBMC	SS6P δ 2	clone 3	CACDT <u>WLD</u> WGI <u>PG</u> GG TDKLIF	V δ 2;D δ 2F3;D δ 2F1;J δ 1	6/26 – 23%	p=0.0003	Yes
		clone 16	CACDT <u>VRGTA</u> SSWDTRQ	V δ 2;J δ 3	4/26 – 15%	p=0.0138	Yes
PBMC	SS7P δ 2	clone 2	CACD <u>GLRY</u> TGG LGD <u>SER</u> TDKLIF	V δ 2;D δ 2F1;D δ 2F2;J δ 1	3/19-15.8%	p=0.0595	No
PBMC	SS16P δ 2	clone 1	CACDT <u>IV</u> GGY LG TDKLIF	V δ 2;D δ 2F1;J δ 1	8/33 – 24%	p<0.0001	Yes
		clone 4	C <u>GLAPL</u> GG <u>STY</u> TDKLIF	V δ 2;D δ 2F1;J δ 1	4/33 – 12%	p=0.0141	Yes
Biopsy	SS4T δ 2	clone 2	CACDT <u>VRGTA</u> SSWDTR	V δ 2;J δ 3	4/22-18%	p=0.0135	Yes
Biopsy	SS16T δ 2	clone 1	CACDT <u>IV</u> GGY LG TDKLIF	V δ 2;D δ 2F1;J δ 1	20/25-80%	p<0.0001	Yes

Statistical analysis by binomial distribution (p<0.05 is statistically significant)

TABLE 35 – COMPILED SUMMARY TABLE OF CLONAL EXPANSION PROFILE OF V γ - AND V δ -CHAIN TCR TRANSCRIPTS IN SSc SAMPLES FROM THE PREVIOUS AND CURRENT STUDIES

Patient	Specimen	Vgamma I	Vgamma II	Vdelta 1	Vdelta 2
SSc-1	PBMC	Clonal expansion (p<0.0001)	Polyclonal	Clonal expansion (p<0.0001)	Clonal expansion (p=0.0135)
SSc-2	PBMC	Clonal expansion (p=0.012)	Clonal expansion (p<0.0001)	Clonal expansion (P=0.0022)	Polyclonal
SSc-3	PBMC	Clonal expansion (p=0.002)	Clonal expansion (p<0.0001)	Polyclonal	Polyclonal
	Skin biopsy	Non productive rearrangements	Clonal expansion (p<0.0001)	Clonal expansion (p<0.002)	Clonal expansion p<0.0001
SSc-4	PBMC	Polyclonal	Polyclonal	Clonal expansion (p=0.0022)	Polyclonal
	Skin biopsy	No sample	Clonal expansion (p=0.011)	Clonal expansion (p<0.0001)	Clonal expansion p=0.0135
SSc-5	PBMC	Polyclonal	Clonal expansion (p=0.013)	Clonal expansion (p=0.0016)	Polyclonal
	Skin biopsy	Clonal expansion (p=0.002)	Non productive rearrangements	Polyclonal	Clonal expansion (p<0.0001)
SSc-6	PBMC	Clonal expansion (p=0.0013)	Clonal expansion (p<0.0001)	Polyclonal	Clonal expansion (p=0.0003)
	Skin biopsy	Clonal expansion (p<0.0001)	Clonal expansion (p<0.0001)	Clonal expansion (p<0.0017)	Clonal expansion (p<0.0001)
SSc-7	PBMC	No sample	Clonal expansion (p=0.0023)	Clonal expansion (p<0.0001)	Polyclonal (p=0.0595)
	Skin biopsy	No sample	No sample	Clonal expansion (p<0.0001)	No sample
SSc-11	Skin biopsy	No sample	No sample	Clonal expansion (p<0.0001)	No sample
SSc-16	Skin biopsy	Polyclonal	Clonal expansion (p<0.0001)	Polyclonal	Clonal expansion (p<0.0001)
	PBMC	Polyclonal	Clonal expansion p<0.0001	Polyclonal	Clonal expansion (p<0.0001)

*Results from the current study are highlighted (grey) in the table.

TABLE 36 – COMPILED TABLE OF PRESENCE OR ABSENCE OF V γ - AND V δ -CHAIN TCR TRANSCRIPTS CLONAL EXPANSIONS IN SSc SAMPLES FROM THE PREVIOUS AND CURRENT STUDIES

Patient	Specimen	Vγ I	Vγ II (Vγ9)	Vδ 1	Vδ 2
SSc-1	PBMC	Yes	No	Yes	Yes
SSc-2	PBMC	Yes	Yes	Yes	No
SSc-3	PBMC	Yes	Yes	No	No
	Skin biopsy	NP*	Yes	Yes	Yes
SSc-4	PBMC	No	No	Yes	No
	Skin biopsy	NS**	Yes	Yes	Yes
SSc-5	PBMC	No	No	No	No
	Skin biopsy	Yes	NP*	No	Yes
SSc-6	PBMC	Yes	Yes	No	Yes
	Skin biopsy	Yes	Yes	Yes	Yes
SSc-7	PBMC	NS**	Yes	Yes	No
	Skin biopsy	NS**	NS**	Yes	NS**
SSc-11	Skin biopsy	NS**	NS**	Yes	NS**
SSc-16	Skin biopsy	No	Yes	No	Yes
	PBMC	No	Yes	No	Yes

Work began by Dr. Weon-Ju Jung; NP* (Non-productive rearrangements); NS** (No patient sample for analysis)

In the PBMC from patient SS-1 (see table 25), V δ 2 chain analysis revealed 4 out of 22 (18.1%) transcripts had identical CDR3 regions (transcript #2), representing a statistically significant clonal expansion; CACDT EET TDKLIF, V δ 2;J δ 1, $p=0.0135$. There were two clones with 3 out of 22 (13.6%) identical transcripts, but these represented a statistically insignificant clonal expansion ($p>0.05$). In addition, there were twelve clones, each with unique CDR3 regions (1 out of 22). For patient SS-2 (see table 26), there were no statistically significant clonal expansions, however two clones had 2 out of 21 (9.5%) identical transcripts, but these represented a statistically insignificant clonal expansion ($p>0.05$). The remaining seventeen transcripts had unique CDR3 regions (1 out of 21). Hence the V δ 2 transcripts in the patient represented a polyclonal expansion. The PBMC from patient SS-3 (see table 27), also had no statistically significant clonally-expanded transcripts, but had seventeen clones, each with a unique CDR3 region (1 out of 20), and a clone with 3 out of 20 (15%) identical transcripts that represented a statistically insignificant clonal expansion ($p>0.05$). Hence, the V δ 2 transcripts in this patient also were polyclonal. In the V δ 2 analysis of transcripts in the PBMC from patient SS-4 (see table 29), all twenty clones each had unique CDR3 regions (1 out of 20), hence indicating a polyclonal population. Patient SS-6 (see table 30) had 6 out of 26 (23%) identical transcripts (transcript #3), representing a statistically significant clonal expansion; CACDT WLD WGI PG GG TDKLIF, V δ 2;D δ 2F3;D δ 2F1;J δ 1, $p=0.0003$; and 4 out of 26 (15%) identical transcripts (transcript #16), representing another statistically significant clonal expansion; CACDT VRGTA SSWDTRQ, V δ 2;J δ 3, $p=0.0138$. There was one clone with 2 out of 26 (8%) identical transcripts, but this represented a statistically insignificant clonal expansion ($p>0.05$). The remaining fourteen transcripts had unique CDR3 regions (1 out of 26). For patient SS-7 (see

table 31), V δ 2 chain analysis of the PBMC revealed a lack of clonally-expanded transcripts, with two clones having 3 out of 19 (15.9%), or 2 out of 19 (10.5%) identical transcripts, representing a statistically insignificant clonal expansion ($p > 0.05$). The remaining fourteen transcripts had unique CDR3 regions (1 out of 19). The PBMC from patient SS-16 (see table 32), had 8 out of 33 (24%) identical transcripts (transcript #1), representing a statistically significant clonal expansion; CACDT IV GGY LG TDKLIF, V δ 2;D δ 2F1;J δ 1, $p < 0.0001$; and 4 out of 33 (12%) identical transcripts (transcript #4), representing another statistically significant clonal expansion; C GLAPL GG STY TDKLIF, V δ 2;D δ 2F1;J δ 1, $p = 0.0141$. The remaining eleven clones, each had a unique CDR3 region (1 out of 33).

The skin biopsy sample of patient SS-4 (see table 28), revealed 4 out of 22 (18%) identical transcripts (transcript #2); CACDT VRGTA SSWDTR, V δ 2;J δ 3, 4/22-18%, $p = 0.0135$, representing a statistically significant clonal expansion. Three clones had 2 out of 22 (9%) identical transcripts, which was a statistically insignificant clonal expansion ($p > 0.05$). The remaining twelve clones, each had unique CDR3 transcripts (1 out of 22). Similar to the V γ 9 transcripts from patient SS-16, the same interesting finding was apparent, with the presence of a transcript that was expanded in both the SSc lesion skin biopsy and the PBMC (see table 32 and 33 respectively) from SS-16. There were 20 out of 25 (80%) transcripts with identical CDR3 regions (transcript #1) in the skin biopsy, and as described previously, 8 out of 23 (24%) transcripts with identical CDR3 regions (transcript #1) in the PBMC, representing a statistically significant clonal expansion; CACDT IV GGY LG TDKLIF, V δ 2;D δ 2F1;J δ 1, $p < 0.0001$, in both cases. Other V δ 2 transcripts present in the skin biopsy of SS-16 were different from those present in the PBMC, and included one clone, with 2 out of 25 (8%) identical transcripts, representing statistically insignificant clonal expansion

($p > 0.05$). The remaining three clones each had unique CDR3 region transcripts (1 out of 25). As observed in the other chains analyzed, the deduced amino acid sequence of all V δ 2 transcripts analyzed revealed neither amino acid sequence homology nor the presence of conserved motifs, when compared using BLAST searches to transcripts in the GENBANK database, suggesting these were unique, previously unidentified, clones.

Combining the results of the previous and current studies on the clonal expansion profile of $\gamma\delta$ TCR transcripts, isolated from skin biopsy and PBMC of patients with SSc of recent onset, there is clear evidence of oligoclonal expansion, via the presence of multiple identical transcripts of V γ and/or V δ subsets (see table 35). Specifically, in the skin biopsy samples, statistically significant clonal expansions were observed in V γ I transcripts from 2 out of 3 patients, V γ 9 transcripts from 4 out of 4 patients, V δ 1 transcripts from 4 out of 6 patients, and V δ 2 transcripts from 5 out of 5 patients. In the peripheral blood samples statistically significant clonal expansions were observed in V γ I transcripts from 4 out of 7 patients, V γ 9 transcripts from 5 out of 7 patients, V δ 1 transcripts from 5 out of 8 patients, and V δ 2 transcripts from 3 out of 8 patients.

The peripheral blood and skin biopsy of each patient had evidence of statistically significant clonal expansions in one or more of the V γ - or V δ chain subsets (see table 35). The PBMC of SS-1 had statistically significant clonal expansions in the V γ I, V δ 1 and V δ 2 chains, but had a statistically insignificant clonal expansion in the V γ 9 chain. The PBMC of SS-2 had statistically significant clonal expansions in the V γ I, V γ 9, and V δ 1 chains, but the V δ 2 chain was polyclonal. The PBMC of SS-3 had statistically significant clonal expansions in the V γ I and V γ 9 chains but the V δ 1 chain was polyclonal and the V δ 2 chain had a

statistically insignificant clonal expansion. However, the skin biopsy of SS-3 had statistically significant clonal expansions in the V γ 9, V δ 1, and V δ 2 chains, with non-productive rearrangements in the V γ I chains. The PBMC of SS-4 had statistically significant clonal expansion in the V δ 1 chain, while the V γ I, V γ 9, and V δ 2 chains were polyclonal, however, the skin biopsy had statistically significant clonal expansions in the V γ 9, V δ 1, and V δ 2 chains, with no sample available for V γ I analysis. The PBMC of SS-5 had statistically significant clonal expansions in the V γ 9 and V δ 1 chains, a polyclonal V γ I population, and a statistically insignificant clonal expansion of the V δ 2 chain. However, the skin biopsy of SS-5 had statistically significant clonal expansion in the V γ I and V δ 2 chains, a polyclonal V δ 1 population, and non-productive rearrangements of the V γ 9 chain. The PBMC of SS-6 had statistically significant clonal expansions in the V γ I, V γ 9 and V δ 2 chains, while the V δ 1 chain was polyclonal, however, in the skin biopsy, all four chains (V γ I, V γ 9, V δ 1, and V δ 2) has statistically significant clonal expansions. The PBMC of SS-7 had statistically significant V γ 9 and V δ 1 chain clonal expansions, a statistically insignificant V δ 2 chain expansion, and no sample available for V γ I chain analysis. The skin biopsy of SS-7 and SS-11 had statistically significant V δ 1 chain expansion, but no sample available for analysis of the rest of the γ - and δ -chains. Finally, both the PBMC and skin biopsy of SS16 had statistically significant clonal expansions in the V γ 9 and V δ 2 chains, and polyclonal V γ I and V δ 1 chain populations.

These findings are strongly suggestive of an antigen-driven clonal expansion of $\gamma\delta$ TCR+ T-cells in the skin lesional samples and peripheral blood from patients with SSC. Interestingly, two patients (SS-7 and SS-16) exhibited the presence of a clonally-expanded

transcript, identical in both the PBMC and the skin biopsy. Patient SS-7 (see table 19 and 20), had an identical V δ 1 transcript clonally-expanded in both the skin biopsy and the PBMC and patient SS-16, had an identical V γ 9 transcript clonally-expanded in both the skin biopsy and the PBMC, as well as a V δ 2 transcript that exhibited statistically significant clonal expansion in both the PBMC and the skin biopsy. The significance of this specific finding, as well as the presence of clonally-expanded $\gamma\delta$ TCR+ T-cells in skin biopsy and PBMC samples from patients with SSc of recent onset, is currently unknown, in great part because the antigens recognized by clonally expanded $\gamma\delta$ TCR+ T-cells in SSc skin lesions and/or PBMC is still elusive.

The nucleic acid and the deduced amino acid sequence obtained were compared to those in the GENBANK database using the online BLAST program and revealed that the sequences analyzed from the skin biopsy and peripheral blood of SSc patients, were typical of γ - and δ -chain TCR. No homologies were found between the TCR clones sequenced in this study and those in the GENBANK database, suggesting that they had not been previously identified, and, therefore were novel.

As previously alluded to, the antigen-binding site of $\gamma\delta$ TCR is formed primarily from the three complementarity-determining regions (CDRs) of the V γ and V δ chains composing the receptor. CDR1 and CDR2 domains are encoded by germ line V genes, but the CDR3 is generated during somatic rearrangement of V(D) and J fragments, with N-nucleotide additions at the V(D)J junctions. Sequence diversity in antigen receptors is highly concentrated in the CDR3 domains. CDR3 length diversity in the V δ chain is a feature that provides empirical evidence of antigen recognition properties of $\gamma\delta$ TCR+ T-cells, as being similar to B-cells (MHC-unrestricted recognition of whole protein epitopes). In this study,

comparison of the most clonally-expanded V γ and V δ transcript CDR3 transcripts from either skin biopsy or peripheral blood showed the absence of non-germline encoded (i.e. N-nucleotides) conserved sequences in γ - or δ -chain TCR transcripts were identified. The CDR3 δ sequences were diverse in nucleotide content and in length. The CDR3 lengths of the clonally-expanded V γ I chains from both the skin biopsy and PBMC of the SSc patients, ranged from 11-14 amino acids; V γ 9 from 13-14 amino acids, V δ 1 from 14-22 amino acids, V δ 2 from 15-21 amino acids (see below).

Gamma 1 PBMC

GS3P1.2 C A T W D S R K N Y K K L F V γ 5;j γ 2.3;C γ 2

Gamma 1 SKIN BIOPSY

GS6T1.2 C A T W D R P N Y K K L F V γ 5;j γ 2.3;C γ 2
 GS6T1.1 C A T W D R P Y Y K K L F V γ 5;j γ 2.3;C γ 2
 GS6T1.12 E S G L S P D K Q L D V γ 5;C γ 2
 GS6T1.8 C A T W D G Y K K L F V γ 5;j γ 2.3;C γ 2

Gamma 2 SKIN BIOPSY

GS16T2.1 C A L W E V L A A G L G K K V γ 9;j γ 1.2;C γ 1

Gamma 2 PBMC

GS7P2.13 C A L W E V W Y Y K K L F V γ 9;j γ 2.3;C γ 2
 GS16P2.2 C A L W E V L A A G L G K K V γ 9;j γ 1.2;C γ 1

Delta 1 PBMC

DS1P1.8 C A L G N K P H W G Y A S F T Y T D K L I F V δ 1;D δ 2F3;j δ 1
 DS2P1.6 C A L E L H P F Y W G I L Y T D K L I F V δ 1;D δ 2F3;j δ 1
 DS4P1.5 C A L G D L P R G F L R L G I P S D K L I F V δ 1;D δ 1F3;D δ 2F2;j δ 1
 DS7P1.1 C A L G E L P F L L W G I T D K L I F V δ 1;D δ 1F3;D δ 2F3;j δ 1

Delta 1 SKIN BIOPSY

DS7T1.4 C A L G G T Y Y T D K L I F V δ 1;j δ 1
 DS7T1.5 C A L G E L P F L L W G I T D K L I F V δ 1;D δ 1;D δ 2;j δ 1
 DS11T1.2 C A L G N K P H W G Y A S F T Y T D K L I F V δ 1;D δ 2F3;j δ 1

Delta 2 PBMC

DS1P2.2 C A C D T E E T T D K L I F V δ 2;j δ 1
 DS6P2.3 C A C D T W L D W G I P G G G T D K L I F V δ 2;D δ 2F3;D δ 2F1;j δ 1
 DS6P2.16 C A C D T V R G T A S S W D T R V δ 2;j δ 3
 DS16P2.1 C A C D T I V G G Y L G T D K L I F V δ 2;D δ 2F1;j δ 1
 DS16P2.4 C G L A P L G G S T Y T D K L I F V δ 2;D δ 2F1;j δ 1

Delta 2 SKIN BIOPSY

DS4T2.2 C A C D T V R G T A S S W D T R V δ 2;j δ 3
 DS16T2.1 C A C D T I V G G Y L G T D K L I F V δ 2;D δ 2F1;j δ 1

4.2.5 Sequencing results of γ - and δ -chain TCR Transcripts from normal skin

In the current study, γ - and δ -TCR chain transcripts from commercially available normal skin RNA isolated from an adult female donor, NS-1 (63 year old), and from a female fetal donor, FNS-2 (18-20 weeks old) were obtained, amplified, cloned, and sequenced. The predicted amino acid sequence of the transcripts was generated using the ExPASy proteomics server. Results revealed the presence of substantial proportions of multiple identical V γ 9, V δ 1 and V δ 2 transcripts in NS-1, and multiple identical V γ 9 transcripts in the FNS-2, both representing statistically significant clonal expansions. No conserved sequences were identified in the γ - or δ -chain transcripts, nor were there any homologies after comparison to γ - or δ -chain transcripts in the GENBANK database using BLAST searches, suggesting that these clones were previously unidentified and were therefore novel.

TABLE 37 – PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ I SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE SKIN SAMPLE RNA FROM A NORMAL DONOR (NS-1)

Clone	Variable region	N-region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
GN1T1.6	C A T W TGTGCCACCTGG	T P R H ACCCCTAGGCAT	K K L F AAGAAACTCTTT	V γ 4;J γ 2.3;C γ 2 4/36-11%	0.0142	0.0901
GN1T1.39	C A T W D R TGTGCCACCTGGGACAGG	R G CGAGGT	Y K K L F TATAAGAAACTCTTT	V γ 5;J γ 2.3;C γ 2 4/36-11%	0.0142	0.0901
GN1T1.8	C A T W D G TGTGCCACCTGGGATGGG	P G CCGGGT	Y Y K K L F TATTATAAGAAACTCTTT	V γ 4;J γ 2.3;C γ 2 3/36-8.3%	0.0604	0.1857
GN1T1.11	C A T W TGTGCCACCTGG	Y TAT	S S D W I K T AGTAGIGATTGGATCAAGACG	V γ 2;J γ 2.1;C γ 2 2/36-5.5%	0.1865	0.2785
GN1T1.38	C A T W TGTGCCACCTGG	Q V L CAAGTGCTC	Y Y K K L F TATTATAAGAAACTCTTT	V γ 5;J γ 2.3;C γ 2 2/36-5.5%	0.1865	0.2785
GN1T1.45	C A T W D R TGTGCCACCTGGGACAGG	P A W P C S D CCGGCGTGGCCGTGTAGCGAT	K K L F AAGAAACTCTTT	V γ 5;J γ 2.3;C γ 2 2/36-5.5%	0.1865	0.2785
GN1T1.2	C A T W D R TGTGCCACCTGGGACAGG	P T T G CCTACCACTGGT	W F K I F TGGTTCAAGATATTT	V γ 3;J γ 1.1;C γ 2 1/36-2.8%	0.3731	NS
GN1T1.5	C A T W TGTGCCACCTGG	P S CCCTCT	Y Y K K L F TATTATAAGAAACTCTTT	V γ 4;J γ 2.3;C γ 2 1/36-2.8%	0.3731	NS
GN1T1.9	C A T W TGTGCCACCTGG	R AGA	N Y Y K K L F AATTATTATAAGAAACTCTTT	V γ 3;J γ 2.3;C γ 2 1/36-2.8%	0.3731	NS
GN1T1.14	C A T W D G TGTGCCACCTGGGACGGG	P G R CCGGGGAGG	F K I F TTCAAGATATTT	V γ 2;J γ 1.1;C γ 2 1/36-2.8%	0.3731	NS
GN1T1.15	C A T W D G TGTGCCACCTGGGATGGG	P S CCATCG	N Y Y K K L S AATTATTATAAGAAACTCTCT	V γ 4;J γ 2.3;C γ 2 1/36-2.8%	0.3731	NS
GN1T1.16	C A T W TGTGCCACCTGG	E T N GAAACAAAT	K K L F AAGAAACTCTTT	V γ 2;J γ 2.3;C γ 2 1/36-2.8%	0.3731	NS

TABLE 37 – PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ 1 SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE SKIN SAMPLE RNA FROM A NORMAL DONOR (NS-1) continued

Clone	Variable region	N-region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
GN1T1.18	C A T W D TGTGCCACCTGGGAC	M ATG	Y K K L F TATAAGAAACTCTTT	V γ 3;J γ 2.3;C γ 2 1/36-2.8%	0.3731	NS
GN1T1.19	C A T W D G TGTGCCACCTGGGATGGG	R N CGTAAT	Y K K L F TATAAGAAACTCTTT	V γ 4;J γ 2.3;C γ 2 1/36-2.8%	0.3731	NS
GN1T1.21	C A T TGTGCCACC	Y D TACGAT	Y Y K K L F TATTATAAGAAACTCTTT	V γ 4;J γ 2.3;C γ 1/36-2.8%	0.3731	NS
GN1T1.24	C A T W D TGTGCCACCTGGGAT	T T G ACCACTGGT	W F K I F TGGTTCAAGATATTT	V γ 4;J γ 1.1;C γ 2 1/36-2.8%	0.3731	NS
GN1T1.25	C A T W D R TGTGCCACCTGGGACAGG	P R CCACGT	K K L F AAGAAACTCTTT	V γ 3;J γ 2.3;C γ 2 1/36-2.8%	0.3731	NS
GN1T1.26	C A T W TGTGCCACCTGG	T P R H R ACCCCTAGGCATAGG	K L F AAACTCTTT	V γ 4;J γ 2.3;C γ 2 1/36-2.8%	0.3731	NS
GN1T1.29	C A TGTGCC	V R S GTACGCTCG	N Y Y K K L F AATTATTATAAGAAACTCTTT	V γ 4;J γ 2.3;C γ 2 1/36-2.8%	0.3731	NS
GN1T1.34	C A T W D R TGTGCCACCTGGGACAGA		Y Y K K L F TATTATAAGAAACTCTTT	V γ 3;J γ 2.3;C γ 1 1/36-2.8%	0.3731	NS
GN1T1.47	C A T W TGTGCCACCTGG	C S TGTAGT	S D W I K T AGTGATTGGATCAAGACG	V γ 5;J γ 2.1;C γ 2 1/36-2.8%	0.3731	NS
GN1T1.65	C A T W D R TGTGCCACCTGGGACAGA	R CGT	Y Y K K L F TATTATAAGAAACTCTTT	V γ 5;J γ 2.3;C γ 2 1/36-2.8%	0.3731	NS
GN1T1.71	C A T W TGTGCCACCTGG	E A Y GAGGCTTAT	Y Y K K L F TATTATAAGAAACTCTTT	V γ 5;J γ 2.3;C γ 2 1/36-2.8%	0.3731	NS
GN1T1.76	C A T W D R TGTGCCACCTGGGACCGG		N Y Y K K L F AATTATTATAAGAAACTCTTT	V γ 5;J γ 2.3;C γ 2 1/36-2.8%	0.3731	NS

TABLE 37 – PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ 1 SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE SKIN SAMPLE RNA FROM A NORMAL DONOR (NS-1) continued

Clone	Variable region	N-region	Junctional region	C-region	Identity/Frequency	Statistical analysis	
						1/n	2/n
GN1T1.64	E S G L S P GAATCAGGACTCAGTCCA			D K Q L D GATAACAACCTTGAT	V γ 5;C γ 2 1/36-2.8%	0.3731	NS

**TABLE 38 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V γ II (V γ 9) SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN SKIN
SAMPLE RNA FROM A NORMAL DONOR (NS-1)**

Clone	Variable region	N-region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
GN1T2.2	C A L W E TGTGCCTTGTGGGAG	P L CCACTC	Y Y K K L F TATTATAAGAACTCTTT	Vγ9; Jγ2.3; Cγ2 20/25-80%	<0.0001	<0.0001
GN1T2.4	C A L W E V TGTGCCTTGTGGGAGGTG	N I AATATT	Y Y K K L F TATTATAAGAACTCTTT	V γ 9; J γ 2.3; C γ 2 2/25-8%	0.1877	0.2821
GN1T2.5	C A L W E TGTGCCTTGTGGGAG	A T GCGACA	Y Y K K L F TATTATAAGAACTCTTT	V γ 9; J γ 2.3; C γ 1 2/25-8%	0.1877	0.2821
GN1T2.1	C A L W E V TGTGCCTTGTGGGAGGTT	F Y TTTTAT	Y Y K K L F TATTATAAGAACTCTTT	V γ 9; J γ 2.3; C γ 2 1/25-4%	0.3754	

TABLE 39 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF Vδ1 SUBGROUP δ-CHAIN TCR TRANSCRIPTS IN THE SKIN SAMPLE RNA FROM A NORMAL DONOR (NS-1)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DN1T1.7	C A L G E L S G L P T L A V G D T R I A TGTGCTCTTGGGGAA	CTTTCGGGC CTCCT ACGTTAGCAGTG GGGGATACG CGTATAGCT	L T A Q L TTGACAGCACAAC	Vδ1;Dδ1F1;Dδ2F2;Jδ2 12/17-70.6%	<0.0001	<0.0001
DN1T1.46	C A L G A R F L P S R E K S L G V A P L TGIGCTCTTGGG GCCCGTTC CTCCT TCCCGGAAAAAAGT CIGGGG GTCGCGCCTTA		K L I F AAACTCATCTTT	Vδ1;Dδ1.1;Dδ2F2;Jδ1 3/17-17.6%	0.0594	0.2452
DN1T1.5	C A L G E R L A L R F P S Y W G I P G G E TGIGCTCTTGGGGAA CGGTGGCCCTACGGTTCCCAAGTTAC TGGGGGATA CCTGGTGGGGAG		T D K L I F ACCGATAAACTCATCTTT	Vδ1;Dδ2F3;Jδ1 2/17-11.8%	0.1894	0.2869

TABLE 40 – PRODUCTIVELY-REARRANGED CDR3 SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN SKIN SAMPLE RNA FROM A NORMAL DONOR (NS-1)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DN1T2.2	C A C TGTGCCTGC	P T G V S G G CCC ACTGGG GTAAGC GGTGGG	L I F G CTCATCTTTGGA	V δ 2;D δ 2F1;D δ 2F1;J δ 1 14/31-45%	<0.0001	<0.0001
DN1T2.1	C A C D T TGTGCCTGTGACACT	G D GGGGAT	T R Q ACCCGACAG	V δ 2;D δ 2F2;J δ 3 6/31-19%	0.0004	0.0100
DN1T2.4	C A C D T TGTGCCTGTGACACC	G A A T GGGGCCGCCACT	S W D T R Q TCCTGGGACACCCGACAG	V δ 2;J δ 3 3/31-9.6%	0.0602	0.1865
DN1T2.6	C A C D TGTGCCTGTGAC	A L L G D L R GCCCTA CTGGGGGAC CTCCGC	T D K L I F G ACCGATAAACTCATCTTTGGA	V δ 2;D δ 2F2;J δ 1 2/31-6.4%	0.1870	0.2798
DN1T2.7	C A C D T TGTGCCTGTGACACC	V V G D T D S R T GTG GTGGGGGAT ACGGATTCCAGGACC	W D T R Q TGGGACACCCGACAG	V δ 2;D δ 3;J δ 3 2/31-6.4%	0.1870	0.2798
DN1T2.12	C A C D TGTGCCTGTGAC	G P L G V G D T S GGACCC TTGGGG GTGGGGGAC ACCTCC	D K L I F G GATAAACTCATCTTTGGA	V δ 2;D δ 2F2;D δ 3;J δ 1 2/31-6.4%	0.1870	0.2798
DN1T2.13	C A C D TGTGCCTGTGAC	P L G G G Q S K Y CCC CTAGGT GGGGGT CAATCAAAGTAC	T D K L I F G ACCGATAAACTCATCTTTGGA	V δ 2;D δ 2F2;D δ 3;J δ 1 1/31-3.2%	0.3739	NS
DN1T2.22	C A C D T TGTGCCTGTGACACC	V G M G D T T GTGGGAATG GGGGATACG ACT	T D K L I F G ACCGATAAACTCATCTTTGGA	V δ 2;D δ 2F2;J δ 1 1/31-3.2%	0.3739	NS

TABLE 41 – PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ I SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE SKIN FROM A NORMAL 18-20week FEMALE FETAL DONOR (FNS-2)

Clone	Variable region	N-region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
GFN2T1.5	C A T W D G TGTGCCACCTGGGACGGG	P CCT	T T G W F K ACCACTGGTTGGTTCAAG	V γ 2;J γ 1.1;C γ 1 3/14 - 21%	0.0587	0.1947
GFN2T1.11	C A T W D TGTGCCACCTGGGAC	A GCT	G W F K GGTTGGTTCAAG	V γ 4;J γ 1.1;C γ 1 3/14 - 21%	0.0587	0.1947
GFN2T1.21	C A T W D G TGTGCCACCTGGGATGGG	P CCC	T G W F K ACTGGTTGGTTCAAG	V γ 4;J γ 1.1;C γ 1 2/14 - 14%	0.1908	0.2921
GFN2T1.31	C A T W D G TGTGCCACCTGGGACGGG	P CCT	G W F K GGTTGGTTCAAG	V γ 2;J γ 1.1;C γ 1 2/14 - 14%	0.1908	0.2921
GFN2T1.4	C A T W D G TGTGCCACCTGGGACGGG	P N CCGAAT	Y K K L F TATAAGAAACTCTTT	V γ 2;J γ 2.3;C γ 2 1/14 - 7%	NS	NS
GFN2T1.8	C A T W D G TGTGCCACCTGGGACGGG	Q A CAAGCT	Y Y K K L F TATTATAAGAAACTCTTT	V γ 2;J γ 2.3;C γ 1 1/14 - 7%	NS	NS
GFN2T1.10	C A T W D TGTGCCACCTGGGAT		Y Y K K L F TATTATAAGAAACTCTTT	V γ 4;J γ 2.3;C γ 2 1/14 - 7%	NS	NS
GFN2T1.29	C A T W D G TGTGCCACCTGGGACGGG	F TTT	T T G W F K ACCACTGGTTGGTTCAAG	V γ 2;J γ 1.1;C γ 1 1/14 - 7%	NS	NS

TABLE 42 – PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V γ II SUBGROUP γ -CHAIN TCR TRANSCRIPTS IN THE SKIN FROM A NORMAL 18-20week FEMALE FETAL DONOR (FNS-2)

Clone	Variable region	N-region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
GFN2T2.1	C A L W E V TGTGCCTTGTGGGAGGTG		Q E L G K K CAAGAGTTGGGCAAAAAA	Vγ9;Jγ1.2;Cγ1 17/26 – 65.4%	<0.0001	<0.0001
GFN2T2.3	C A L W E TGTGCCTTGTGGGAG		E L G K K GAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1 1/26 – 3.8%	NS	NS
GFN2T2.5	C A L W E TGTGCCTTGTGGGAG	V K GTAAAG	L G K K TTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1 1/26 – 3.8%	NS	NS
GFN2T2.10	C A L W E V TGTGCCTTGTGGGAGGTG	H CAT	Y K K L F TATAAGAAACTCTTT	V γ 9;J γ 2.3;C γ 1 1/26 – 3.8%	NS	NS
GFN2T2.12	C A L W TGTGCCTTGTGG	V V GTCGTA	Q E L G K K CAAGAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1 1/26 – 3.8%	NS	NS
GFN2T2.15	C A L W E V TGTGCCTTGTGGGAGGTG	Q CAG	L G K K TTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1 1/26 – 3.8%	NS	NS
GFN2T2.17	C A L W E TGTGCCTTGTGGGAG	V GTA	E L G K K GAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1 1/26 – 3.8%	NS	NS
GFN2T2.20	C A L W E V TGTGCCTTGTGGGAGGTG	V GTA	E L G K K GAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1 1/26 – 3.8%	NS	NS
GFN2T2.23	C A L W E V TGTGCCTTGTGGGAGGTG	Q V CAGGTG	L G K K TTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1 1/26 – 3.8%	NS	NS
GFN2T2.25	C A L W E TGTGCCTTGTGGGAG		Q E L G K K CAAGAGTTGGGCAAAAAA	V γ 9;J γ 1.2;C γ 1 1/26 – 3.8%	NS	NS

TABLE 43 – PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V δ 1 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE SKIN FROM A NORMAL 18-20week FEMALE FETAL DONOR (FNS-2)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DFN2T1.24	C A L G E TGTGCTCTGGGGAA	G G GGGGC	S W D T R Q TCCTGGGACACCCGACAG	V δ 1;D δ 2F1;J δ 3 3/13-23%	NS	NS
DFN2T1.28	C A L TGTGCTCT	T G G ACTGGGGGA	S W D T R Q TCCTGGGACACCCGACAG	V δ 1;D δ 2F1;J δ 3 2/13-15%	NS	NS
DFN2T1.2	C A L G E S P Y L L T L P P Y W G I S L G L P		D K L I	V δ 1;D δ 1;D δ 3;J δ 1 1/13-7.7%	NS	NS
DFN2T1.3	C A L G E R Y Y W G I R X TGTGCTCTGGGGAA	CGTTACTAC TGGGGGATACGG NAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;D δ 2F3;J δ 1 1/13-7.7%	NS	NS
DFN2T1.23	C A L G TGTGCTCTTGA	Y TAT	L T A Q L F TTGACAGCACAACTCTTC	V δ 1;J δ 2 1/13-7.7%	NS	NS
DFN2T1.32	C A L G E F G G Y TGTGCTCTGGGGAA	TTT GGGGGATAC	S W D T R Q TCCTGGGACACCCGACAG	V δ 1;D δ 2F1;J δ 3 1/13-7.7%	NS	NS
DFN2T1.34	C A L G E L Y TGTGCTCTGGGGAA	CTGTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 1;J δ 1 1/13-7.7%	NS	NS
DFN2T1.35	C A L G E P G G Y TGTGCTCTGGGGAA	CCG GGGGGATAC	S W D T R Q TCCTGGGACACCCGACAG	V δ 1;D δ 2F1;J δ 3 1/13-7.7%	NS	NS
DFN2T1.38	C A L G E X G G Y TGTGCTCTGGGGAA	NTG GGGGGATAC	S W D T R Q TCCTGGGACACCCGACAG	V δ 1;D δ 2F1;J δ 3 1/13-7.7%	NS	NS
DFN2T1.40	C A L G E L X G D T G G TGTGCTCTGGGGAA	CTAANG GGGGAT ACTGGGGC	S W D T R Q TCCTGGGACACCCGACAG	V δ 1;D δ 2;D δ 2;J δ 3 1/13-7.7%	NS	NS

TABLE 44 – PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE SKIN FROM A NORMAL 18-20week FEMALE FETAL DONOR (FNS-2)

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DFN2T2.2	C A C D T TGTGCCTGTGACACC	W G TGGGGG	S S W D T R AGCTCCTGGGACACCCGA	V δ 2;D δ 2F3;J δ 3 2/24 - 8.3%	NS	NS
DFN2T2.1	C A C TGTGCCTGT	G T G G GGT ACTGGGGGA	S S W D T R AGCTCCTGGGACACCCGA	V δ 2;D δ 2F1;J δ 3 1/24 - 4.2%	NS	NS
DFN2T2.3	C A C D T TGTGCCTGTGACACC	G G Y GGGGGATAC	S W D T R TCCTGGGACACCCGA	V δ 2;D δ 2F1;J δ 3 1/24 - 4.2%	NS	NS
DFN2T2.4	C A C D TGTGCCTGTGAC	N T G G Y AAT ACTGGGGGATAC	S W D T R TCCTGGGACACCCGA	V δ 2;D δ 2F1;J δ 3 1/24 - 4.2%	NS	NS
DFN2T2.5	C A C D T TGTGCCTGTGACACC	G I R GGGATACGC	S W D T R TCCTGGGACACCCGA	V δ 2;D δ 2F3;J δ 3 1/24 - 4.2%	NS	NS
DFN2T2.6	C A C D TGTGCCTGTGAC	A L D G L D Y G H GCACTAGACGGCCTCGATTACGGCCAT	K L I F AAACTCATCTTT	V δ 2;J δ 1 1/24 - 4.2%	NS	NS
DFN2T2.7	C A C D T TGTGCCTGTGACACC	G G Y GGGGGATAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F1;J δ 1 1/24 - 4.2%	NS	NS
DFN2T2.8	C A C D TGTGCCTGTGAC	L L G V V CTA CTGGGG GTTGTC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/24 - 4.2%	NS	NS
DFN2T2.10	C A C D TGTGCCTGTGAC	Y W G TAC TGGGGG	S S W D T R AGCTCCTGGGACACCCGA	V δ 2;D δ 2F3;J δ 3 1/24 - 4.2%	NS	NS
DFN2T2.11	C A C D T TGTGCCTGTGACACC	G Y GGGTAC	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F1;J δ 1 1/24 - 4.2%	NS	NS
DFN2T2.12	C A C D T TGTGCCTGTGACACC	G G GGGGGA	L T A Q L F TTGACAGCACAACCTCTTC	V δ 2;D δ 2F1;J δ 2 1/24 - 4.2%	NS	NS
DFN2T2.13	C A C D TGTGCCTGTGAC	K V L G D T R AAAGTA CTGGGGGATACA CGC	S W D T R TCCTGGGACACCCGA	V δ 2;D δ 2F2;J δ 3 1/24 - 4.2%	NS	NS

TABLE 44 – PRODUCTIVELY-REARRANGED CDR3 REGION SEQUENCES OF V δ 2 SUBGROUP δ -CHAIN TCR TRANSCRIPTS IN THE SKIN FROM A NORMAL 18-20week FEMALE FETAL DONOR (FNS-2) continued

Clone	Variable region	N-Diversity region	Junctional region	Identity/Frequency	Statistical analysis	
					1/n	2/n
DFN2T2.14	C A C D tgtgcctgtgac	W G tggggg	S S W D T R agctcctgggacaccga	V δ 2;D δ 2F3;J δ 3 1/24 - 4.2%	NS	NS
DFN2T2.16	C A C D T TGTGCCTGTGACACC	W G I TGGGGGATA	D K L I F GATAAACTCATCTTT	V δ 2;D δ 2F3;J δ 1 1/24 - 4.2%	NS	NS
DFN2T2.17	R A C D CGTGCCTGTGAC	I L G E A ATA CTGGGG GAAGCT	L T A Q L F TTGACAGCACAACCTTTC	V δ 2;D δ 2F2;J δ 2 1/24 - 4.2%	NS	NS
DFN2T2.18	C A C D TGTGCCTGTGAC	T G G Y ACTGGGGGATAC	S W D T R TCCTGGGACACCCGA	V δ 2;D δ 2F1;J δ 3 1/24 - 4.2%	NS	NS
DFN2T2.19	C A C D TGTGCCTGTGAC	I L G D ATA CTGGGGGAT	L T A Q L F TTGACAGCACAACCTTTC	V δ 2;D δ 2F2;J δ 2 1/24 - 4.2%	NS	NS
DFN2T2.22	C A C D T TGTGCCTGTGACACC	A G G Y GCT GGGGGATAC	S W D T R TCCTGGGACACCCGA	V δ 2;D δ 2F1;J δ 3 1/24 - 4.2%	NS	NS
DFN2T2.23	C A C D T TGTGCCTGTGACACC	V L G D T R GTA CTGGGGGATACG CGC	S W D T R TCCTGGGACACCCGA	V δ 2;D δ 2F2;J δ 3 1/24 - 4.2%	NS	NS
DFN2T2.24	C A C D TGTGCCTGTGAC	V L G D T GTA CTGGGGGATACC	S W D T R TCCTGGGACACCCGA	V δ 2;D δ 2F2;J δ 3 1/24 - 4.2%	NS	NS
DFN2T2.25	C A C D TGTGCCTGTGAC	I L G D ATA CTGGGGGAT	T D K L I F ACCGATAAACTCATCTTT	V δ 2;D δ 2F2;J δ 1 1/24 - 4.2%	NS	NS
DFN2T2.26	C A C D T TGTGCCTGTGACACC	G G L G D T GGAGGG CTGGGGGATACC	W D T R TGGGACACCCGA	V δ 2;D δ 2F2;J δ 3 1/24 - 4.2%	NS	NS

In NS-1, statistically significant clonal expansions were seen in the V γ I, V γ 9, V δ 1 and V δ 2 transcripts, (see table 37-40). Specifically, the V γ I chain analysis (table 37) revealed two clones with 4 out of 36 (11%) identical CDR3 transcripts (transcript #6 and #39), representing a statistically significant clonal expansion; CATW TPRH KKLF, V γ 4;J γ 2.3;C γ 2, and CATWDR RG YKKLF, V γ 5;J γ 2.3;C γ 2, $p=0.0142$, respectively. There were two clones with 3 out of 36 (8.3%) identical transcripts, but these represented a statistically insignificant clonal expansion ($p>0.05$). Three clones had 2 out of 21 (5.5%), identical transcripts, but these also represented a statistically insignificant clonal expansion ($p>0.05$). In addition, there were nineteen clones, each with unique CDR3 regions (1 out of 22), indicative of polyclonal expansions. The V γ 9 chain analysis of NS-1 (table 38) revealed 20 out of 25 (80%) transcripts with identical CDR3 regions (transcripts #2), representing a statistically significant clonal expansion; CALWE PL YYKKLF, V γ 9;J γ 2.3;C γ 2, $p<0.0001$. Two clones had 2 out of 25 (8%) identical transcripts, but these represented a statistically insignificant clonal expansion ($p>0.05$). The remaining transcript had a unique CDR3 region (1 out of 25). The V δ 1 chain analysis of NS-1 (table 39) revealed 12 out of 17 (70.6%) transcripts with identical CDR3 regions (transcript #7), representing a statistically significant clonal expansion; CALGE LSG LP TLAV GDT RIA LTAQL, V δ 1;D δ 1F1;D δ 2F2;J δ 2, $p<0.0001$. One clone had 3 out of 17 (17.6%) identical transcripts, and another clone had 2 out of 17 (11.8%) identical transcripts, both representing statistically insignificant clonal expansions ($p>0.05$). The V δ 2 chain analysis of NS-1 (table 40) revealed 14 out of 31 (45%) transcripts with identical CDR3 regions (transcript #2), and 6 out of 31 (19%) transcripts with identical CDR3 regions (transcript #1), both representing statistically significant clonal expansions; CAC P TG VS GG LIFG, V δ 2;D δ 2F1;D δ 2F1;J δ 1, $p<0.0001$ and CACDT GD TRQ,

V δ 2;D δ 2F2;J δ 3, $p=0.0004$, respectively. The remaining transcripts all represented statistically insignificant clonal expansions ($p<0.05$).

In FNS-2, a statistically significant clonal expansion was only seen in the V γ 9 transcripts. Specifically, the V γ I chain analysis (see table 41) revealed the presence of two clones with 3 out of 14 (21%) identical transcripts ($p=0.0596$), two clones with 2 out of 14 (14%) identical transcripts ($p=0.1908$), and four unique transcripts. In contrast, the V γ 9 chain analysis (see table 42) revealed the presence of 17 out of 26 (65.4%) identical transcripts (transcript #1), representing a statistically significant clonal expansion; CALWEV QELGKK, V γ 9;J γ 1.2;C γ 1, $p<0.0001$. The remaining nine V γ 9 transcripts were unique. The V δ 1 chain analysis (see table 43) revealed the presence of a clone with 3 out of 13 (23%) identical transcripts, and another clone with 2 out of 13 (15%) identical transcripts, both representing statistically insignificant clonal expansion ($p>0.05$). The remaining eight V δ 1 transcripts had unique CDR3 region transcripts. The V δ 2 chain analysis (see table 44) revealed the presence of statistically insignificant clonal expansion in a clone, with 2 out of 24 (8.3%), and 21 unique transcripts ($p>0.05$). Minimal N-nucleotide addition was observed in the majority of the γ - and δ -chain transcripts from the fetal donor, and D δ -region segments were intact with minimal junctional exonuclease activity, which were all features consistent with a early T-cell development possibly prior to strong expression of tdt enzyme.

The deduced amino acid sequence of all γ - and δ -chain transcripts analyzed in both NS-1 and FNS-2 revealed neither amino acid sequence homology nor the presence of conserved motifs, when compared using BLAST searches to transcripts in the GENBANK database, suggesting these were unique, previously unidentified, clones.

4.3 T-CELL RECEPTOR EXPRESSION STUDY

4.3.1 Full length γ - and δ -chain TCR transcript construction

To determine if $\gamma\delta$ TCR, clonally expanded in SSc skin biopsies, recognized highly implicated putative SSc antigens, the γ - and δ -chain from an SSc patient (SS-16) that exhibited statistically significant clonal expansion were expressed in a TCR-negative T-cell line. The skin biopsy of SS-16 had statistically significant clonal expansion of one transcript each of the V γ 9 and V δ 2, which are physiological $\gamma\delta$ TCR pairs, *in vivo*, while the remaining transcripts were unique. Furthermore, SS-16 had the same V γ 9 and V δ 2 transcript clonally-expanded in the PBMC. Identification of the antigen recognized by these V γ 9 and V δ 2 transcript pair, may facilitate a better understanding of the role of $\gamma\delta$ TCR+ T-cells in not only the skin lesion of this patient, but also in the persistence of the clonal expansion in the peripheral blood.

To initiate the expression of the most clonally-expanded (MCE) V γ 9 and V δ 2 from SS-16, as well as non clonally-expanded V γ 9 and V δ 2 transcripts, as controls for the antigen recognition study, full length copies of each transcript was constructed (see figure 9).

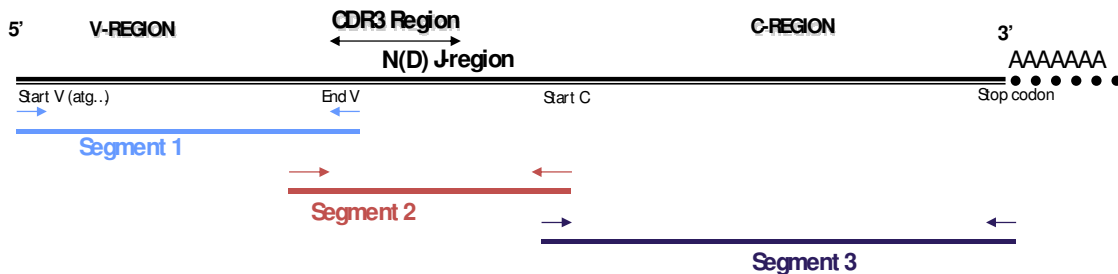


Figure 9 – General summary of full length construction, depicting the 3 overlapping segments spanning the full length gene of the TCR chain; Segment 1 (light blue) codes for most of the V-region, Segment 2 (red) codes for the CDR3 region (end V- N(D)-J-region) and the start of the C-region, and segment 3 codes for the C-region.

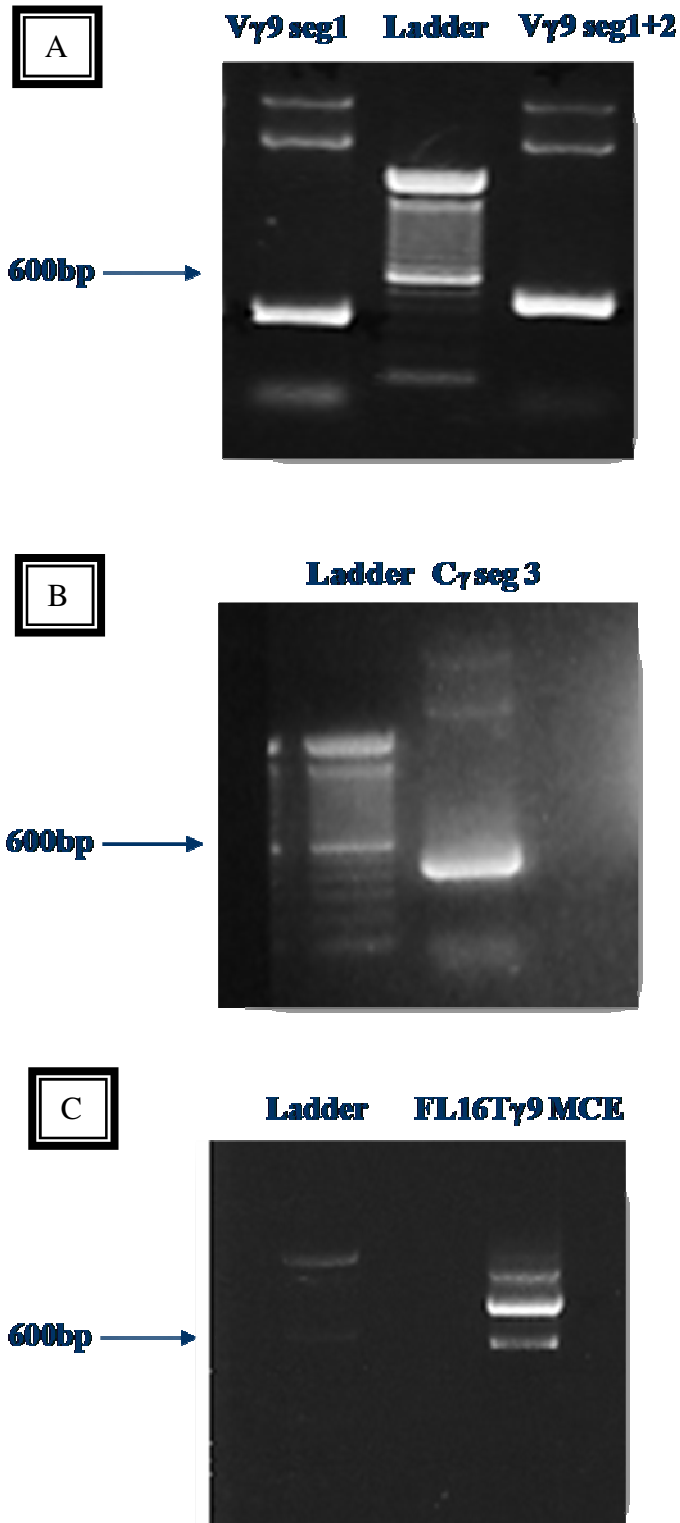


Figure 10 – Example of generation of full length TCR chain, specifically the full length copy of the most clonally-expanded V γ 9 chain from SS-16 skin biopsy; Three

overlapping segments that spanned the entire gene were generated, **figure 10A** depicts the gel picture of segment 1 (V γ 9 coding segment) in lane 1, and ligation product of segment 1 and 2 (specific CDR3 coding segment) in lane 3; **figure 10B** depicts the gel picture of segment 3 (C γ coding segment) in lane 1; **figure 10C** depicts the gel picture of the ligation product of segment 1+2 with segment 3 in lane 2, representing the full length transcript (951 bp).

A

Segment 1

```

ctgcttaagcatgctcgagcggccgccagtgatggatatctgcagaattcgcccttatg
C L S M L E R P P V - W I S A E F A L M
ctgtcactgctccacacatcaacgctggcagtccttggggctctgtgtgtataggtgca
L S L L H T S T L A V L G A L C V Y G A
ggtcacctagagcaacctcaaatttccagtaactaaaacgctgtcaaaaacagcccgcctg
G H L E Q P Q I S S T K T L S K T A R L
gaatgtgtggtgctctggaataacaatttctgcaacatctgtatattggtatcgagagaga
E C V V S G I T I S A T S V Y W Y R E R
cctggtgaagtcatacagttcctggtgtccatttcatatgacggcactgtcagaaaggaa
P G E V I Q F L V S I S Y D G T V R K E
tccggcattccgtcaggcaaatttgaggtggataggatacctgaaacgtctacatccact
S G I P S G K F E V D R I P E T S T S T
ctcaccattcacaatgtagagaaaacaggacatagctacctactactgtgccttgtgggag
L T I H N V E K Q D I A T Y Y C A L W E
gtgcattataagaaactctttgggttgatgtgtggagcagtgacagcataagggcgaaatt
V H Y K K L F G L M C G A V T A - G R I

```

B

Segment 1+2

```

ggcacttcagaagcatgctcgagcggccgccagtgatggatatctgcagaattcgccctt
H F R S M L E R P P V - W I S A E F A L
atgtgtcactgctccacacatcaacgctggcagtccttggggctctgtgtgtataggt
M L S L L H T S T L A V L G A L C V Y G
gcaggtcacctagagcaacctcaaatttccagtaactaaaacgctgtcaaaaacagcccgc
A G H L E Q P Q I S S T K T L S K T A R
ctggaatgtgtagtgtctggaataacaatttctgcaacatctgtatattggtatcgagag
L E C V V S G I T I S A T S V Y W Y R E
agacctggtgaagtcatacagttcctggtgtccatttcatatgacggcactgtcagaaag
R P G E V I Q F L V S I S Y D G T V R K
gaatccggcattccgtcaggcaaatttgaggtggataggatacctgaaacgtctacatcc
E S G I P S G K F E V D R I P E T S T S
actctcaccattcacaatgtagagaaaacaggacatagctacctactactgtgccttgtgg
T L T I H N V E K Q D I A T Y Y C A L W
gaggtgctggcggcgggggttgggcaaaaaaatcaaggtatattggtcccggaaacaaagctt
E V L A A G L G K K I K V F G P G T K L
ataattacagataaacaacttgatgcagatgtttccccaagcccactatTTTTTcttctg
I I T D K Q L D A D V S P S P L F F F L

```



Segment 3

```

ctggaattcgcccttgataaacaacttgatgcagatggttcccccaagcccactatTTTT
L E F A L D K Q L D A D V S P K P T I F
cttccttcaattgctgaaacaaagctccagaaggctggaacatacctttgtcttcttgag
L P S I A E T K L Q K A G T Y L C L L E
aaatTTTTccctgatggttattaagatacattggcaagaaaagaagagcaacacgattctg
K F F P D V I K I H W Q E K K S N T I L
ggatcccaggaggggaacacccatgaagactaacgacacatacatgaaatTTtagctggTTa
G S Q E G N T M K T N D T Y M K F S W L
acggTgccagaaaagtcactggacaaagaacacagatgtatcgtcagacatgagaataat
T V P E K S L D K E H R C I V R H E N N
aaaaacggagttgatcaagaaattatcttccctccaataaagacagatgtcatcacaatg
K N G V D Q E I I F P P I K T D V I T M
gatcccagagacaattggtcaaaagatgcaaatgatacactactgctgcagctcacaac
D P R D N C S K D A N D T L L L Q L T N
acctctgcatattacatgtacctcctcctgctcctcaagagtgTggTctatTTTTgccatc
T S A Y Y M Y L L L L L K S V V Y F A I
atcacctgctgtctgcttagaagaacggctttctgctgcaatggagagaaatcataaaag
I T C C L L R R T A F C C N G E K S - K

```

Figure 11 – Sequence analysis of the V γ 9 gene segments obtained. Color-coded depiction of the different regions of the TCR regions; V-region (red), N-region (blue), J-region (yellow), C-region (purple); **figure 11A** depicts segment 1, **figure 11B** depicts segment 1+2, **figure 11C** depicts segment 3.

Segment1+2+3 – 16T γ 9MCE Full length construct

```

atgctgtcactgctccacacatcaacgctggcagtccttggggctctgtgtgtatatggt
M L S L L H T S T L A V L G A L C V Y G
gcaggTcacctagagcaacctcaaatttccagTactaaaacgctgtcaaaaacagccccg
A G A H L E Q P Q I A S S T K T L S K T A R
ctggaatgtgtgtgtctggaataacaatttctgcaacatctgtatatTTggtatcgagag
L E C V V S G I T I S A T S V Y W Y R E
agacctggtgaagtcatcacagttcctgggtgtccatttcatatgacggcactgtcagaaag
R P G E V I Q F L V S I S Y D G T V R K
gaatccggcattccgtcaggcaaatttgaggTggataggatacctgaaacgtctacatcc
E S G I P S G K F E V D R I P E T S T S
actctcaccattcacaatgtagagaaacaggacatagctacctactactgtgccttTggg
T L T I H N V E K Q D I A T Y Y C A L W
gaggTgctggcggcggggTtgggcaaaaaaatcaaggTatTTggtcccggaaacaaagctt
E V L A A G L G K K I K V F G P G T K L
ataattacagataaacaacttgatgcagatggttcccccaagcccactatTTTTcttctc
I I T D K Q L D A D V S P K P T I F L P
tcaattgctgaaacaaagctccagaaggctggaacatacctttgtcttcttgagaaattt
S I A E T K L Q K A G T Y L C L L E K F
ttccctgatggttattaagatacattggcaagaaaagaagagcaacacgattctgggatcc
F P D V I K I H W Q E K K S N T I L G S
caggaggggaacacccatgaagactaacgacacatacatgaaatTTtagctggTTaacggTg
Q E G N T M K T N D T Y M K F S W L T V
ccagaaaagtcactggacaaagaacacagatgtatcgtcagacatgagaataataaaaac
P E K S L D K E H R C I V R H E N N K N

```

```

ggagttgatcaagaaattatctttcctccaataaagacagatgtcatcacaatggatccc
G V D Q E I I F P P I K T D V I T M D P
agagacaattggtcaaaagatgcaaatagatacactactgctgcagctcacaacacctct
R D N C S K D A N D T L L L Q L T N T S
gcatattacatgtacctcctcctgctcctcaagagtggtgtctatTTTgcatcatcacc
A Y Y M Y L L L L L K S V V Y F A I I T
tgctgtctgcttagaagaacggctttctgctgcaatggagagaaatcataa
C C L L R R T A F C C N G E K S -

```

Figure 12 – Color-coded depiction of the different regions of the TCR regions of the error-free, complete full length copy of the most clonally-expanded V γ 9 chain of SS-16.

V γ 9 chain of SS16 - 316 amino acids (951 bp)

```

MLSL LH TSTLAVLGALCVYGAGHLEQPQISSTKTL SKTARLECVVSGITISATSVYW
YRERPGEVIQFLVSI SYDGTVRKESGIPSGKFEVD RIPETSTSTLTIHNVEKQDIATY
YCALWEVLAAGLGKKIKVFGPGTKLIITDKQLDADVSPKPTIFLPSIAETKLQKAG
TYLCLLEKFFPDVIKIHVQEKKSNTILGSQEGNTMKTNDTYMKFSWLTVPKSLD
KEHRCIVRHENNKNGVDQEIFPPIKTDVITMDPRDNCSKDANDTLLLQLTNTSA
YYMYLLLLLKSVVYFAITCCLLRRTAFCCNGEKS

```

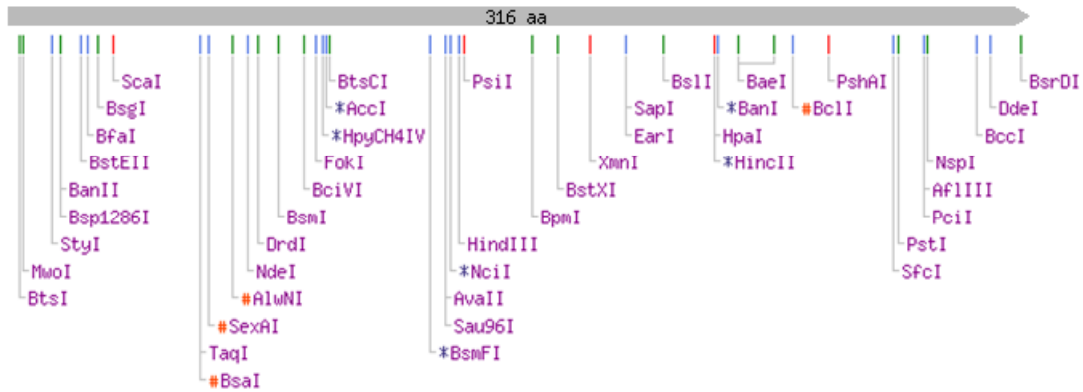


Figure 13 – restriction digest check (NEBcutter) to determine restriction enzyme optimal for expression vector construction (must be absent from within the gene).

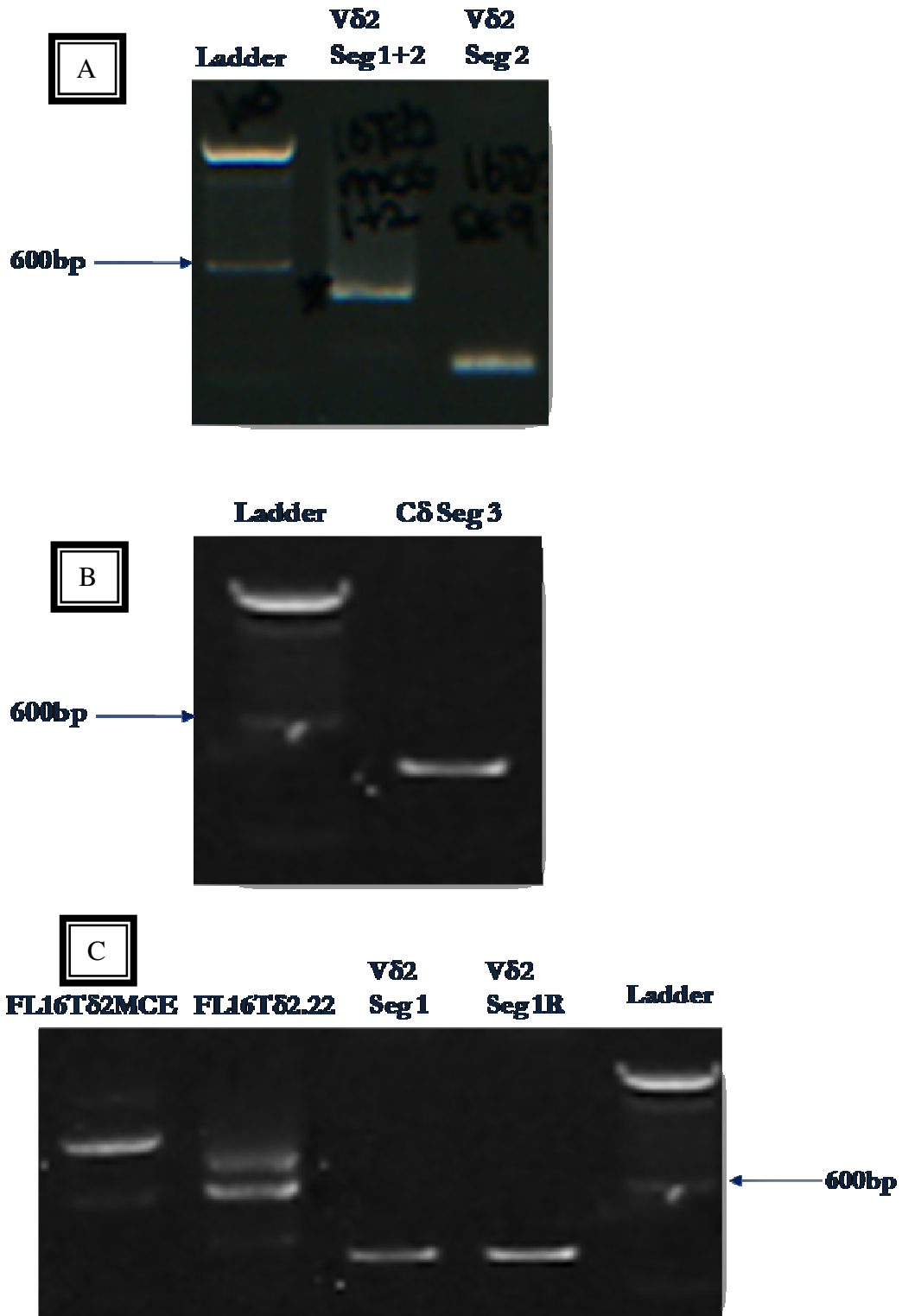


Figure 14 – Example of generation of full length TCR chain, specifically the full length copy of the most clonally-expanded Vδ2 chain from SS-16 skin biopsy; Three

overlapping segments that spanned the entire gene were generated, **figure 14A** depicts the gel picture of the ligation product of segment 1+2 in lane 1, and segment 2 (specific CDR3 coding segment) in lane 2; **figure 14B** depicts the gel picture of segment 3 (C δ coding segment) in lane 1; **figure 14C** depicts the gel picture of the full length copy of 16T δ 2MCE in lane 1 (879 bp).

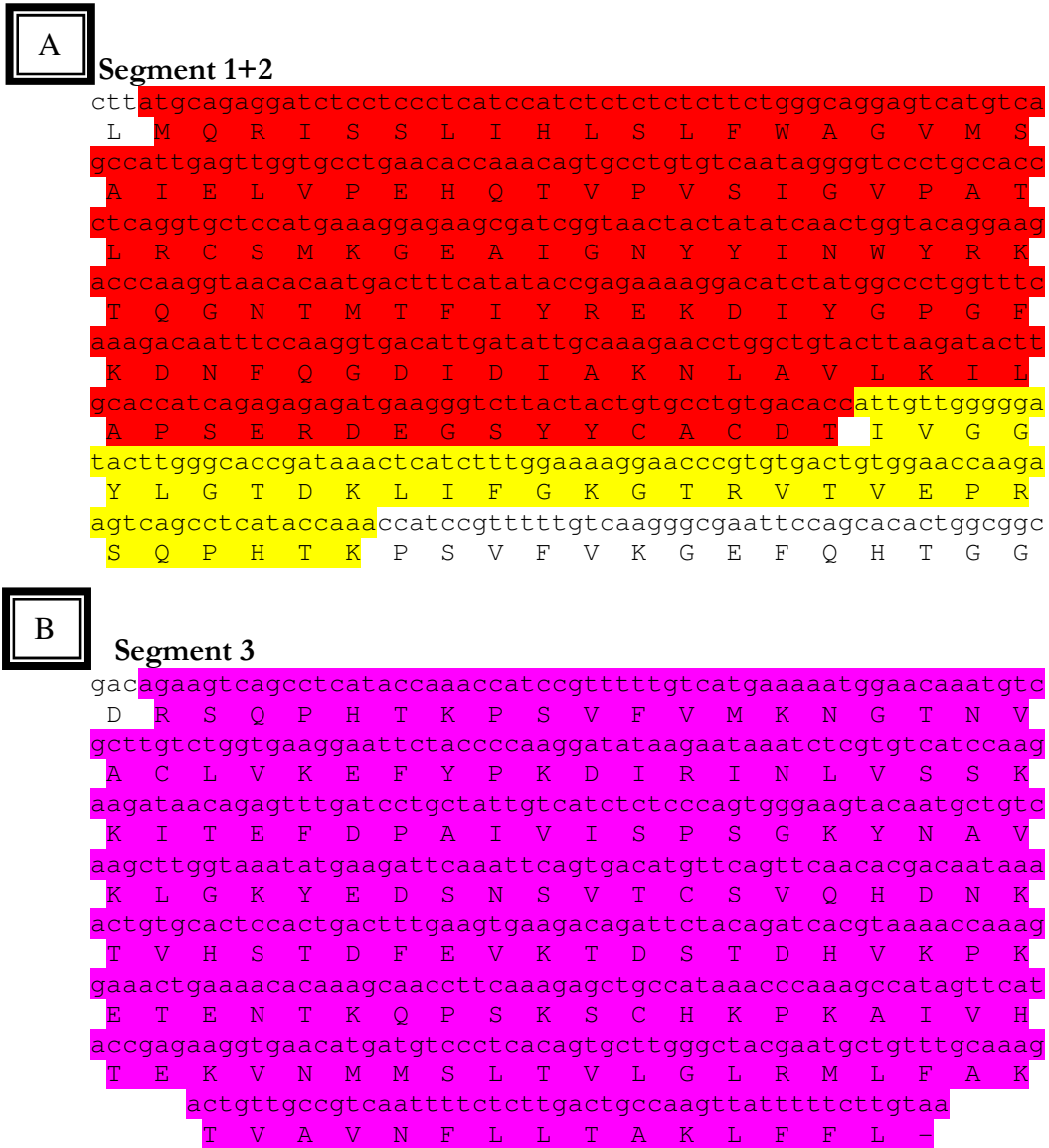


Figure 15 – Sequence analysis of the V δ 2 gene segment obtained. Color-coded depiction of the different regions of the TCR regions; V-region (red), N-region (blue), D-region (green), J-region (yellow), C-region (purple); **figure 15A** depicts segment 1+2, **figure 15B** depicts segment 3.

Segment 1+2+3

atgcagaggatctcctccctcatccatctctctctctctctctctgggcaggagtcatgtcagcc
M Q R I S S L I H L S L F W A G V M S A
attgagttggtgcctgaacaccaaacagtgcctgtgtcaataggggtccctgccaccctc
I E L V P E H Q T V P V S I G V P A T L
aggtgctccatgaaaggagaagcgatcggtactactatatcaactggtacaggaagacc
R C S M K G E A I G N Y Y I N W Y R K T
caaggtaacacaatgactttcatataccgagaaaaggacatctatggccctggtttcaaa
Q G N T M T F I Y R E K D I Y G P G F K
gacaatttccaaggtgacattgatattgcaaagaacctgggtgtacttaagatacttgca
D N F Q G D I D I A K N L A V L K I L A
ccatcagagagagatgaagggcttactactgtgcctgtgacaccattggtgggggatac
P S E R D E G S Y Y C A C D T I V G G Y
ttgggcaccgataaactcatctttggaaaaggaacccgtgtgactgtggaaccaagaagt
L G T D K L I F G K G T R V T V E P R S
cagcctcataccaaaccatccgtttttgtcatgaaaaatggaacaaatgtcgttgtctg
Q P H T K P S V F V M K N G T N V A C L
gtgaaggaattctacccaaggatataagaataaatctcgtgtcatccaagaagataaca
V K E F Y P K D I R I N L V S S K K I T
gagtttgatcctgctattgtcatctctcccagtggggaagtacaatgctgtcaagcttgg
E F D P A I V I S P S G K Y N A V K L G
aaatatgaagattcaaattcagtgacatggttcagttcaacacgacaataaaaactgtgcac
K Y E D S N S V T C S V Q H D N K T V H
tccactgactttgaagtgaagacagattctacagatcacgtaaaaccaaaggaaactgaa
S T D F E V K T D S T D H V K P K E T E
aacacaaagcaaccttcaaagagctgccataaaaccaaagccatagttcataccgagaag
N T K Q P S K S C H K P K A I V H T E K
gtgaatatgatgtccctcacagtgcttgggctacgaatgctgtttgcaaagactgttgcc
V N M M S L T V L G L R M L F A K T V A
gtcaattttctcttgactgcccaagttattttcttgtaa
V N F L L T A K L F F L -

Figure 16 – Color-coded depiction of the different regions of the TCR regions of the error-free, complete full length copy of the most clonally-expanded V β 2 chain of SS-16.

V δ 2 chain of SS16 - 292 amino acids (879 bp)

MQRISLIHLISLFWAGVMSAIELVPEHQTVPVSIGVPATLRCSMKGEAIGNYYINWY
 RKTQGNTMTFIYREKDIYGPFGKDNFQGDIDIAKNLAVLKILAPSERDEGSYYCA
 CDTIVGGYLGTDKLIKFGKGTTRVTVEPRSQPHTKPSVFMKNGTINVAQLVKEFYF
 KDIRINLVSSKKITEFDPAIVISPSGKYNAVKLGKYEDSNSVTCVQHDNKTVHST
 DFEVKTDSTDHVKPKETENTKQPSKSKCHKPKAIVHTEKVNMMSLTVLGLRMLFA
 KTVAVNFLTAKLFFL

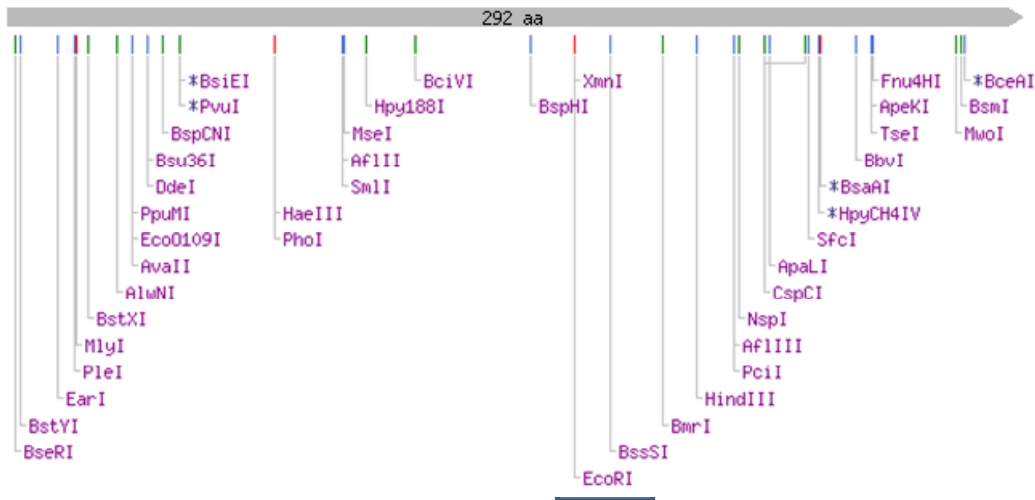


Figure 17 – Restriction digest check (NEBcutter) to determine restriction enzyme optimal for expression vector construction (must be absent from within the gene) - The restriction enzyme, *EcoR I* was excluded from the expression vector construction of the V δ 2, due to the presence of a site in the J δ region (see the blue underline).

Full length construction of the V γ 9 transcripts (see figures 10, 11, and 12) and the V δ 2 transcripts (see figure 14, 15, and 16) as well as restriction enzyme site checks (see figures 13 and 17) was done both for the most clonally-expanded γ - and δ -chain from SS-16, as described above. Full length copies of non clonally-expanded transcripts, for use as controls in the antigen recognition study, were also generated, and included a V γ I transcript (transcript #5), two other V γ 9 transcripts (transcripts #7 and #23) and four V δ 2 transcripts (transcripts #3, #10, #11, and #22).

4.3.2 Expression vector construction

The expression vector construction was a four step process; 1) obtaining TCR chain full length copies from TOPO® TA cloning vector by PCR (see figure 18), 2) Restriction digest of PCR products, 3) Restriction digest and de-phosphorylation of the expression plasmid, and 4) T4-mediated ligation of TCR chain full length copies to the expression vectors (see figure 19).

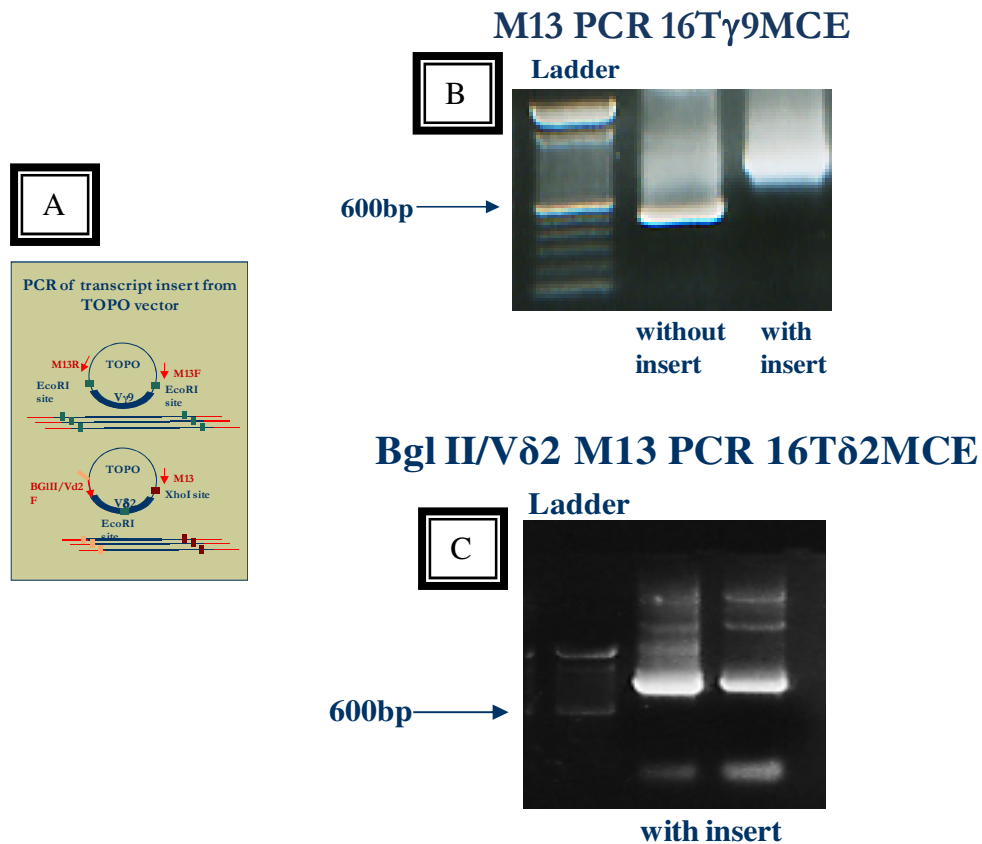


Figure 18 – First step of expression vector construction; figure 18A depicts a diagram of M13 PCR of the TCR chain product from the TOPO cloning vector, **figure 18B** depicts a gel picture of the most clonally-expanded 16T γ 9 PCR product, without the insert (lane 1) and with the insert (lane 2), **figure 18C** depicts a gel of the most clonally-expanded 16T δ 2 PCR product, both lanes with the insert.

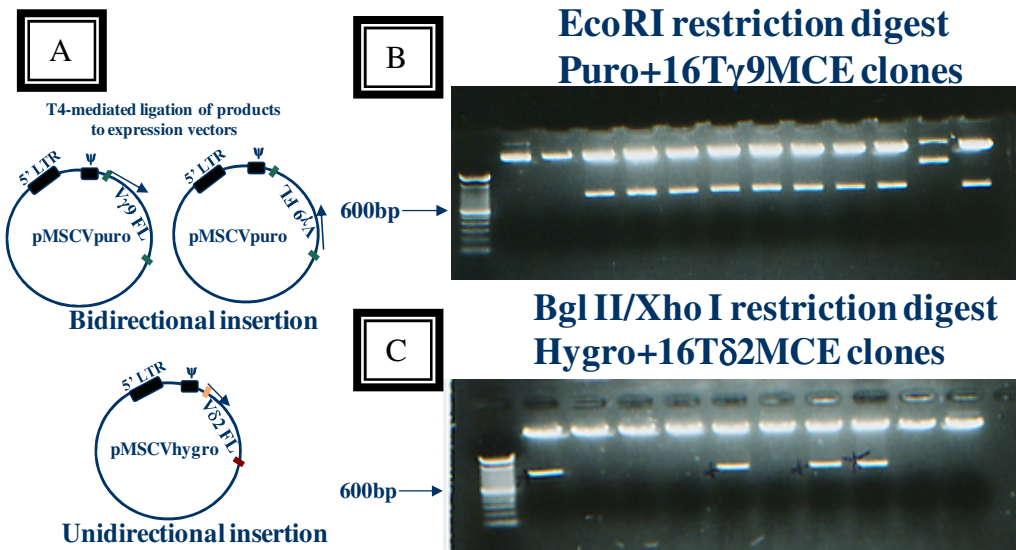


Figure 19 – Fourth step of pMSCV expression vector construction; figure 19A depicts a schematic diagram of T4-mediated ligation to the expression vector, **figure 19B** depicts a gel picture of the most clonally-expanded 16T γ 9 restriction digest product, showing presence or absence of insert in the puromycin vector, **figure 19C** depicts a gel picture of the most clonally-expanded 16T δ 2 restriction digest product, showing presence or absence of the insert in the hygromycin vector.

Expression vector constructs were made containing both the full length copies of the most clonally-expanded V γ 9 and V δ 2 transcripts from the skin biopsy of SS-16, as well as for the full length copies of two non-clonally expanded transcripts per chain, including V γ 9 transcripts #7 and #23, and V δ 2 transcripts #11 and #22. These constructs were, puromycin+SS16T γ 9MCE, puromycin+SS16T γ 9.7, puromycin+16T γ 9.23, hygromycin+SS16T δ 2MCE, hygromycin+SS16T δ 2.11, and hygromycin+SS16T δ 2.22.

4.3.3 Transfection of packaging cell line

The expression vectors were subsequently used to transfect the packaging cell lines to facilitate retrovirus production. To verify presence of the both vector and the specific γ - and δ -chains in the cell line, an RT-PCR was performed to amplify the transcripts of interest,

first using the kit provide pMSCV primers, with sites flanking the multiple cloning site of the expression vector, and using gene-specific primers.

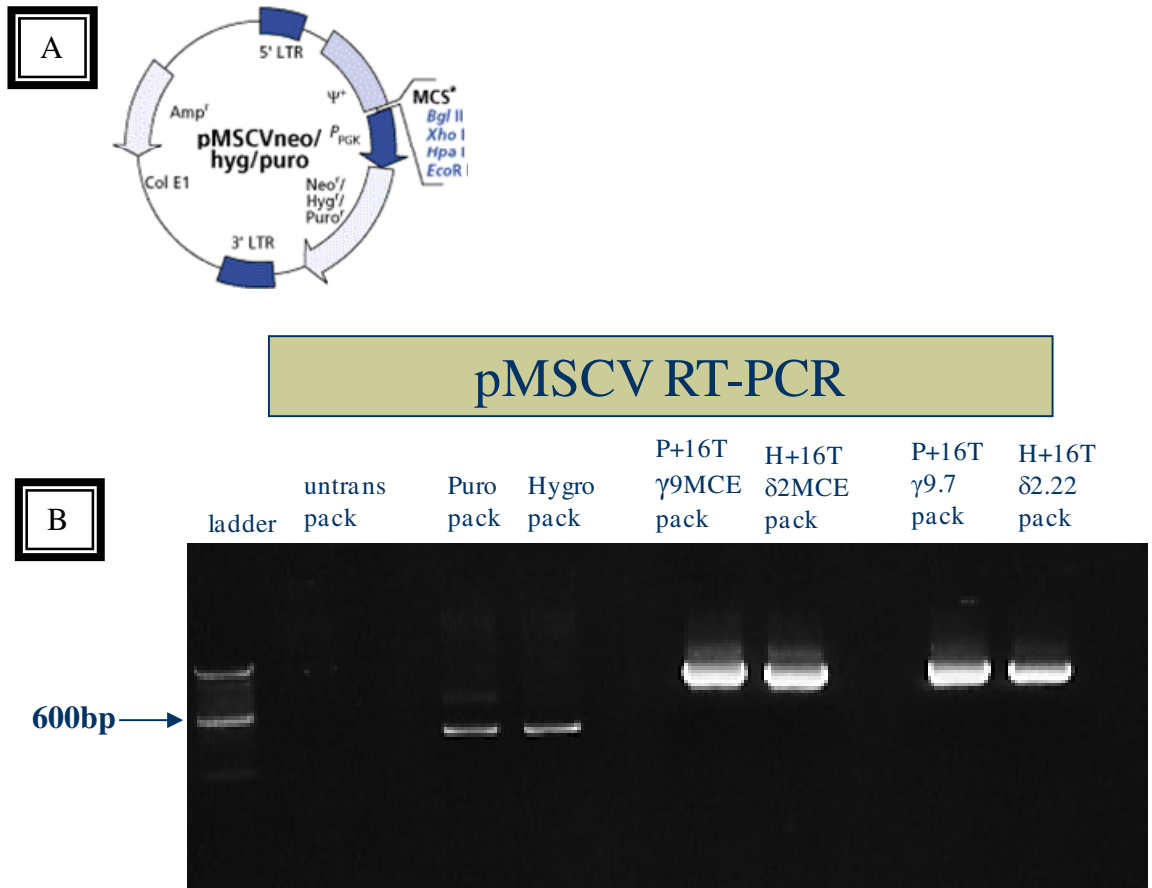


Figure 20 – Transfection of the packaging cell line with different expression vector constructs; figure 20A depicts the Clontech pMSV vector schematic diagram, figure 20B depicts a gel picture depicting post-transfection results, after RT-PCR using primers of the pMSCV vector sites flanking the multiple cloning site, to verify the presence of the vector.

There was a lack of vector presence in the untransfected packaging cell line (lane 1), the first negative control and the puromycin and hygromycin vector only transfected packaging cell lines (lane 3 and 4 respectively), showed the presence of the vector, as the low sized bands (350 bp). In addition, the packaging cell lines with the TCR chain gene insert (lanes 6, 7, 9, and 10), also showed the presence of the vector, however they were larger sized bands, since they contained an additional 850-1000 bp of genetic material inserted into

the multiple cloning site. Specifically the most clonally-expanded γ - and δ -chain (lanes 6 and 7, respectively), as well as a non clonally-expanded γ - and δ -chain (lanes 9 and 10 respectively) from SS-16, verifying the presence of the genes of interest, transfected into the packaging cell lines.

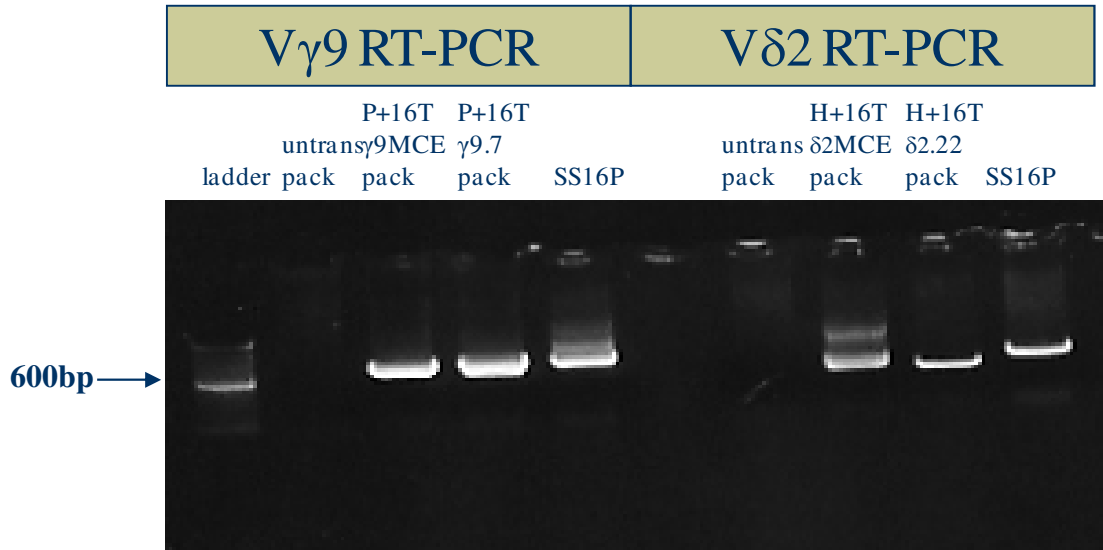


Figure 21 – Gel picture depicting post-transfection results, after RT-PCR using primers specific for either the V γ 9 and V δ 2 chain gene.

The RT-PCR results showed a lack of presence of the V γ 9 and V δ 2 gene-specific transcripts in the untransfected package (lane 1 and lane 6 respectively), the negative control, which corroborated the lack of expression vector transcripts (see figure 22). As expected, there was the presence of both of the V γ 9 transcripts (lane 2 and 3) and both V δ 2 transcripts (lane 7 and 8) of SS-16, in the appropriately transfected packaging cell lines. The positive controls were V γ 9 and V δ 2 transcripts from the PBMC of SS-16 (lane 4 and 9 respectively). All V γ 9 and V δ 2 full length transcripts generated were inserted into individual

expression vectors and used to transfect packaging cells, generating multiple cell lines, each producing retroviral vectors containing a V γ 9 or V δ 2 gene.

4.3.4 Transduction of mutant Jurkat T-cell line

The retrovirus generated by the packaging cell lines were harvested, filter-sterilized and used to transduce the mutant TCR-negative J.RT3-T3.5 Jurkat T-cell line, to generate multiple T-cell lines, including the negative controls (untransduced and mock vector only) that lacked TCR genes, a T-cell line transduced with the full length copies of the most clonally-expanded V γ 9 and V δ 2 chains from SS-16 (16TMCE), and other T-cell lines of the different combinations of the full length copies of non clonally-expanded V γ 9 and V δ 2 chains from SS-16. Successful expression was determined both by analyzing mRNA expression, via RT-PCR, and by verifying surface TCR expression by flow cytometry using antibodies specific to the $\gamma\delta$ TCR heterodimer, conjugated to a fluorochrome for detection.

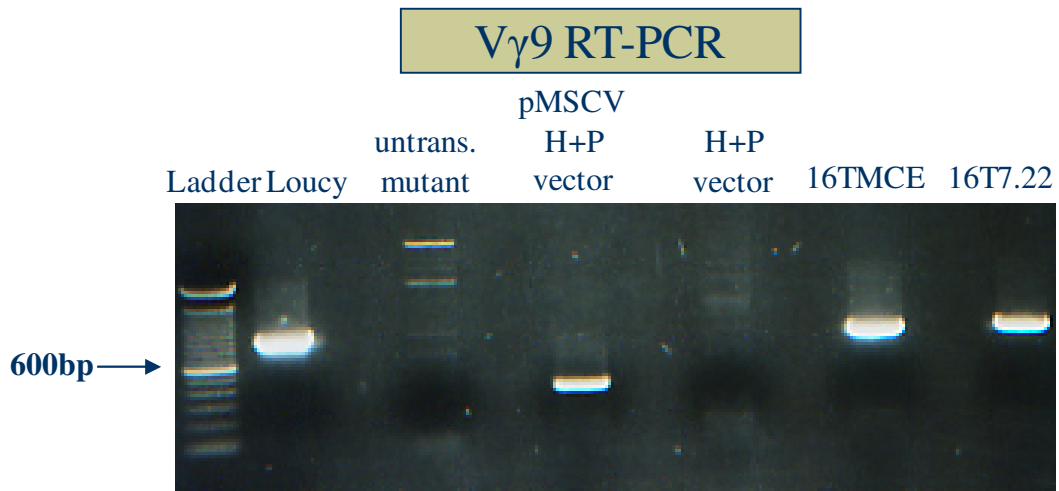


Figure 22 – Gel picture of V γ 9 gene RT-PCR of transduced Jurkat T-cell lines

The RT-PCR, using gene-specific primers, verified the presence of mRNA transcripts of the V γ 9 TCR chain in the transduced cell lines (lane 5 and 6), and the positive

control, the Loucy $\gamma\delta$ TCR+ T-cell line, from a $\gamma\delta$ TCR+ T-cell leukemia (lane 1). There was a lack of V γ 9 transcripts in the negative controls, specifically, the untransduced Jurkat T-cell line (lane 2), and the mock vector only transduced T-cell line (lane 4), which showed the presence of the expression vector with a concomitant RT-PCR using vector specific pMSCV primers (lane 3).

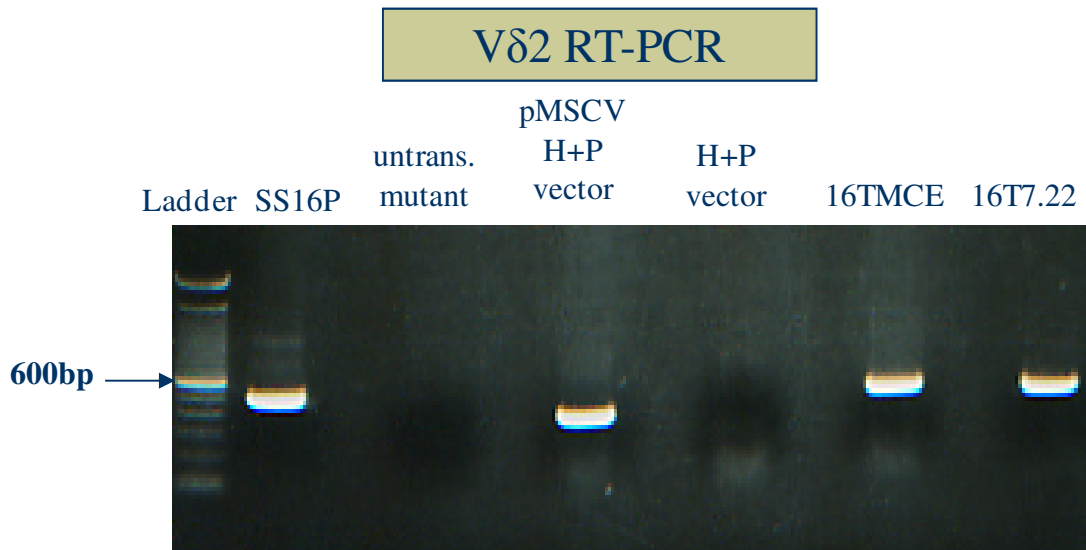


Figure 23 – Gel picture of V δ 2 gene RT-PCR of transduced Jurkat T-cell lines

The RT-PCR of the same samples analyzed in figure 24, using gene-specific primers, verified the presence of mRNA transcripts of the V δ 2 TCR chain in the transduced cell lines (lane 5 and 6), and the positive control, the PBMC of SS-16 (lane 1). There was a lack of V δ 2 transcripts in the negative controls, specifically, the untransduced Jurkat T-cell line (lane 2), and the mock vector only transduced T-cell line (lane 4), which showed the presence of the expression vector with an RT-PCR using vector specific pMSCV primers (lane 3).

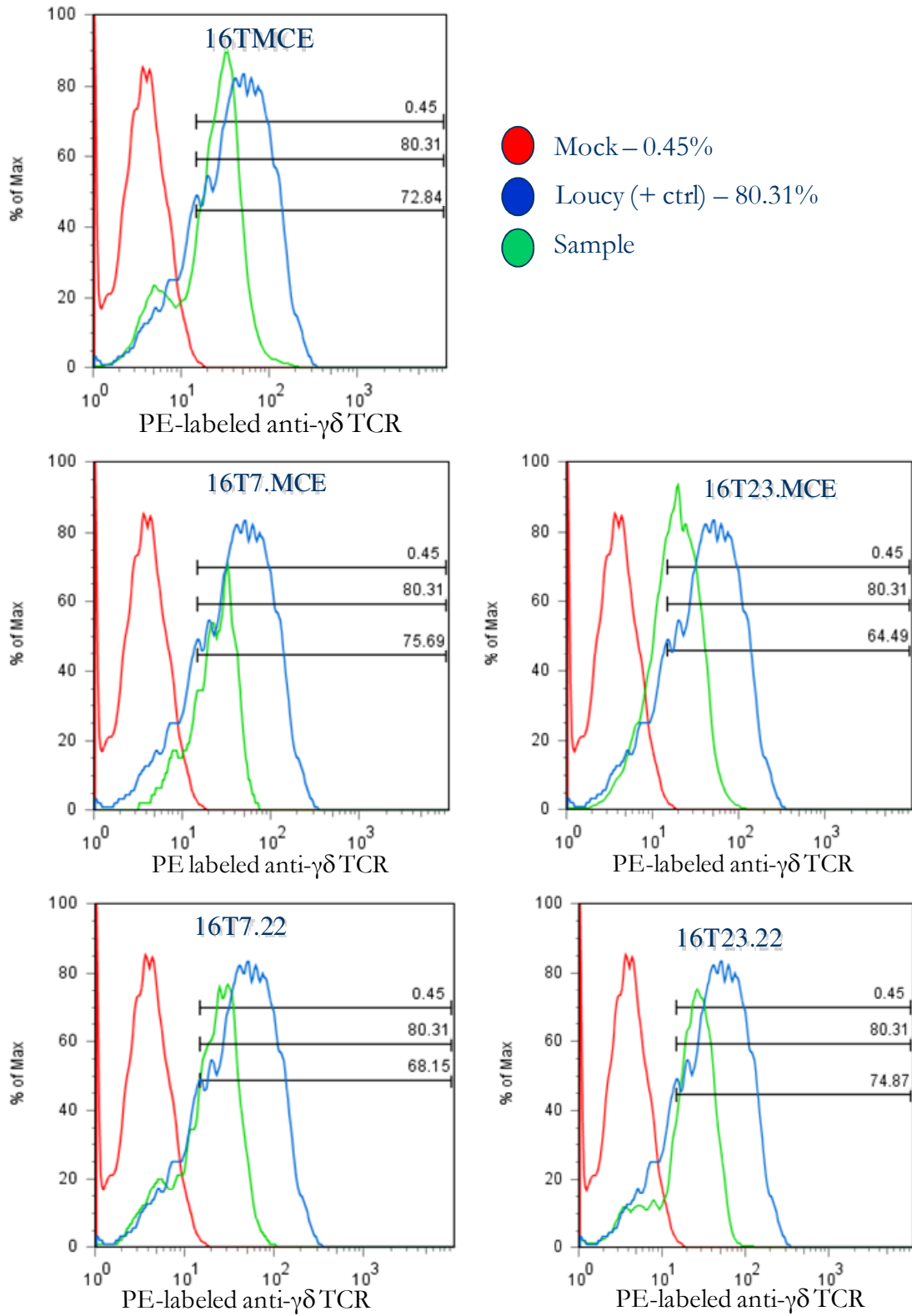


Figure 24 – Surface expression of the $\gamma\delta$ T-cell receptor of transduced cell lines -

Surface $\gamma\delta$ TCR transduced cells (green histogram) from different transduced Jurkat T-cell lines using phycoerythrin (PE) labeled anti- $\gamma\delta$ TCR antibody; negative control was the mock transduced cells (red) and positive control $\gamma\delta$ TCR+ T-cell line, Loucy, (blue).

In total, five experimental Jurkat T-cell lines were generated including a T-cell line expressing full length copies of the most clonally-expanded V γ 9 and V δ 2 transcripts from SS-16 (16TMCE), and different combination of full length copies of non clonally-expanded transcripts from SS-16 including, 16T7.22, 16T23.22, 16T7MCE, 16T23MCE. The negative control T-cell lines generated included the mock vector only cell line (hygromycin and puromycin), and single chain T-cell lines 16T γ 9MCE, 16T δ 2MCE, 16T γ 9.7, 16T γ 9.23, 16T δ 2.22.

4.3 ANTIGEN RECOGNITION STUDY

To determine if $\gamma\delta$ TCR+ T-cells from SSc patient skin or PBMC recognize putative SSc antigens, two different parameters were assessed. The first was measurement of calcium flux after stimulation of the transduced $\gamma\delta$ TCR+ Jurkat T-cell lines, especially the cell line expressing the most clonally-expanded TCR transcripts isolated from skin biopsy from an SSc patient (SS-16), with whole recombinant proteins of the antigens. The second parameter was measurement of cytotoxicity against human endothelial cell lines (HUVEC and HLMVEC), by the transduced $\gamma\delta$ TCR+ Jurkat T-cell lines, to assay for recognition of endothelial cell surface antigens.

4.3.1 Measurement of intracellular calcium flux with antigen stimulation

The transduced V γ 9V δ 2 T-cell lines were assessed for recognition of putative antigens via measuring calcium flux. The antigens assessed were positive controls, i.e. OKT3

(10 µg/mL) for anti-CD3 stimulation, and IPP (50 µM) for Vγ9Vδ2-specific TCR stimulation; experimental SSc putative antigens (15 µg/mL) as soluble human recombinant proteins; DNA topoisomerase I, centromere protein A, centromere protein B, hsp 27, and hsp 90, as well as the viral lysate of hCMV (AD619 strain).

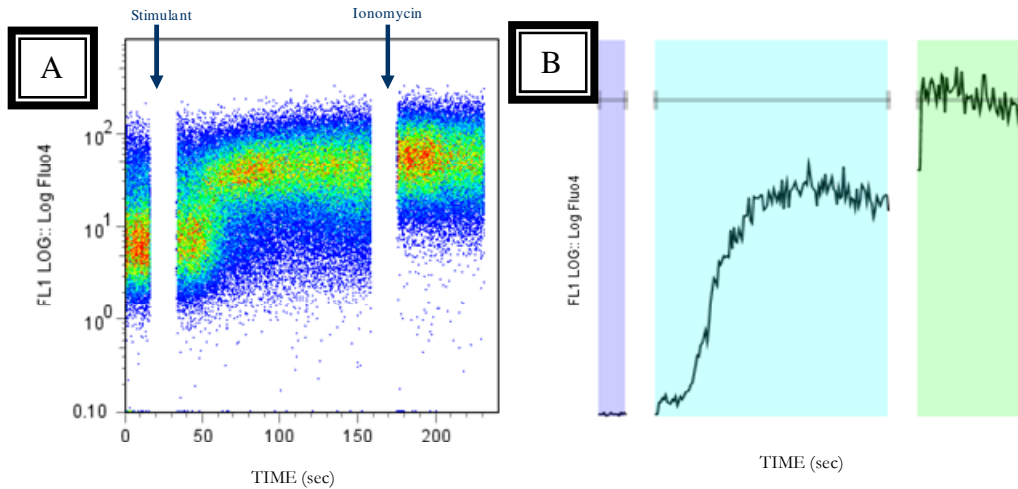


Figure 25 – Calcium flux in a wildtype Jurkat T-cell line in response to positive control OKT3 stimulation (anti-CD3 antibody; 5 µg/mL) – showing the change in calcium over time after antigen stimulation as well as the maximum calcium flux (F_{\max}) after subsequent ionomycin (2 µM) stimulation; dot plot (figure 25A) and mean fluorescent line (figure 25B).

To optimize the intracellular calcium flux protocol, a wildtype Jurkat T-cell line (with a functional TCR/CD3 complex) was stimulated with the positive control antigen, OKT3, an antibody against the CD3 complex of the TCR complex, which cross-links the CD3 molecules, initiating a positive activating to the T-cell. As expected, an almost instantaneous change in intracellular calcium was observed after addition of the antigen, (see figure 27). The subsequent ionomycin addition facilitated normalizing for the maximum dye binding capacity of the cell. All calcium flux data, obtained from the mean fluorescent line data was subsequently expressed as $\%F_{\max}$ i.e. $(F-F_0)/F_{\max}$ where F is the peak fluorescent after antigen stimulation, F_{\max} is the maximum fluorescence after ionomycin stimulation and F_0 is the

baseline fluorescence. The calcium flux in the wildtype Jurkat after stimulation was 65% of F_{\max} (see figure 28).

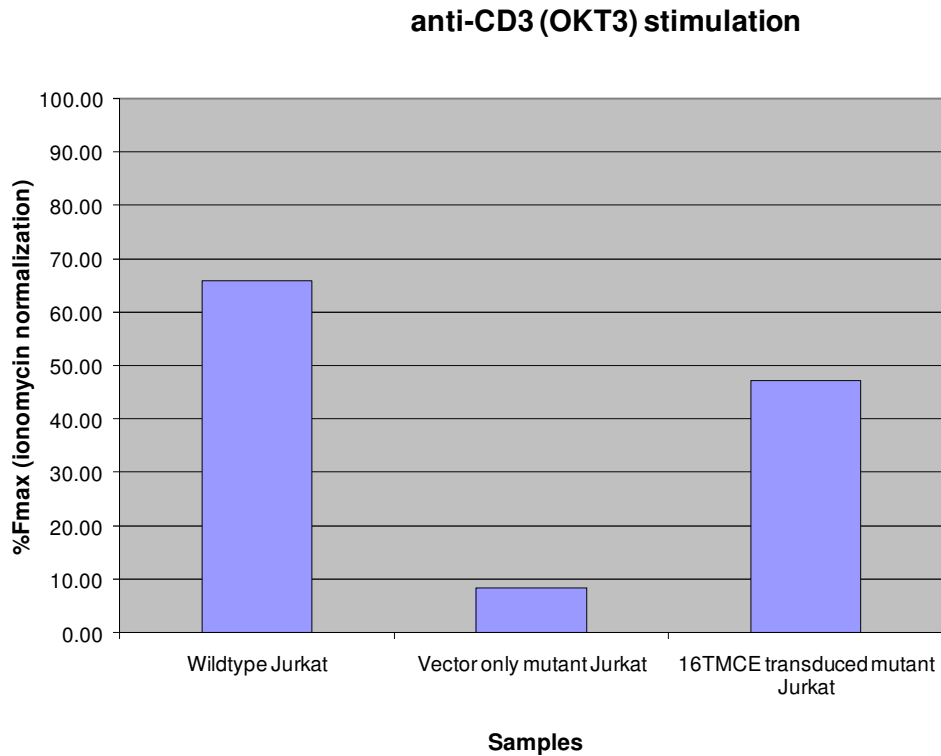


Figure 26 – Calcium flux, depicted as % F_{\max} , in the wildtype Jurkat and the transduced mock vector only and 16TMCE mutant Jurkat T-cells, with 5 $\mu\text{g}/\text{ml}$ of OKT3 for CD3 stimulation.

Positive control (OKT3) stimulation of the experimental transduced Jurkat T-cells showed a change in calcium fluorescence over time, with the peak fluorescence after fluorescence being 45% of F_{\max} , compared to the negative control, i.e. the mock vector only mutant Jurkat control, verifying the presence of the functional surface TCR/CD3 complex.

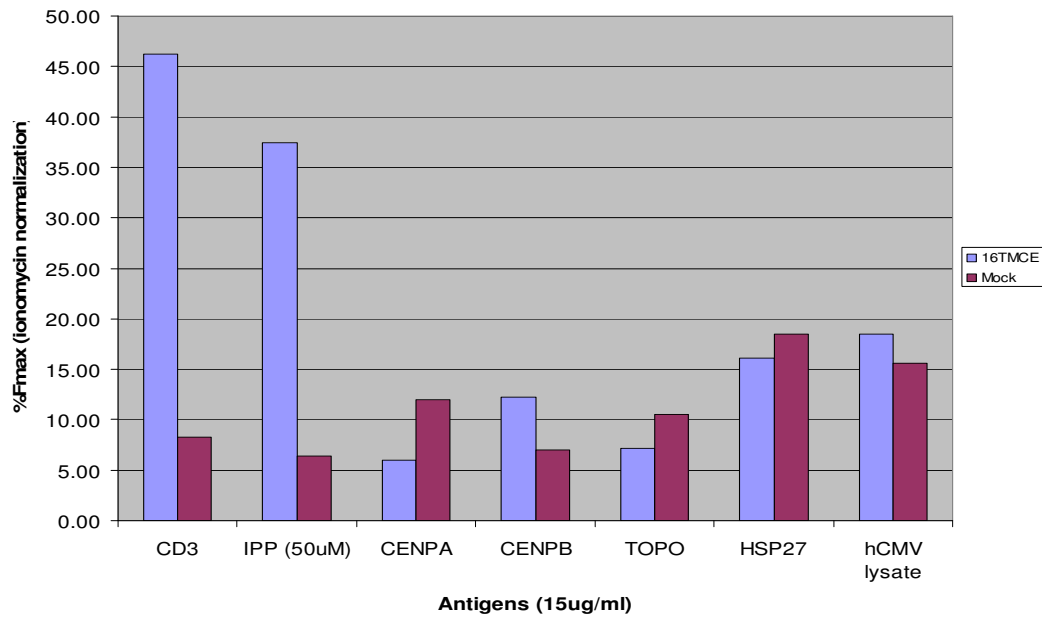
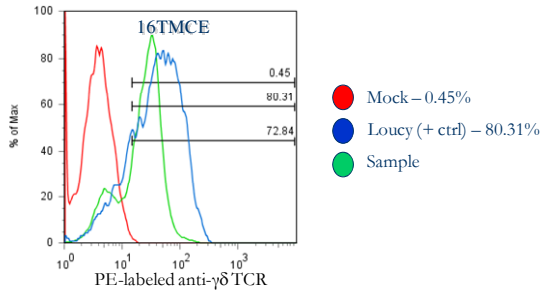


Figure 27 – Calcium flux of 16TMCE (blue bars) compared to the mock vector only control (red bars) in response to the positive control stimulants and the putative SSc antigens; an insert is showing surface expression of $\gamma\delta$ TCR in 16TMCE on the day of calcium flux assay.

Stimulation of the transduced 16TMCE Jurkat T-cell line with the positive control stimulants, OKT3 (5 $\mu\text{g}/\text{mL}$) for CD3 crosslinking, and IPP (50 μM), a $V\gamma9V\delta2$ -specific TCR-dependent agonist, resulted in calcium flux over time, validating a functional TCR/CD3 complex on the surface of the T-cell line. The mock vector only transduced cell line, lacking a TCR or CD3, had minimal change in calcium fluorescence, showing a lack of calcium flux, as expected of the negative control cell line. Stimulation with recombinant whole proteins of all the putative SSc antigens analyzed (each at 15 $\mu\text{g}/\text{mL}$), including DNA topoisomerase I, centromere proteins A and B, and the viral lysate of hCMV, and the

heatshock protein, hsp 27, (hsp 90 assessed in initial trial – see figure 28) resulted in no significant calcium flux after stimulation, compared to the mock vector only negative control. Therefore, there was no evidence of recognition of the putative antigens assessed by the most clonally expanded TCR from the skin biopsy of SS-16.

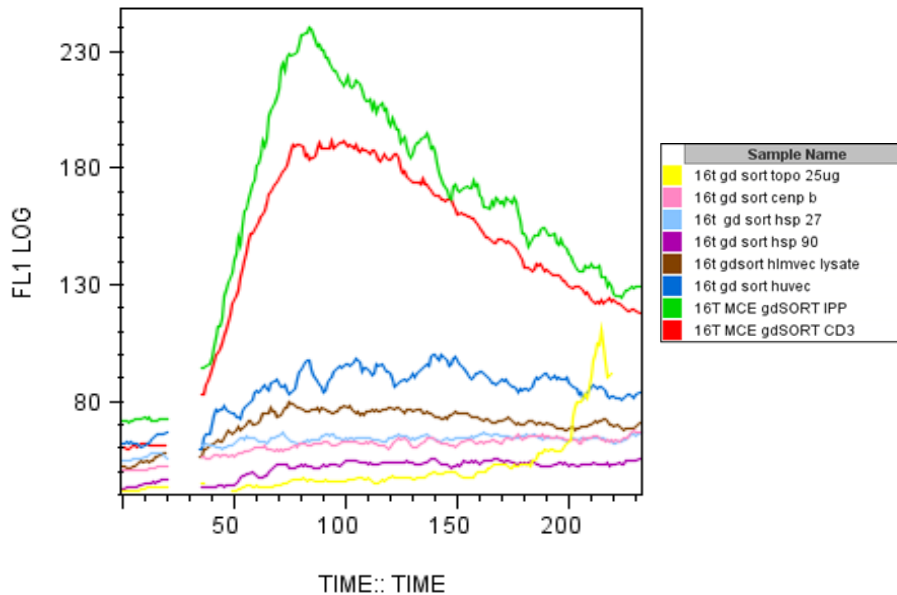


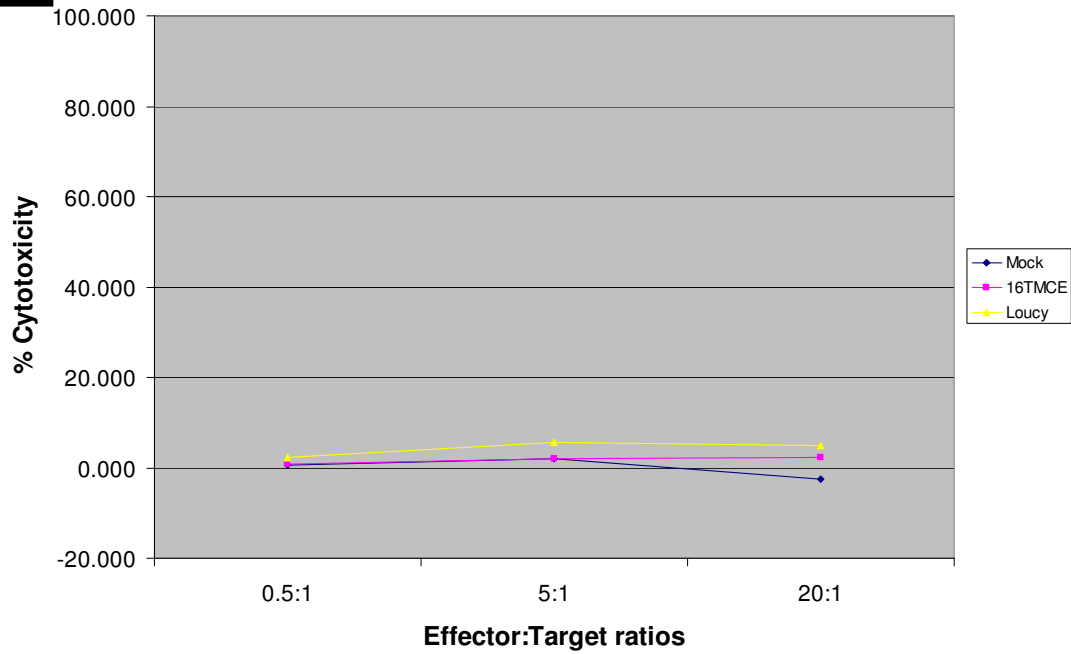
Figure 28 – Initial trial without ionomycin normalization – Change in calcium fluorescence over time of the transduced cell line, 16TMCE in response to the positive control stimulants and the putative SSc antigens, depicting the mean fluorescent line.

4.3.2 Measurement of endothelial cell cytotoxicity

Cytotoxicity against human endothelial cell (HUVEC and HLMVEC) lines by the most clonally-expanded transduced Jurkat T-cell line (16TMCE), was assessed, by measurement of cytoplasmic lactate dehydrogenase release. After co-incubation of the most-clonally-expanded transduced $\gamma\delta$ TCR+ T-cell line with both HLMVEC (figure 31A) and HUVEC (figure 31B), no cytotoxicity against the human endothelial cell lines. Therefore, no evidence of recognition by the most clonally expanded TCR transcript from the skin biopsy of SS-16, of surface antigens of the human endothelial cell lines assessed

A

HLMVEC (20,000 targets) Cytotoxicity



B

HUVEC (20,000 targets) Cytotoxicity

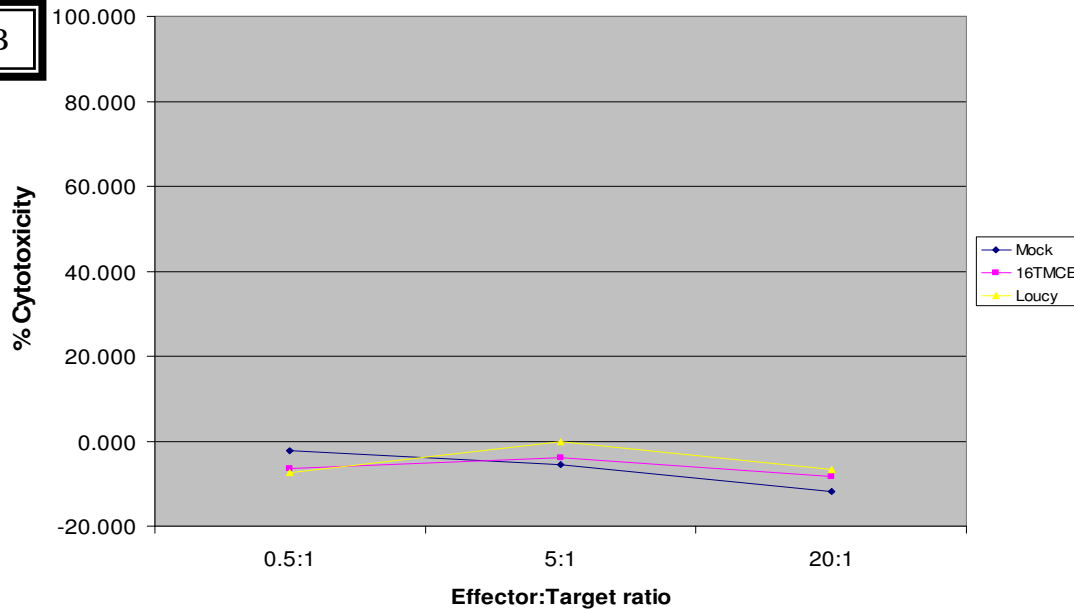


Figure 29 - Percent human endothelial cell cytotoxicity after co-incubation of 16TMCE (pink line) with microvascular HLMVEC (figure 29A) and macrovascular HUVEC (figure 29B) human endothelial cell lines, compared with the Loucy $\gamma\delta$ TCR+ T-cell line, and the negative control, mock vector transduced T-cell line.

CHAPTER 5 CONCLUSION AND DISCUSSION

5.1 RESULTS OF STUDIES

5.1.1 $\gamma\delta$ TCR+ T-cells are clonally-expanded in lesional skin biopsy and peripheral blood of patients with systemic sclerosis.

Transcripts isolated from the lesional skin biopsies or peripheral blood mononuclear cells (PBMC) from patients with early SSc disease, were cloned and sequenced, after cDNA synthesis, and V-specific PCR amplification. We report the presence of substantial, statistically significant (by binomial distribution) proportions of multiple identical V γ - and V δ -chain transcripts in lesional skin biopsies of patients with SSc in both the previous and the current study, strongly suggestive of a $\gamma\delta$ TCR+ T-cell clonal expansion. Specifically, 4 out of 7 patients with SSc exhibited varying degrees of multiple identical V γ I TCR chains in the peripheral blood, and 3 out of 4 patients with SSc exhibited multiple identical V γ I TCR chain in the lesional skin biopsy (see summary table 9). Likewise, 7 out of 8 patients analyzed with SSc had multiple identical V γ 9 TCR chain transcripts in the peripheral blood, as did the lesional skin biopsy of all 4 patients analyzed (see summary table 14). However, in previous studies with PBMC from normal donors, used as methodological controls, all V γ I or V γ 9 TCR chains transcripts were unique (data not shown).

In addition, the PBMC and lesional skin biopsy of 5 out of 8 patients, and 6 out of 7 patients with SSc, respectively, exhibited multiple identical V δ 1 TCR chain transcripts (see summary table 24). Furthermore, 6 out of 8 patients with SSc had multiple identical V δ 2 transcripts in the peripheral blood, as did all patients with SSc analyzed (5 out of 5) in the lesional skin biopsy (see summary table 34). However, in previous studies with PBMC from normal donors, used as methodological controls, all V δ 2 TCR chains transcripts were unique

(data not shown). Interestingly, multiple identical V δ 1 TCR chain transcripts were demonstrated in the PBMC from 2 out of 3 normal donors in our study (data not shown) as well as reported in published reports (Shen J et al., 1998). The significance of this finding is still elusive; however, it is important to note that with the random pairing of the unique V γ chains and the V δ chains, unique $\gamma\delta$ TCR specificities are still generated.

These γ - and δ -chains from the patients with SSc analyzed were unique specificities, with no conserved sequences identified in the γ - or δ -chain transcripts, nor were there any homologies after comparison to γ - or δ -chain transcripts in the GENBANK database using BLAST searches, suggesting that these clones were previously unidentified and were therefore novel.

These findings of dominant V δ TCR chain clones present in the lesional skin biopsy and peripheral blood of patients with SSc, corroborated the findings with the V γ sequence analyses and strengthens the evidence suggestive of a $\gamma\delta$ TCR+ T-cell clonal expansion, in response to a specific antigen(s), occurring in the context of SSc disease. In some patients (SS-7 and SS-16), identical V γ and V δ transcripts were present and expanded in both the peripheral blood and lesional skin biopsy. Patient SS-7 showed the presence of multiple identical copies of a V δ 1 TCR chain transcript present in both the lesional skin biopsy (clone DS7T1.5) and the peripheral blood (clone DS7P1.1) (see tables 19 and 20). Interestingly, in patient SS-16, a significant clonal expansion of a V γ 9 and V δ 2 TCR chain clone was present and identical in both the peripheral blood (GS16P2.2, DS16P2.1) and the lesional skin biopsy (GS16T2.1, DS16T2.1), while all other transcripts analyzed were unique to between both samples (see tables 12, 13 for V γ 9 data of SS-16 lesional skin biopsy tissue and peripheral blood, respectively and tables 32, 33 for V δ 2 data of SS-16 lesional skin biopsy

tissue and peripheral blood, respectively). This suggests that the same V γ 9V δ 2 T-cell clone may have undergone an antigen driven clonal expansion in both peripheral blood and the SSc skin lesion. Although sample contamination is always a concern, the cloning and sequencing of the lesional skin transcripts and the PBMC occurred at different times, as the peripheral blood of patient SS-16 was analyzed approximately 5 months after the lesional skin biopsy study.

Compelling evidence of the presence of specific antigen(s)-driven clonal expansions, *in vivo*, has been demonstrated in the skin lesions and peripheral blood patients with SSc, however the inciting antigen(s) remains to be elucidated. These putative antigen(s) could be an autoantigen or a foreign antigen from pathogens that may trigger autoimmunity through the mechanism of molecular mimicry, the cross-reactivity of a lymphocyte receptor with a microbial antigen and a self antigen. The varying degrees of clonal expansion observed in patients with SSc may represent the heterogeneity of SSc pathogenesis, possibly different inciting antigen(s), as well as the different clinical profiles (length of disease, treatments etc) of the SSc patients analyzed.

5.1.2 The approach: Full length V γ and V δ TCR chain construction and retroviral-mediated expression of specific $\gamma\delta$ TCR in TCR-mutant Jurkat T-cells

With the demonstration of statistically significant clonal expansions of V γ -and V δ TCR chain transcripts in the peripheral blood and lesional skin biopsies of patients with SSc, an approach was developed to facilitate further study into the antigen(s) recognized by these clonally $\gamma\delta$ TCR transcripts. This approach of $\gamma\delta$ TCR reconstitution in Jurkat reporter cells facilitated the use of clonally-expanded $\gamma\delta$ T-cell receptor transcripts, initially isolated from the lesional skin biopsy or peripheral blood of patients with SSc, with known CDR3 region gene sequence, to determine their cognate antigen. Briefly, full length copies of TCR

transcripts were constructed based on the genetic information obtained in the context of the clonal expansion study, which demonstrated the presence or absence of $\gamma\delta$ TCR clonal expansion in lesional skin biopsy and PBMC of patients with SSc. The full length transcripts, after verification of the sequence integrity by cloning and sequencing, were then expressed in TCR-mutant Jurkat T-cell line using a commercially available retroviral expression system kit (Clontech). The $V\gamma$ or $V\delta$ TCR chain were inserted into respective expression vectors, provided in the kit, in the correct orientation and used to transfect the packaging cell line for production of retrovirus that contained the $V\gamma$ - or $V\delta$ TCR chain transcript. TCR-mutant Jurkat T-cells were transduced with the retrovirus-containing supernatants of the packaging cell line to facilitate gene transfer, and mRNA expression of the $V\gamma$ and $V\delta$ transcripts was verified by RT-PCR (see figures 22 and 23). Surface expression was verified by flow cytometry, using a fluorescently-labeled antibody against the $\gamma\delta$ TCR heterodimer (see figure 24). More importantly, functional analysis of the expressed $\gamma\delta$ TCR was performed using positive controls (anti-CD3 antibody and IPP) to stimulate the transduced $\gamma\delta$ TCR+ T-cell line in a TCR/CD3 complex-dependent fashion (see figures 25 and 26).

The importance of this approach is that it allowed for analysis of TCR transcripts specificities using multiple different parameters, without the limitations that may occur with direct use of $\gamma\delta$ TCR+ T-cell isolated from lesional skin biopsy samples or peripheral blood of patients. In addition, it provided a way to physically preserve the important genetic information from the samples from patients, even after the patient samples are depleted, that can be used in multiple different applications, for further study and understanding into the disease pathogenesis. A similar approach was employed in a study on polymyositis, an autoimmune disease of skeletal muscle. The authors first identified a monoclonal population

of $\gamma\delta$ TCR+ T-cells from lesional skeletal muscle biopsy from a patient with polymyositis by cloning and sequencing. This expanded $\gamma\delta$ TCR was expressed in a mouse hybridoma cell line, and the transformed cells were shown to be cytotoxic against a human skeletal muscle cell line (O'Hanlon, Messersmith et al. 1995; Wiendl, Malotka et al. 2002). With the generation of soluble forms of the clonally-expanded $\gamma\delta$ TCR transcripts, the authors were also able to identify a specific cytosolic protein in human skeletal muscle cells that was recognized by this $\gamma\delta$ TCR, with a labeling approach similar to the use of antibodies probes (Dornmair, Schneider et al. 2004).

5.1.3 Antigen recognition by the clonally expanded, expressed $\gamma\delta$ TCR was not yet identified, after assaying by different parameters

The transduced cell line expressing the clonally expanded V γ and V δ TCR chain transcripts from the lesional skin biopsy (and peripheral blood) of patient SS-16 was used in the subsequent antigen recognition studies. Unique V γ and V δ transcripts from the same patient were used, in different combinations as negative controls. Patient SS-16 presented an intriguing choice for the study because of the same strong clonal expansion of a γ - and δ -chain transcript, present in both the lesional skin biopsy and the peripheral blood. The clonal expansion was of the V γ 9 (see tables 12 and 13) and V δ 2 TCR subsets (see tables 32 and 33), while the V γ I (see tables 7 and 8), and V δ 1 TCR subsets (see tables 22 and 23) were unique transcripts, highly suggestive of a V γ 9V δ 2 TCR+ T-cell clone expanding *in vivo*, in response to an antigen. In addition, a V γ 9 and V δ 2 transcript was strongly clonally-expanded, while the remaining V γ 9 and V δ 2 transcripts were unique, therefore making the choice of V γ and V δ chain combination for expression more obvious and eliminating the need for different combinations of clonally-expanded V γ 9 and V δ 2 transcripts in attempt to generate the combination that was activated *in vivo* (see tables 12, 13, 32 and 33).

5.1.3.1 Intracellular calcium flux in response to soluble whole proteins

The transduced V γ 9V δ 2 T-cell lines were assessed for recognition of putative antigens via measuring intracellular calcium flux after stimulation with the putative SSc antigens. The putative SSc antigens assessed (15 μ g/mL) were soluble human recombinant proteins of DNA topoisomerase I, centromere protein A, centromere protein B, hsp 27, and hsp 90, as well as the viral lysate of hCMV (AD619 strain). The positive controls, included OKT3 (10 μ g/mL) for anti-CD3 stimulation, and IPP (50 μ M) for V γ 9V δ 2-specific TCR stimulation, described in different studies (Bukowski, Morita et al. 1998; Lafont, Liautard et al. 2001; Bieback, Breer et al. 2003; Hebbeler, Cairo et al. 2007; Puan, Jin et al. 2007). The concentration of 15 μ g/mL was used as a starting point based on various published reports on studies done using different concentrations soluble whole proteins to stimulate human $\gamma\delta$ TCR+ T-cells (Sciammas, Johnson et al. 1994). None of the studies used a calcium flux assay as the outcome parameter for antigen recognition after stimulation of isolated $\gamma\delta$ TCR+ T-cells or PBMC with antigens. However, using other parameters such as proliferation, the whole protein concentration ranged from 5 μ g/mL to 25 μ g/mL that resulted in a response by $\gamma\delta$ TCR+ T-cells. In this study, Jurkat T-cells transduced with the clonally-expanded $\gamma\delta$ TCR transcripts from the skin biopsy (and PBMC) of patient SS-16 showed no evidence of calcium flux, compared to the vector only control T-cell line, in response to stimulation with the putative SSc antigens assessed (see figures 27 and 28), although the positive control stimulation, using anti-CD3 antibody and IPP (the V γ 9V δ 2 TCR specific non-peptide phosphoantigen) validated the presence of a functional $\gamma\delta$ TCR/CD3 complex expressed on the surface of the Jurkat T-cells (see figures 25 and 26) and surface expression of the $\gamma\delta$ TCR was positive on the day of the calcium assay (see figure 27).

The lack of recognition by the transduced $\gamma\delta$ TCR reflects several possibilities, including the fact that the $\gamma\delta$ TCR, clonally-expanded in this patient with SSc, 1) may not recognize the putative SSc antigens assessed, 2) may recognize other antigens that are implicated in the disease, 3) may recognize antigens that are currently unknown, 4) may recognize one of the assessed antigens but in a modified form *in vivo* that is not reflected in the study. The clonally-expanded transcript from SS-16 was used to develop and optimize the parameters of this study. Studies of transcripts isolated from additional patients with SSc are needed, to determine whether the putative SSc antigens assayed in this study are recognized by clonally-expanded $\gamma\delta$ TCRs in patients with SSc. Other putative SSc antigens also need to be analyzed to determine if they are recognized by clonally-expanded $\gamma\delta$ TCR from patients with SSc, an investigation that is greatly facilitated by the approach developed in this study.

5.1.3.2 Endothelial cell cytotoxicity by the transduced $\gamma\delta$ TCR+ T-cells

Kahaleh et al, reported an enhanced TCR-mediated interaction of $\gamma\delta$ TCR+ T-cells from the peripheral blood of patients with SSc with human dermal endothelial cells *in vitro* (Kahaleh, Fan et al. 1999), which may represent an initial response to endothelial cells, or an immune response as a consequence of endothelial cell damage. These $\gamma\delta$ TCR+ T-cells showed increased proliferation than control cells, endothelial cell cytotoxicity, and expression of granzyme A (Kahaleh, Fan et al. 1999). The $\alpha\beta$ TCR+ T-cells from patients with SSc and normal control did not show evidence of endothelial cytotoxicity or proliferation.

In this study, it was hypothesized that a specific $\gamma\delta$ TCR+ T-cell cytotoxic response against normal endothelial cells may be occurring in the context of SSc disease. Therefore

the presence of a potential endothelial cytotoxicity by clonally-expanded $\gamma\delta$ TCR+ T-cells in SSc pathogenesis was investigated. Jurkat T-cells transduced with the clonally-expanded $\gamma\delta$ TCR transcripts from patient SS-16 were effector cells in a cytotoxicity assay, measuring the cytosolic lactate dehydrogenase release upon coincubation with the endothelial cell targets, the microvascular human lung microvascular endothelial cell line (HLMVEC) and the macrovascular human umbilical vein endothelial cell line (HUVEC). Result showed no evidence of cytotoxicity against the HLMVEC and HUVEC cell lines (see figures 29). This may represent a true lack of recognition of HLMVEC and HUVEC by the expanded $\gamma\delta$ TCR from SS-16 lesional skin biopsy (and peripheral blood) or a limitation of the study where the endothelial cell lines may not reflect the true endothelial cell phenotype in the context of SSc pathogenesis. Future studies may include investigating cytotoxicity against human dermal endothelial cells (HDEC), or endothelial cells from patients with SSc, which may be undergoing a pathogenic process not captured in the *in vitro* study using normal endothelial cell lines.

Endothelial cell destruction is a main characteristic feature of SSc, and manifests clinically as Raynaud's phenomenon, as well as capillary destruction at the nail beds, and may be an initiating event in SSc pathogenesis. Causes of endothelial cell damage are still elusive but may involve an aberrant immune response, including a possible cross reactivity of surface endothelial antigens with hCMV proteins (Lunardi, Bason et al. 2000), or direct damage by lymphocytes. Apoptosis of endothelial cells have also been demonstrated by hCMV antibodies, which are increased in patients with SSc (Lunardi, Dolcino et al. 2007). Anti-endothelial cell antibodies have also been described in patients with SSc and induce endothelial cell apoptosis as well as induce fibrillin-1 expression (Ahmed, Tan et al. 2006),

and may represent a subset of anti-centromere protein autoantibodies (Servettaz, Tamby et al. 2006). More clinically subtle consequences of endothelial cell damage may result in the other features of SSc pathogenesis, including fibrosis. Endothelin-1(ET-1) is released from endothelial cells, and is both a potent vasoconstrictor and fibroblast mitogen. Studies have demonstrated increased ET-1 in the serum of patients with SSc, specifically lcSSc subsets with hypertensive lung or renal disease, and patients with dcSSc, compared to normal controls or disease controls (Vancheeswaran, Azam et al. 1994; Vancheeswaran, Magoulas et al. 1994; Kazzam, Waldenstrom et al. 1997; Denton, Black et al. 2006). Shi-wen et al demonstrated that ET-1 is a downstream mediator of the profibrotic effects of TGF- β in lung fibroblasts and contributes to the ability of TGF- β to promote a profibrotic phenotype in human lung fibroblasts, consistent with the notion that endothelin receptor antagonism may be beneficial in controlling fibrogenic responses in lung fibroblasts (Shi-wen, Kennedy et al. 2007).

5.2 SIGNIFICANCE AND FUTURE DIRECTIONS

Autoimmunity, which is the immune recognition of and response to self antigens may have some normal physiologic significance, especially in the elimination of stressed, transformed, or precancerous host cells and microtumors. However, autoimmune disease may ensue when an aberrant recognition of normal cell antigen(s) by a lymphocyte occurs, especially when coupled with enhanced inflammatory responses (in the case of SSc, possibly due to endothelial cell damage), which may have resulted from this recognition or may have been coincidentally concurrent with the autorecognition. The apparent persistence of clonally-expanded $\gamma\delta$ TCR+ T-cells in the peripheral blood of patients with SSc, but not

normal controls, represents further evidence of an alteration in immune system homeostasis occurring in the context of SSc disease. Physiologically, the majority of lymphocytes that clonally-expand in response to a specific antigen are promptly reduced by apoptosis after elimination of the antigen, while only small subsets of memory cells persist. This elimination prevents prolonged immune cell activity that may become aberrant and result in chronic inflammation, possible epitope spreading, autoimmune disease, and further tissue damage. Epitope spreading is a phenomenon where an immune response against a foreign inciting antigen targets distinct self antigen(s) exposed via tissue damage in the context of chronic inflammation, long after the inciting antigen has been eliminated, possibly resulting in autoimmune disease. The persistence of clonally-expanded $\gamma\delta$ TCR+ T-cells in patients with SSc therefore may represent evidence of immune dysfunction, possibly due to a continued exposure to the inciting antigen(s), or a resistance to apoptosis in $\gamma\delta$ TCR+ T-cells. Published reports have described a resistance to apoptosis in peripheral blood $\alpha\beta$ TCR+ and $\gamma\delta$ TCR+ T-cell in patients with SSc, evidenced by decreased death overtime after antigenic stimulation, compared to normal controls, as well as a significant increase in serum levels of sFas, increased bcl-2 expression and a significant decrease in caspase-3 activity after anti-Fas antibody treatment (Cipriani, Fulminis et al. 2006).

There was a lack of antigen recognition by the expressed $\gamma\delta$ TCR using two different parameters assayed, namely intracellular calcium flux in response stimulation with soluble human recombinant forms of putative SSc antigens, and human endothelial cell cytotoxicity. However the antigens analyzed in this study, although highly implicated in SSc disease based on published reports and clinical findings are by no means exhaustive, as there are a myriad of different antigenic possibilities, implicated in SSc pathogenesis. The major hindrance to

study of $\gamma\delta$ TCR+ T-cell biology and function in both health and disease pathogenesis is because of an incomplete knowledge of $\gamma\delta$ TCR+ T-cell antigen recognition. Unlike the in-depth knowledge of antigen recognition of B-cells (i.e. linear or conformational epitopes of whole proteins) and $\alpha\beta$ TCR+ T-cells (MHC-peptide complexes), antigen recognition by $\gamma\delta$ TCR+ T-cells has not been as straightforward. Emerging evidence seem to suggest subset-specific antigen recognition, that may straddle the recognition properties of B-cells, $\alpha\beta$ TCR+T-cells, and potentially novel recognition mechanisms (non-peptide phosphoantigens), but that knowledge is incomplete. Subsets of $\gamma\delta$ TCR+ T-cells may recognize whole proteins, while others may be MHC-restricted. This antigen recognition, in addition to being subset specific, also may depend on the specific localization sites of $\gamma\delta$ TCR+ T-cells. It is well established that $\gamma\delta$ TCR+ T-cells are a minor subset of T-cells in peripheral blood and lymph, as well as in most lymphoid organs, however there is predominance in different epithelial tissues. A subset of V γ 9V δ 2 TCR+ T-cells, not V δ 1+ TCR+ T-cells, may recognize phosphoantigens (a finding also shown in this study), particularly those with J γ 1.2, in a TCR-dependent fashion. Therefore, there is a dual reactivity of these cells, as it has been described that the binding site for phosphoantigens is distinct from the antigen binding site, hence these cells also respond to another specific antigen.

In this study, comparison of the most clonally-expanded V γ and V δ transcript CDR3 transcripts from either skin biopsy or peripheral blood showed the absence of non-germline encoded conserved sequences in γ - or δ -chain TCR transcripts. The CDR3 δ sequences were diverse in nucleotide content and in length. Interestingly, specific motifs have been identified for conferring antigen-binding specificity for $\gamma\delta$ TCR+ T-cells, in other studies. Shin et al. (2005) showed that $\gamma\delta$ TCR+ T-cells expressing TCRs that had a specific CDR3 δ motif

could bind the ligand analyzed (the T22 tetramer), whereas those lacking this motif could not, even though the TCRs assessed were all V γ 4/V δ 5+. This motif consists of a tryptophan (W) encoded by the V δ or D δ 1 gene segments and the sequence serine–glutamic acid–glycine–tyrosine–glutamic acid (SEGYE), followed by a P nucleotide–encoded leucine (L). Their results suggested that the W-(S)EGYEL motif correlates much better than V gene usage with antigen recognition (Shin, El-Diwany et al. 2005). Chen et al. (2003), using peptides generated from the CDR3 regions of γ - and δ - TCR isolated from tumor-infiltrating lymphocytes as probes, were able to probe tumor protein extracts (as epitopes in a 12-mer random peptide phage-display library), and identify specific proteins, including human mutS homolog 2 (hMSH2) and heat shock protein (hsp) 60, as well as nine peptides that bind the $\gamma\delta$ TCR+ T-cells, and functionally activate them *in vitro* (Chen, See et al. 2003). Hsp 60 has been identified as $\gamma\delta$ TCR+ T-cell ligands in other studies (Chauhan, Singh et al. 2007) and served to validate the approach.

Some studies, including those in our laboratory have demonstrated the presence of restricted diversity in $\gamma\delta$ TCR+ T-cells skin from normal donors. The significance of this finding is unknown, in large part due to the lack of knowledge of the antigen the may be inciting this recognition. Speculations have been made in regards to the role of $\gamma\delta$ TCR+ T-cells in normal skin, including in wound repair, and immunosurveillance of stressed or tumorigenic epithelial cells, and maintaining epithelial integrity. This makes identification of the specific antigen(s) that may have incited the $\gamma\delta$ TCR+ T-cell clonal expansion in the lesional skin biopsy and peripheral blood of patients with SSc, of important significance in understanding the pathogenesis of SSc disease as well as in development of therapeutic approaches.

REFERENCES CITED

- Abraham, D. J. and J. Varga (2005). "Scleroderma: from cell and molecular mechanisms to disease models." Trends Immunol **26**(11): 587-95.
- Adams, B. D. (2003). "Scleroderma renal crisis." Ann Emerg Med **42**(5): 713-4.
- Ahmed, S. S., F. K. Tan, F. C. Arnett, L. Jin, Y. J. Geng (2006). "Induction of apoptosis and fibrillin 1 expression in human dermal endothelial cells by scleroderma sera containing anti-endothelial cell antibodies." Arthritis Rheum **54**(7): 2250-62.
- Aljurf, M., A. Ezzat, M., O. Musa (2002). "Emerging role of gammadelta T-cells in health and disease." Blood Rev **16**(4): 203-6.
- Arnett, F. C., R. F. Howard, F. Tan, J. M. Moulds, W. B. Bias, E. Durban, H. D. Cameron, G. Paxton, T. J. Hodge, P. E. Weathers, J. D. Reveille (1996). "Increased prevalence of systemic sclerosis in a Native American tribe in Oklahoma. Association with an Amerindian HLA haplotype." Arthritis Rheum **39**(8): 1362-70.
- Arnett, F. C., J. D. Reveille, R. Goldstein, K. M. Pollard, K. Leaird, E. A. Smith, E. C. Leroy, M. J. Fritzler (1996). "Autoantibodies to fibrillar in systemic sclerosis (scleroderma). An immunogenetic, serologic, and clinical analysis." Arthritis Rheum **39**(7): 1151-60.
- Arnett, F. C. (1995). "HLA and autoimmunity in scleroderma (systemic sclerosis)." Int Rev Immunol **12**(2-4): 107-28.
- Banning, U., J. Krutmann, D. Korholz (2006). "The role of IL-4 and IL-12 in the regulation of collagen synthesis by fibroblasts." Immunol Invest **35**(2): 199-207.
- Bar, J., M. Ehrenfeld, J. Rozenman, M. Perelman, Y. Sidi, H. Gur (2001). "Pulmonary-renal syndrome in systemic sclerosis." Semin Arthritis Rheum **30**(6): 403-10.
- Baroni, S. S., M. Santillo, F. Bevilacqua, M. Luchetti, T. Spadoni, M. Mancini, P. Fraticelli, P. Sambo, A. Funaro, A. Kazlauskas, E. V. Avvedimento, A. Gabrielli (2006). "Stimulatory autoantibodies to the PDGF receptor in systemic sclerosis." N Engl J Med **354**(25): 2667-76.
- Bason, C., R. Corrocher, C. Lunardi, P. Puccetti, O. Olivieri, D. Girelli, R. Navone, R. Beri, E. Millo, A. Margonato, N. Martinelli, A. Puccetti (2003). "Interaction of antibodies against cytomegalovirus with heat-shock protein 60 in pathogenesis of atherosclerosis." Lancet **362**(9400): 1971-7.
- Beranek, J. T. (2001). "Pathogenesis of heart fibrosis in systemic sclerosis." Int J Cardiol **80**(2-3): 261-2.

- Black, C. (1996). "Systemic sclerosis: current pathogenetic concepts and future prospects for targeted therapy." Lancet **347**(9013): 1453-8.
- Brandes, M., K. Willmann, B. Moser (2005). "Professional antigen-presentation function by human gammadelta T Cells." Science **309**(5732): 264-8.
- Boin, F. and L. Hummers. (2008). "Sclerodermal-like fibrosing disorder." Rheum Dis Clin N Am **34**:199-220
- Casale, R., G. Frazzitta, C. Fundaro, P. Balbi, A. Del Rosso, L. Bertinotti, M. Matucci-Cerinic (2004). "Blink reflex discloses CNS dysfunction in neurologically asymptomatic patients with systemic sclerosis." Clin Neurophysiol **115**(8): 1917-20.
- Chauhan, S. K., M. Singh, S. Nityanand (2007). "Reactivity of gamma/delta T cells to human 60-kd heat-shock protein and their cytotoxicity to aortic endothelial cells in Takayasu arteritis." Arthritis Rheum **56**(8): 2798-802.
- Chen, K., A. See, S. Shumack (2003). "Epidemiology and pathogenesis of scleroderma." Australas J Dermatol **44**(1): 1-7
- Chizzolini, C., E. Raschi, R. Rezzonico, C. Testoni, R. Mallone, A. Gabrielli, A. Facchini, N. Del Papa, M. O. Borghi, J. M. Dayer, P. L. Meroni (2002). "Autoantibodies to fibroblasts induce a proadhesive and proinflammatory fibroblast phenotype in patients with systemic sclerosis." Arthritis Rheum **46**(6): 1602-13.
- Cipriani, P., A. Fulminis, E. Pingiotti, A. Marrelli, V. Liakouli, R. Perricone, A. Pignone, M. Matucci-Cerinic, R. Giacomelli (2006). "Resistance to apoptosis in circulating alpha/beta and gamma/delta T lymphocytes from patients with systemic sclerosis." J Rheumatol **33**(10): 2003-14.
- Clements, P. J. (2000). "Systemic sclerosis (scleroderma) and related disorders: clinical aspects." Baillieres Best Pract Res Clin Rheumatol **14**(1): 1-16.
- D'Angelo, R. and R. Miller (1997). "Pregnancy complicated by severe preeclampsia and thrombocytopenia in a patient with scleroderma." Anesth Analg **85**(4): 839-41.
- Dariavach, P. and M. P. Lefranc (1989). "First genomic sequence of the human T-cell receptor delta 2 gene (TRDV2)." Nucleic Acids Res **17**(12): 4880.
- Dau, P. C. and J. P. Callahan (1994). "Immune modulation during treatment of systemic sclerosis with plasmapheresis and immunosuppressive drugs." Clin Immunol Immunopathol **70**(2): 159-65.

- Del Galdo, F., G. G. Maul, S. A. Jimenez, C. M. Artlett (2006). "Expression of allograft inflammatory factor 1 in tissues from patients with systemic sclerosis and in vitro differential expression of its isoforms in response to transforming growth factor beta." Arthritis Rheum **54**(8): 2616-25.
- Denton, C. P. and C. M. Black (2004). "Scleroderma--clinical and pathological advances." Best Pract Res Clin Rheumatol **18**(3): 271-90.
- Dhillon, V., S. McCallum, D. Wilks, B. Twomey, D. Latchman, D. Isenberg (1993). "The differential expression of heat shock proteins in rheumatic disease." Br J Rheumatol **32**(10): 883-92.
- Dornmair, K., C. K. Schneider, J. Malotka, G. Dechant, H. Wiendl, R. Hohlfeld (2004). "Antigen recognition properties of a Vgamma1.3Vdelta2-T-cell receptor from a rare variant of polymyositis." J Neuroimmunol **152**(1-2): 168-75.
- Elkayam, O., M. Oumanski, M. Yaron, D. Caspi (2000). "Watermelon stomach following and preceding systemic sclerosis." Semin Arthritis Rheum **30**(2): 127-31.
- Fanning, G. C., K. I. Welsh, C. Bunn, R. Du Bois, C. M. Black (1998). "HLA associations in three mutually exclusive autoantibody subgroups in UK systemic sclerosis patients." Br J Rheumatol **37**(2): 201-7.
- Ferrarini, M., E. Ferrero, L. Dagna, A. Poggi, M. R. Zocchi (2002). "Human gammadelta T cells: a nonredundant system in the immune-surveillance against cancer." Trends Immunol **23**(1): 14-8.
- Fischhoff, D. K. and D. Sirois (2000). "Painful trigeminal neuropathy caused by severe mandibular resorption and nerve compression in a patient with systemic sclerosis: case report and literature review." Oral Surg Oral Med Oral Pathol Oral Radiol Endod **90**(4): 456-9.
- Fujii, H., Y. Shimada, M. Hasegawa, K. Takehara, S. Sato (2004). "Serum levels of a Th1 chemoattractant IP-10 and Th2 chemoattractants, TARC and MDC, are elevated in patients with systemic sclerosis." J Dermatol Sci **35**(1): 43-51.
- Gabrielli, A., S. Svegliati, G. Moroncini, M. Luchetti, C. Tonnini, E. V. Avvedimento (2007). "Stimulatory autoantibodies to the PDGF receptor: a link to fibrosis in scleroderma and a pathway for novel therapeutic targets." Autoimmun Rev **7**(2): 121-6.
- Gabrielli, A., C. Di Loreto, R. Taborro, M. Candela, P. Sambo, C. Nitti, M. G. Danieli, F. DeLustro, J. R. Dasch, G. Danieli (1993). "Immunohistochemical localization of intracellular and extracellular associated TGF beta in the skin of patients with systemic sclerosis (scleroderma) and primary Raynaud's phenomenon." Clin Immunol Immunopathol **68**(3): 340-9.

- Giacomelli, R., P. Cipriani, A. Fulminis, G. Barattelli, M. Matucci-Cerinic, S. D'Alo, G. Cifone, G. Tonietti (2001). "Circulating gamma/delta T lymphocytes from systemic sclerosis (SSc) patients display a T helper (Th) 1 polarization." Clin Exp Immunol **125**(2): 310-5.
- Giacomelli, R., M. Matucci-Cerinic, P. Cipriani, I. Ghersetich, R. Lattanzio, A. Pavan, A. Pignone, M. L. Cagnoni, T. Lotti, G. Tonietti (1998). "Circulating Vdelta1+ T cells are activated and accumulate in the skin of systemic sclerosis patients." Arthritis Rheum **41**(2): 327-34.
- Grassi, W., P. D. Medico, F. Izzo, C. Cervini (2001). "Microvascular involvement in systemic sclerosis: capillaroscopic findings." Semin Arthritis Rheum **30**(6): 397-402.
- Harvey, G. R., S. Butts, A. L. Rands, Y. Patel, N. J. McHugh (1999). "Clinical and serological associations with anti-RNA polymerase antibodies in systemic sclerosis." Clin Exp Immunol **117**(2): 395-402.
- Harvey, G. R. and N. J. McHugh (1999). "Serologic abnormalities in systemic sclerosis." Curr Opin Rheumatol **11**(6): 495-502.
- Hasegawa, M., S. Sato, K. Takehara (1999). "Augmented production of chemokines (monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1alpha (MIP-1alpha) and MIP-1beta) in patients with systemic sclerosis: MCP-1 and MIP-1alpha may be involved in the development of pulmonary fibrosis." Clin Exp Immunol **117**(1): 159-65.
- Hayakawa, I., M. Hasegawa, K. Takehara, S. Sato (2004). "Anti-DNA topoisomerase IIalpha autoantibodies in localized scleroderma." Arthritis Rheum **50**(1): 227-32.
- Hebbar, M., P. Lassalle, A. Janin, D. Vanhee, S. Bisiau, P. Y. Hatron, A. B. Tonnel, B. Gosselin (1995). "E-selectin expression in salivary endothelial cells and sera from patients with systemic sclerosis. Role of resident mast cell-derived tumor necrosis factor alpha." Arthritis Rheum **38**(3): 406-12.
- Hill C. L., A. M. Nguyen, D. Roder, P. Roberts-Thomson (1998). "Risk of cancer in patients with scleroderma: a population-based cohort study." Ann Rheum Dis **57**: 682-686.
- Hirakata, M., Y. Okano, U. Pati, A. Suwa, T. A. Medsger, Jr., J. A. Hardin, J. Craft (1993). "Identification of autoantibodies to RNA polymerase II. Occurrence in systemic sclerosis and association with autoantibodies to RNA polymerases I and III." J Clin Invest **91**(6): 2665-72.
- Hu, P. Q., N. Fertig, T. A. Medsger, Jr., T. M. Wright (2004). "Molecular recognition patterns of serum anti-DNA topoisomerase I antibody in systemic sclerosis." J Immunol **173**(4): 2834-41.

- Ihn, H., N. Yazawa, M. Kubo, K. Yamane, S. Sato, M. Fujimoto, K. Kikuchi, Y. Soma, K. Tamaki (2000). "Circulating levels of soluble CD30 are increased in patients with localized scleroderma and correlated with serological and clinical features of the disease." J Rheumatol **27**(3): 698-702.
- Ioannides, C. G., K. Itoh, F. E. Fox, R. Pahwa, R. A. Good, C. D. Platsoucas (1987). "Identification of a second T-cell antigen receptor in human and mouse by an anti-peptide gamma-chain-specific monoclonal antibody." Proc Natl Acad Sci U S A **84**(12): 4244-8.
- Jarvis, M. A. and J. A. Nelson (2007). "Human cytomegalovirus tropism for endothelial cells: not all endothelial cells are created equal." J Virol **81**(5): 2095-101.
- Jimenez, S. A. and O. Batuman (1993). "Immunopathogenesis of systemic sclerosis: possible role of retroviruses." Autoimmunity **16**(3): 225-33.
- Joachims, M. L., J. L. Chain, S. W. Hooker, C. J. Knott-Craig, L. F. Thompson (2006). "Human alpha beta and gamma delta thymocyte development: TCR gene rearrangements, intracellular TCR beta expression, and gamma delta developmental potential--differences between men and mice." J Immunol **176**(3): 1543-52.
- Jones, E. Y., M. Salio, V. Cerundolo (2007). "T cell receptors get back to basics." Nat Immunol **8**(10): 1033-5.
- Jung W-J, A. I. Nwaneshiudu, A. R. Myers, E. Oleszak, C. D. Platsoucas " Gamma-delta T-cell receptors expressing T-cells are clonally expanded in the peripheral blood and skin biopsies of patients with systemic sclerosis of recent onset." Manuscript in preparation.
- Kahaleh, M. B., P. S. Fan, T. Otsuka (1999). "Gammadelta receptor bearing T cells in scleroderma: enhanced interaction with vascular endothelial cells in vitro." Clin Immunol **91**(2): 188-95.
- Kalogerou, A., E. Gelou, S. Mountantonakis, L. Settas, E. Zafiriou, L. Sakkas (2005). "Early T cell activation in the skin from patients with systemic sclerosis." Ann Rheum Dis **64**(8): 1233-5.
- Kane, G. C., J. Varga, E. F. Conant, P. W. Spirn, S. Jimenez, J. E. Fish (1996). "Lung involvement in systemic sclerosis (scleroderma): relation to classification based on extent of skin involvement or autoantibody status." Respir Med **90**(4): 223-30.
- Kessel, A., I. Rosner, M. Rozenbaum, D. Zisman, A. Sagiv, Z. Shmuel, E. Sabo, E. Toubi (2004). "Increased CD8+ T cell apoptosis in scleroderma is associated with low levels of NF-kappa B." J Clin Immunol **24**(1): 30-6.

- Kikuchi, K., M. Kubo, S. Sato, M. Fujimoto, K. Tamaki (1995). "Serum tissue inhibitor of metalloproteinases in patients with systemic sclerosis." J Am Acad Dermatol **33**(6): 973-8.
- Kodera, T., T. L. McGaha, R. Phelps, W. E. Paul, C. A. Bona (2002). "Disrupting the IL-4 gene rescues mice homozygous for the tight-skin mutation from embryonic death and diminishes TGF-beta production by fibroblasts." Proc Natl Acad Sci U S A **99**(6): 3800-5.
- Kubo, M., H. Ihn, K. Yamane, K. Tamaki (2001). "Up-regulated expression of transforming growth factor beta receptors in dermal fibroblasts in skin sections from patients with localized scleroderma." Arthritis Rheum **44**(3): 731-4.
- Kurasawa, K., K. Hirose, H. Sano, H. Endo, H. Shinkai, Y. Nawata, K. Takabayashi, I. Iwamoto (2000). "Increased interleukin-17 production in patients with systemic sclerosis." Arthritis Rheum **43**(11): 2455-63.
- Kuwana, M., Y. Okazaki, H. Yasuoka, Y. Kawakami, Y. Ikeda (2004). "Defective vasculogenesis in systemic sclerosis." Lancet **364**(9434): 603-10.
- Kuwana, M., T. A. Medsger, Jr., T. M. Wright (1997). "Highly restricted TCR-alpha beta usage by autoreactive human T cell clones specific for DNA topoisomerase I: recognition of an immunodominant epitope." J Immunol **158**(1): 485-91.
- Kuwana, M., T. A. Medsger, Jr., T. M. Wright (1995). "Detection of anti-DNA topoisomerase I antibody by an enzyme-linked immunosorbent assay using overlapping recombinant polypeptides." Clin Immunol Immunopathol **76**(3 Pt 1): 266-78.
- Kuwana, M., T. A. Medsger, Jr., T. M. Wright (1995). "T cell proliferative response induced by DNA topoisomerase I in patients with systemic sclerosis and healthy donors." J Clin Invest **96**(1): 586-96.
- Laing, T. J., B. W. Gillespie, M. B. Toth, M. D. Mayes, R. H. Gallavan, Jr., C. J. Burns, J. R. Johanns, B. C. Cooper, B. J. Keroack, M. C. Wasko, J. V. Lacey, Jr., D. Schottenfeld (1997). "Racial differences in scleroderma among women in Michigan." Arthritis Rheum **40**(4): 734-42.
- Lawrence, A., D. Khanna, R. Misra, A. Aggarwal (2006). "Increased expression of basic fibroblast growth factor in skin of patients with systemic sclerosis." Dermatol Online J **12**(1): 2.
- Lefranc, M. P., P. Chuchana, P. Dariavach, C. Nguyen, S. Huck, F. Brockly, B. Jordan, G. Lefranc (1989). "Molecular mapping of the human T cell receptor gamma (TRG) genes and linkage of the variable and constant regions." Eur J Immunol **19**(6): 989-94.

- Lefranc, M. P. and T. H. Rabbitts (1989). "The human T-cell receptor gamma (TRG) genes." Trends Biochem Sci **14**(6): 214-8.
- LeRoy, E. C. (1996). "Systemic sclerosis. A vascular perspective." Rheum Dis Clin North Am **22**(4): 675-94.
- Lunardi, C., M. Dolcino, D. Peterlana, C. Bason, R. Navone, N. Tamassia, E. Tinazzi, R. Beri, R. Corrocher, A. Puccetti (2007). "Endothelial cells' activation and apoptosis induced by a subset of antibodies against human cytomegalovirus: relevance to the pathogenesis of atherosclerosis." PLoS ONE **2**(5): e473.
- Lunardi, C., M. Dolcino, D. Peterlana, C. Bason, R. Navone, N. Tamassia, R. Beri, R. Corrocher, A. Puccetti (2006). "Antibodies against human cytomegalovirus in the pathogenesis of systemic sclerosis: a gene array approach." PLoS Med **3**(1): e2.
- Lunardi, C., C. Bason, R. Corrocher, A. Puccetti (2005). "Induction of endothelial cell damage by hCMV molecular mimicry." Trends Immunol **26**(1): 19-24.
- Lunardi, C., C. Bason, R. Navone, E.N. Millo, G. Damonte, R. Corrocher, A. Puccetti (2000). "Systemic sclerosis immunoglobulin G autoantibodies bind the human cytomegalovirus late protein UL94 and induce apoptosis in human endothelial cells." Nat Med **6**(10): 1183-6.
- Maeda, Y., P. Reddy, K. P. Lowler, C. Liu, D. K. Bishop, J. L. Ferrara (2005). "Critical role of host gammadelta T cells in experimental acute graft-versus-host disease." Blood **106**(2): 749-55.
- Magro, C. M., A. N. Crowson, C. Ferri (2007). "Cytomegalovirus-associated cutaneous vasculopathy and scleroderma sans inclusion body change." Hum Pathol **38**(1): 42-9.
- Marie, I., H. Levesque, P. Ducrotte, P. Denis, M. F. Hellot, J. Benichou, N. Cailleux, H. Courtois (2001). "Gastric involvement in systemic sclerosis: a prospective study." Am J Gastroenterol **96**(1): 77-83.
- Mavalia, C., C. Scaletti, P. Romagnani, A. M. Carossino, A. Pignone, L. Emmi, C. Pupilli, G. Pizzolo, E. Maggi, S. Romagnani (1997). "Type 2 helper T-cell predominance and high CD30 expression in systemic sclerosis." Am J Pathol **151**(6): 1751-8.
- McGaha, T., S. Saito, R. G. Phelps, R. Gordon, N. Noben-Trauth, W. E. Paul, C. Bona (2001). "Lack of skin fibrosis in tight skin (TSK) mice with targeted mutation in the interleukin-4R alpha and transforming growth factor-beta genes." J Invest Dermatol **116**(1): 136-43.
- Michelson, S., J. Alcamí, S. J. Kim, D. Danielpour, F. Bachelierie, L. Picard, C. Bessia, C. Paya, J. L. Virelizier (1994). "Human cytomegalovirus infection induces transcription and secretion of transforming growth factor beta 1." J Virol **68**(9): 5730-7.

- Morton, S. J. and R. J. Powell (2000). "Cyclosporin and tacrolimus: their use in a routine clinical setting for scleroderma." Rheumatology (Oxford) **39**(8): 865-9.
- Murray, J. M., J. P. O'Neill, T. Messier, J. Rivers, V. E. Walker, B. McGonagle, L. Trombley, L. G. Cowell, G. Kelsoe, F. McBlane, B. A. Finette (2006). "V(D)J recombinase-mediated processing of coding junctions at cryptic recombination signal sequences in peripheral T cells during human development." J Immunol **177**(8): 5393-404.
- Muryoi, T., K. N. Kasturi, M. J. Kafina, D. S. Cram, L. C. Harrison, T. Sasaki, C. A. Bona (1992). "Antitopoisomerase I monoclonal autoantibodies from scleroderma patients and tight skin mouse interact with similar epitopes." J Exp Med **175**(4): 1103-9.
- Neidhart, M., S. Kuchen, O. Distler, P. Bruhlmann, B. A. Michel, R. E. Gay, S. Gay (1999). "Increased serum levels of antibodies against human cytomegalovirus and prevalence of autoantibodies in systemic sclerosis." Arthritis Rheum **42**(2): 389-92.
- O'Hanlon, T. P., W. A. Messersmith, M. C. Dalakas, P. H. Plotz, F. W. Miller (1995). "Gamma delta T cell receptor gene expression by muscle-infiltrating lymphocytes in the idiopathic inflammatory myopathies." Clin Exp Immunol **100**(3): 519-28.
- Olive, C., P. A. Gatenby, S. W. Serjeantson, (1994). "Restricted junctional diversity of T cell receptor delta gene rearrangements expressed in systemic lupus erythematosus (SLE) patients." Clin Exp Immunol **97**(3): 430-8.
- Otieno, F. G., A. M. Lopez, S. A. Jimenez, J. Gentiletti, C. M. Artlett (2007). "Allograft inflammatory factor-1 and tumor necrosis factor single nucleotide polymorphisms in systemic sclerosis." Tissue Antigens **69**(6): 583-91.
- Parel, Y., M. Aurrand-Lions, A. Scheja, J. M. Dayer, E. Roosnek, C. Chizzolini (2007). "Presence of CD4+CD8+ double-positive T cells with very high interleukin-4 production potential in lesional skin of patients with systemic sclerosis." Arthritis Rheum **56**(10): 3459-67.
- Polisson, R. P., G. S. Gilkeson, E. H. Pyun, D. S. Pisetsky, E. A. Smith, L. S. Simon (1996). "A multicenter trial of recombinant human interferon gamma in patients with systemic sclerosis: effects on cutaneous fibrosis and interleukin 2 receptor levels." J Rheumatol **23**(4): 654-8.
- Prescott, R. J., A. J. Freemont, C. J. Jones, J. Hoyland, P. Fielding (1992). "Sequential dermal microvascular and perivascular changes in the development of scleroderma." J Pathol **166**(3): 255-63.
- Rands, A. L., J. Whyte, B. Cox, N. D. Hall, N. J. McHugh (2000). "MHC class II associations with autoantibody and T cell immune responses to the scleroderma autoantigen topoisomerase I." J Autoimmun **15**(4): 451-8.

- Reveille, J. D. (2006). "The genetic basis of autoantibody production." Autoimmun Rev **5**(6): 389-98.
- Reveille, J. D., M. Fischbach, T. McNearney, A. W. Friedman, M. B. Aguilar, J. Lisse, M. J. Fritzler, C. Ahn, F. C. Arnett (2001). "Systemic sclerosis in 3 US ethnic groups: a comparison of clinical, sociodemographic, serologic, and immunogenetic determinants." Semin Arthritis Rheum **30**(5): 332-46.
- Rose, N. R. and N. Leskovsek (1998). "Scleroderma: immunopathogenesis and treatment." Immunol Today **19**(11): 499-501.
- Sakamoto, A., T. Sumida, T. Maeda, M. Itoh, T. Asai, H. Takahashi, S. Yoshida, T. Koike, H. Tomioka (1992). "T cell receptor V beta repertoire of double-negative alpha/beta T cells in patients with systemic sclerosis." Arthritis Rheum **35**(8): 944-8.
- Sakkas, L. I. and C. D. Platsoucas (2004). "Is systemic sclerosis an antigen-driven T cell disease?" Arthritis Rheum **50**(6): 1721-33.
- Sakkas, L. I., C. Tourtellotte, S. Berney, A. R. Myers, C. D. Platsoucas (1999). "Increased levels of alternatively spliced interleukin 4 (IL-4delta2) transcripts in peripheral blood mononuclear cells from patients with systemic sclerosis." Clin Diagn Lab Immunol **6**(5): 660-4.
- Sakkas, L. I., B. Xu, C. M. Artlett, S. Lu, S. A. Jimenez, C. D. Platsoucas (2002). "Oligoclonal T cell expansion in the skin of patients with systemic sclerosis." J Immunol **168**(7): 3649-59.
- Sallam, H., T. A. McNearney, J. D. Chen, (2006). "Systematic review: pathophysiology and management of gastrointestinal dysmotility in systemic sclerosis (scleroderma)." Aliment Pharmacol Ther **23**(6): 691-712.
- Sambo, P., L. Jannino, M. Candela, A. Salvi, M. Donini, S. Dusi, M. M. Luchetti, A. Gabrielli (1999). "Monocytes of patients with systemic sclerosis (scleroderma) spontaneously release in vitro increased amounts of superoxide anion." J Invest Dermatol **112**(1): 78-84.
- Sato, S., M. Hasegawa, K. Takehara (2001). "Serum levels of interleukin-6 and interleukin-10 correlate with total skin thickness score in patients with systemic sclerosis." J Dermatol Sci **27**(2): 140-6.
- Sato, S., T. Nagaoka, M. Hasegawa, T. Tamatani, T. Nakanishi, M. Takigawa, K. Takehara (2000). "Serum levels of connective tissue growth factor are elevated in patients with systemic sclerosis: association with extent of skin sclerosis and severity of pulmonary fibrosis." J Rheumatol **27**(1): 149-54.

- Seki, H., M. Nanno, P. F. Chen, K. Itoh, C. Ioannides, R. A. Good, C. D. Platsoucas (1989). "Molecular heterogeneity of gamma delta T-cell antigen receptors expressed by CD4-CD8- T-cell clones from normal donors: both disulfide- and non-disulfide-linked receptors are delta TCS1+." Proc Natl Acad Sci U S A **86**(7): 2326-30.
- Seko, Y., N. Takahashi, Y. Tada, H. Yagita, K. Okumura, R. Nagai (2000). "Restricted usage of T-cell receptor Vgamma-Vdelta genes and expression of costimulatory molecules in Takayasu's arteritis." Int J Cardiol **75 Suppl 1**: S77-83; discussion S85-7.
- Seko, Y., S. Minota, A. Kawasaki, Y. Shinkai, K. Maeda, H. Yagita, K. Okumura, O. Sato, A. Takagi, Y. Tada (1994). "Perforin-secreting killer cell infiltration and expression of a 65-kD heat-shock protein in aortic tissue of patients with Takayasu's arteritis." J Clin Invest **93**(2): 750-8.
- Servettaz, A., M. C. Tamby, P. Guilpain, J. Reinbolt, P. Garcia de la Pena-Lefebvre, Y. Allanore, A. Kahan, O. Meyer, L. Guillevin, L. Mouthon (2006). "Anti-endothelial cell antibodies from patients with limited cutaneous systemic sclerosis bind to centromeric protein B (CENP-B)." Clin Immunol **120**(2): 212-9.
- Shen J., D. M. Andrews, F. Pandolfi, L. A. Boyle, C. M. Kersten, R. N. Blatman, J. T. Kurnick (1998) "Oligoclonality of Vdelta1 and Vdelta2 cells in human peripheral blood mononuclear cells: TCR selection is not altered by stimulation with gram-negative bacteria." J Immunol. **160**(6): 3048-55.
- Shin, S., R. El-Diwany, S. Schaffert, E. J. Adams, K. C. Garcia, P. Pereira, Y. H. Chien (2005). "Antigen recognition determinants of gammadelta T cell receptors." Science **308**(5719): 252-5.
- Shi-wen, X., D. Pennington, A. Holmes, A. Leask, D. Bradham, J. R. Beauchamp, C. Fonseca, R. M. du Bois, G. R. Martin, C. M. Black, D. J. Abraham (2000). "Autocrine overexpression of CTGF maintains fibrosis: RDA analysis of fibrosis genes in systemic sclerosis." Exp Cell Res **259**(1): 213-24.
- Sieling, P. A., D. Chatterjee, S. A. Porcelli, T. I. Prigozy, R. J. Mazzaccaro, T. Soriano, B. R. Bloom, M. B. Brenner, M. Kronenberg, P. J. Brennan (1995). "CD1-restricted T cell recognition of microbial lipoglycan antigens." Science **269**(5221): 227-30.
- Spada, F. M., E. P. Grant, P. J. Peters, M. Sugita, A. Melian, D. S. Leslie, H. K. Lee, E. van Donselaar, D. A. Hanson, A. M. Krensky, O. Majdic, S. A. Porcelli, C. T. Morita, M. B. Brenner (2000). "Self-recognition of CD1 by gamma/delta T cells: implications for innate immunity." J Exp Med **191**(6): 937-48.
- Steen, V. D. (1999). "Pregnancy in women with systemic sclerosis." Obstet Gynecol **94**(1): 15-20.

- Steen, V. D. and T. A. Medsger, Jr. (1999). "Fertility and pregnancy outcome in women with systemic sclerosis." Arthritis Rheum **42**(4): 763-8.
- Stummvoll, G. H., M. Aringer, J. S. Smolen, M. Koller, H. P. Kiener, C. W. Steiner, B. Bohle, R. Knobler, W. B. Graninger (2000). "Derangement of apoptosis-related lymphocyte homeostasis in systemic sclerosis." Rheumatology (Oxford) **39**(12): 1341-50.
- Tan, F. K., F. C. Arnett, J. D. Reveille, C. Ahn, S. Antohi, T. Sasaki, K. Nishioka, C. A. Bona (2000). "Autoantibodies to fibrillin 1 in systemic sclerosis: ethnic differences in antigen recognition and lack of correlation with specific clinical features or HLA alleles." Arthritis Rheum **43**(11): 2464-71.
- Tan, F. K., G. M. Tercero, F. C. Arnett, N. Wang, R. Chakraborty (2003). "Examination of the possible role of biologically relevant genes around FBN1 in systemic sclerosis in the Choctaw population." Arthritis Rheum **48**(11): 3295-6.
- Tanaka, Y., C. T. Morita, E. Nieves, M. B. Brenner, B. R. Bloom (1995). "Natural and synthetic non-peptide antigens recognized by human gamma delta T cells." Nature **375**(6527): 155-8.
- Tanaka, Y., S. Sano, E. Nieves, G. De Libero, D. Rosa, R. L. Modlin, M. B. Brenner, B. R. Bloom, C. T. Morita (1994). "Nonpeptide ligands for human gamma delta T cells." Proc Natl Acad Sci U S A **91**(17): 8175-9.
- Valentini, G. and C. Black (2002). "Systemic sclerosis." Best Pract Res Clin Rheumatol **16**(5): 807-16.
- Valentini, G., M. F. Romano, C. Naclerio, R. Bisogni, A. Lamberti, M. C. Turco, S. Venuta (2000). "Increased expression of CD40 ligand in activated CD4+ T lymphocytes of systemic sclerosis patients." J Autoimmun **15**(1): 61-6.
- Vancheeswaran, R., A. Azam, C. Black, M. R. Dashwood (1994). "Localization of endothelin-1 and its binding sites in scleroderma skin." J Rheumatol **21**(7): 1268-76.
- Vancheeswaran, R., T. Magoulas, G. Efrat, C. Wheeler-Jones, I. Olsen, R. Penny, C. M. Black (1994). "Circulating endothelin-1 levels in systemic sclerosis subsets--a marker of fibrosis or vascular dysfunction?" J Rheumatol **21**(10): 1838-44.
- Varga, J. and R. I. Bashey (1995). "Regulation of connective tissue synthesis in systemic sclerosis." Int Rev Immunol **12**(2-4): 187-99.
- White, B. and V. V. Yurovsky (1995). "Oligoclonal expansion of V delta 1+ gamma/delta T-cells in systemic sclerosis patients." Ann N Y Acad Sci **756**: 382-91.

- Wiendl, H., J. Malotka, B. Holzwarth, H. U. Weltzien, H. Wekerle, R. Hohlfeld, K. Dornmair (2002). "An autoreactive gamma delta TCR derived from a polymyositis lesion." J Immunol **169**(1): 515-21.
- Wooley, P. H., S. Sud, A. Langendorfer, C. Calkins, P. J. Christner, J. Peters, S. A. Jimenez (1998). "T cells infiltrating the skin of Tsk2 scleroderma-like mice exhibit T cell receptor bias." Autoimmunity **27**(2): 91-8.
- Workalemahu, G., M. Foerster, C. Kroegel, R. K. Braun (2003). "Human gamma delta-T lymphocytes express and synthesize connective tissue growth factor: effect of IL-15 and TGF-beta 1 and comparison with alpha beta-T lymphocytes." J Immunol **170**(1): 153-7.
- Wu, H., A. R. Clarkson, J. F. Knight (2001). "Restricted gammadelta T-cell receptor repertoire in IgA nephropathy renal biopsies." Kidney Int **60**(4): 1324-31.
- Yanaba, K., K. Komura, M. Kodera, T. Matsushita, M. Hasegawa, K. Takehara, S. Sato (2006). "Serum levels of monocyte chemotactic protein-3/CCL7 are raised in patients with systemic sclerosis: association with extent of skin sclerosis and severity of pulmonary fibrosis." Ann Rheum Dis **65**(1): 124-6.
- Yazawa, N., K. Kikuchi, H. Ihn, M. Fujimoto, M. Kubo, T. Tamaki, K. Tamaki (2000). "Serum levels of tissue inhibitor of metalloproteinases 2 in patients with systemic sclerosis." J Am Acad Dermatol **42**(1 Pt 1): 70-5.
- Yurovsky, V. V., P. A. Sutton, D. H. Schulze, F. M. Wigley, R. A. Wise, R. F. Howard, B. White (1994). "Expansion of selected V delta 1+ gamma delta T cells in systemic sclerosis patients." J Immunol **153**(2): 881-91.
- Yurovsky, V. V., F. M. Wigley, R. A. Wise, B. White (1996). "Skewing of the CD8+ T-cell repertoire in the lungs of patients with systemic sclerosis." Hum Immunol **48**(1-2): 84-97.
- Zhou, X., F. K. Tan, D. M. Milewicz, X. Guo, C. A. Bona, F. C. Arnett (2005). "Autoantibodies to fibrillin-1 activate normal human fibroblasts in culture through the TGF-beta pathway to recapitulate the "scleroderma phenotype"." J Immunol **175**(7): 4555-60.
- Zhou, X., F. K. Tan, M. Xiong, D. M. Milewicz, C. A. Feghali, M. J. Fritzler, J. D. Reveille, F. C. Arnett (2001). "Systemic sclerosis (scleroderma): specific autoantigen genes are selectively overexpressed in scleroderma fibroblasts." J Immunol **167**(12): 7126-33.
- [No authors listed] American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Preliminary criteria for the classification of systemic sclerosis (scleroderma). Subcommittee for scleroderma criteria of the American Rheumatism

Association Diagnostic and Therapeutic Criteria Committee. *Arthritis Rheum.* May 1980;23(5):581-90.