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The Generation, Immortalisation, and Characterisation of Human γδ T cells Derived from the Blood and Cerebrospinal Fluid of MS Patients

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A thesis submitted to the School of Graduate Studies

University of Ottawa

In partial fulfilment of the requirements for the degree of

Doctor of Philosophy

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Abstract

Multiple sclerosis (MS) is believed to be an autoimmune, inflammatory, demyelinating disease of the central nervous system (CNS), resulting in myelin degradation, loss of the myelin forming cell, the oligodendrocyte (ODC), and axonal degeneration. Although the cause of MS is unknown, it is presumed that damage is a result of the immune inflammatory process. Concentrated within MS lesions are infiltrates consisting mostly of T cells and macrophage/ microglia. Although the majority of T cells express αβ T cell receptors (TcR), a substantial number also express γδ TcRs, though such cells are relatively rare in peripheral blood. The role of αβ T cells is confounded by the requirement for CNS antigen presentation and the fact that no discreet myelin Ag has been demonstrated unique to MS. In contrast, γδ T cells can recognise a diverse spectrum of antigen directly, without restriction from MHC molecules or the need for antigen processing, while retaining effector functions that are deadly to the myelin forming ODCs. The hypothesis underlying this work is that γδ T cells play a distinct role in MS pathogenesis by initiating, perpetuating, or regulating the immune response directed against the myelin/ ODC unit.

Initial comparative experiments utilising short term γδ T cell lines from peripheral blood (PB) and cerebrospinal fluid (CSF) of MS and other neurological disease (OND) patients, indicated no significant γδ subtype or cytotoxicity differences between cells derived from PB and CSF compartments or between MS and OND patients. During the course of these studies, it became apparent that the variably short *in vitro* lifespans of PB and CSF γδ T cells represented a major limitation that hampered further comprehensive studies. Efforts to increase their culture lifespans through restimulation regimens were only marginally successful, so an alternate immortalisation strategy using *Herpesvirus saimiri* (HVS) was employed. This study reports for the first time, the successful HVS growth transformation of human γδ T cells derived from both MS PB and CSF. Multiple HVS transformed PB and CSF γδ T cell (*t*-γδ T cell) lines and clones were generated, and have existed in IL-2 dependent culture for periods in excess of 2.5 years without the need for additional stimulation. Comparative analysis of a series of *t*-γδ T cell lines

and their parental non-transformed lines indicated the transformation process did not alter their surface molecule expression, cytokine, or cytotoxicity responses.

Cell surface characterisation of the *t*-γδ T cell lines and clones demonstrated HVS was capable of immortalising a wide spectrum of γδ TcR subtypes, along with identifying *t*-γδ T cell clones displaying rare γδ T cell phenotypes, not commonly studied. MS *t*-γδ T cells uniformly expressed proinflammatory cytokine profiles (IFN-γ, TNF-α), but only minimal IL-2 or anti-inflammatory IL-4 and IL-10. All γδ TcR subtypes displayed identical cytokine pattern expression, suggesting that cytokine expression is independent of γδ T cell subtype. *t*-γδ T cell lines demonstrated a graded cytotoxicity towards a panel of tumour cell targets, ranging from high (U937, Jurkat) to moderate (KG-1) to poor (Colo-205). Similar killing patterns of tumour targets were observed for subtype specific *t*-γδ T cell clones, and antigen recognition studies indicated a graded recognition of tumour cell ligands, together suggesting that both cytolytic function and tumour cell recognition, as was seen with cytokine profiles, are independent of γδ T cell subtype. In contrast, only Vγ9Vδ2 positive *t*-γδ T cells responded to candidate non-peptidic phospholigand and alkylamine antigens. No MS *t*-γδ T cell reactivity was observed to putative MS antigens myelin basic protein, αB-crystallin, or *Chlamydia pneumoniae*.

Direct comparison of PB and CSF *t*-γδ T cells generated from the same MS patient showed that CSF *t*-γδ T cells were almost exclusively comprised of Vγ9Vδ1 cells, whereas predominantly Vγ9Vδ2 cells were found in PB. Despite these population differences, cytokine and cytotoxicity profiles were indistinguishable. PB and CSF *t*-γδ T cells equally recognised tumour cell antigens, but only PB *t*-γδ T cells responded to phospholigand and alkylamine antigens, likely due to the strong PB presence of Vγ9Vδ2 γδ T cells.

Methodology developed in this project has allowed for the long term growth of γδ T cells from both PB and CSF in order to study their phenotypic and functional properties. This will allow for comparative experiments over time from the same individuals as their disease status changes or they undergo therapy, to further understand the role γδ T cells may play in their disease process.

Acknowledgements

I would like to acknowledge the support, guidance, and friendship of my supervisor, Dr. Mark Freedman, who has succeeded in introducing a bench top chemist into the exciting and challenging world of human immunology. Mark's tremendous ability as a clinician, researcher, and family man will always remain a source of inspiration to me throughout my career.

It goes without saying that the often challenging struggle of school and science life is tempered by the people whose lives you share on a daily basis. Throughout the years I have been fortunate enough to have been part of a group of friends that have shared many a laugh, supported one another in times of duress, and celebrated together those occasions that mark time. Thanks to Vi, Nancy, Rana, Darius, and Melanie. A special thanks to Komal, my "little sister" whose tenacious help, coupled with her "bholla" charm has enabled me to accomplish this work

I would especially like to acknowledge the support of my family and friends throughout this endeavour. To my mother, who never once doubted what I might achieve and who has always provided me with the support that only a mother can. No one has stood beside me more strongly and committed, and had to endure more sacrifices without complaint, than my partner, soulmate, and wife- Susie. Unquestionably, her contribution to this work is equal to my own, and I thank her from the bottom of my heart for her unwavering encouragement. Together, we now set forth to face new challenges.

The course of this work witnessed two profound events in my life, separated only by mere weeks. To my late father, Richard, and to my 10 month old son, Ethan, I would like to dedicate this thesis to you both. Although you didn't see me to the finish line, Dad, I know in my heart that you are proud of me and I thank you for instilling in me all that I am today. To Ethan, who often embodies his Grandpa, I look forward to giving you the gift of education that I was so fortunate to receive. Although you are too young to realise it, your inquisitive, innocent, and smiling face made everything better when times were tough, and for this I thank you.

Table of contents

List of abbreviations

ADCC	antibody dependent cell cytotoxicity
Ag	antigen
ATCC	American Type Culture Collection
C'	complement
CNS	central nervous system
CPE	cytopathic effect
cR/A	complete RPMI/ AIM-V mixture
cRPMI	complete RPMI
CSF	cerebrospinal fluid
cSI	cytokine stimulation index
DA	dimethylallyl pyrophosphate
DiO	3,3'-dioctadecyloxacarbocyanine perchlorate
DN	double negative cells (CD4 and CD8 negative)
E.I.	expansion index
E:S	effector to stimulator ratio
E:T	effector to target ratio
EAE	experimental allergic encephalomyelitis
EB	elementary body
FACS	fluorescence activated cell sorting
FasL	Fas ligand
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FS/ SS	forward scatter/ side scatter
g	unit of gravity
Gln	glutamine
HIV	human immunodeficiency virus
HSP	heat shock protein
HTLV-1	human T cell leukemia virus type-1
HVS	*Herpesvirus saimiri*
IB	isobutylamine
ICP	intracytoplasmic cytokine profile
IFN	interferon
Ig	immunoglobulin
IL-	interleukin-
IL-R	interleukin- receptor
iNOS	inducible nitric oxide synthase
IP	isopentenyl pyrophosphate
KAR	killer activating receptor
kD	kilodalton
KIR	killer inhibitory receptor
m.o.i.	multiplicity of infection
mAb	monoclonal antibody
MBP	myelin basic protein
MC	methyl cellulose
MCF	mean channel fluorescence
MHC	major histocompatibility complex
MS	multiple sclerosis

o.n.	overnight
ODC	oligodendrocyte cell
OMK	owl monkey kidney cells
OMP	outer membrane protein
OND	other neurological disease
P/S	penicillin and streptomycin
PB	peripheral blood
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerB	permeabilisation buffer
PFU	plaque forming unit
PHA	phytohemagglutinin
PI	propidium iodide
PMA	phorbol myristate acetate
r.t.	room temperature
RR	relapsing- remitting
SB	staining buffer
SDS	sodium dodecyl sulfate
SI	stimulation index
SP	secondary progressive
T/E	trypsin/ EDTA solution
TC	tissue culture
TCID	tissue culture infectious dose
TcR	T cell receptor
TD	trehalose 6,6' dimycolate
TNF	tumour necrosis factor

List of figures

List of tables

Chapter 1- Introduction

1.1 Synopsis

Multiple sclerosis (MS) is a presumed autoimmune disease of the central nervous system and existing evidence suggests that a minor T cell subset expressing γδ T cell receptors, can potentially play a role in the pathogenesis of the disease. That they are suspected to be players comes primarily from their concentration within MS lesions of upwards to 30% of the total T cells present, which is in stark contrast to the normal γδ T cell circulating level of 3-7% or to similar numbers in other organs such as the spleen. Unlike the major αβ T cell subset, the study of human γδ T cells has been relatively restricted and not much is known regarding their role, function, and characteristics, although this is rapidly changing since γδ T cells are increasingly being implicated in other disease states. It is clear that γδ T cells are distinct from their αβ T cell cousins in fundamental ways such as possessing T cell receptors (TcR) with binding properties more akin to immunoglobulin receptors than to αβ TcRs; the ability to see antigen directly without initial processing; the general lack of MHC restriction; and the recognition of fundamentally different classes of antigen such as non-peptidic phospholigands. What role, if any, these cells play within the central nervous system (CNS) and their contribution towards MS pathogenesis, is even less understood. This is due primarily to the scarcity of CNS γδ T cell samples from MS patients, the low numbers of γδ T cells found within these samples, and their short culture lifespans which limits their study. What is quite evident is that γδ T cells possess specialised antigen recognition features and exert their effector properties in manners quite unlike other immune cells, which make them particularly suited to interacting with CNS components. This work sets out to establish ways to generate long term human γδ T cells both from peripheral blood (PB) and importantly from cerebrospinal fluid (CSF), and to advance the knowledge base of this poorly understood cell type with particular emphasis on MS pathogenesis. This was mainly accomplished using a novel technique to immortalise PB and CSF γδ T cells using viral transformation with *Herpesvirus saimiri* (HVS). This work will demonstrate for the first time, the

successful application of the HVS transformation technology to MS PB and CSF γδ T cells, identify immortalised γδ T cell subtypes not described *a priori*, characterise a number of γδ T cell effector functions revealing possible γδ T cell phenotype/ function correlations, identify classes of antigen recognised by MS γδ T cells, and comprehensively begin to compare PB vs. CSF γδ T cells for phenotypic and/ or functional differences. This work represents a major step forward in the study of MS PB and CSF γδ T cells since the immortalisation process provides for a continuous stream of consistent, well defined cells in which to rigorously study, and concurrently establishes a γδ T cell cellular database from which to draw upon at future time points.

1.2 Multiple sclerosis

A disease, which would be diagnosed today as multiple sclerosis, was first described some 160 years ago and characterised by multiple, dense, discoloured foci within the central nervous system. These observations were associated with a clinical history of numbness and weakness followed by progressive paralysis, bladder dysfunction, and exacerbated symptoms related to hot temperatures. It remained for Charcot (1), using microscopic staining techniques, to methodically describe the overwhelming myelin loss associated with the relative preservation of axons within these brain lesions and termed this disease "sclérose en plaques". While many of these initial observations are still commonly associated with "modern day" MS, it is now accepted that MS is a complex disease or family of diseases and presents in numerous forms or variants (2), is subject to both genetic and environmental influences (3-5), and disease pathogenesis may be highly variable and patient specific with a common pathological endpoint (6; 7).

MS is now widely recognised as a CNS specific inflammatory demyelinating disease and the resulting neurological dysfunction, a consequence of myelin and axon destruction as well as decreased regenerative capacities by oligodendrocytes (ODC) (8-11). The disease, with a world wide prevalence of approximately 1 million cases, is usually diagnosed in young adults between the ages of 20-40 but is most probably prevalent yet undetected at younger ages. Similar to other suspected autoimmune diseases, MS has a gender related bias of approximately 2-3:1 for

women for reasons that are not well understood, but related to gender differences in immune responsiveness, response to infection, hormonal function and sex-linked genetic factors (12). The disease course of MS is highly variable, with the most common form of MS (~85%), termed relapsing-remitting (RR), characterised clinically by periods of acute neurological dysfunction followed by disease remission with an associated full or partial restoration of neurological ability. The RR phase often transitions, for poorly understood reasons, into a more progressive secondary phase (SP) without remission, and is associated with increasing permanent neurological deficits. In contrast, a small segment of the RR population demonstrates mild disease manifestations and low neurological deficits over prolonged periods of time (benign MS). The remaining clinical forms of MS comprise primary progressive disease (10-15%) which is characterised by progressive disease without a relapsing-remitting course (13) and the rare acute Marburg variant which leads to fulminant disease and death within months (2).

Despite intensive research efforts, the primary cause of MS remains elusive and disease pathogenesis is only beginning to be unravelled. Two widely held beliefs are: 1) MS is a viral disease and CNS demyelination is the end result of host clearance of virus; 2) MS is an autoimmune disease and demyelination is the result of host reactivity to autoantigen(s). Although a myriad of viruses such as influenza, measles, Epstein-Barr virus, human coranavirus, several retroviruses, and most recently human Herpesvirus-6 have been investigated (14-18), no data has been found to conclusively support a viral aetiology in MS. The strongest evidence that environmental factors such as viral infections play a role in the aetiology of MS, comes from epidemiological studies. MS prevalence follows a distinct geographical distribution with a high to low north-south gradient and migration studies indicate disease susceptibility correlates with geographic location (19). Furthermore, a number of human and animal viral infections lead to spontaneous demyelination (15; 20); clinical relapse often follows viral infections (21); and genetic studies in homozygous twins demonstrate MS concordance rates of only 30% implicating the potential for environmental effects (22). The more widely held view that MS is an autoimmune disease also suffers from a lack of an etiological autoantigen(s) and autoimmune proponents cite pathobiological, genetic, clinical, and animal model evidence to support this

viewpoint. Immunopathological studies typically have shown infiltrating lymphocytes and monocytes within MS lesions which concentrate around zones of active demyelination (23-25). Genetic linkage studies investigating germline regions influencing disease susceptibility, have identified the major histocompatibility complex as an important source of susceptibility genes (26-28). Although it was clear that disease susceptibility was multigenic and complex, the HLA-DR2 haplotype was specifically associated with MS susceptibility (3). Clinical evidence supporting an autoimmune process comes from the successful application of immunomodulatory, anti-inflammatory, and immunosuppressive therapies to modulate disease activity (29). Furthermore, an MS-like syndrome known as experimental allergic encephalomyelitis (EAE) can be induced in susceptible animals by immunisation with numerous CNS antigens, and the disease can be induced in naïve animals by the adoptive transfer of purified autoreactive $CD4^+$ $\alpha\beta$ T cells (30). The viral and autoimmune hypotheses need not be mutually exclusive and indeed the prevalent view is that MS is an autoimmune disease triggered by infectious agents in genetically susceptible individuals. As autoimmune initiators, infectious agents can lead to the modulation of the blood-brain barrier causing it to become "leaky" and allowing CNS access to autoreactive cells (31); in the face of a specific immune response, infectious agents provoke the exposure of cryptic CNS antigens via epitope spreading mechanisms (32), or they break natural tolerance to CNS antigens either by activating autoreactive cells in a superantigen fashion (33; 34) or by generating immune responses that mimic CNS antigen(s) (14; 35; 36). The identification of pathogenic substrates that invoke molecular mimicry in this fashion is challenging given the now accepted high degree of degeneracy that exists for TcR recognition of peptide (37).

1.3 MS pathogenesis

The hallmark of MS pathogenesis is the perivenular inflammatory influx of immune cells into the brain parenchyma with an associated loss of myelin and the myelin forming oligodendrocyte cell. Recent evidence also demonstrates that nerve axons are also targets of destruction and that axonal transection occurs frequently and at early time points, and may be a

key factor relating to permanent neurological deficits in MS (38). MS lesions are classified as either active, chronic active, or chronic inactive based on the distribution and density of the inflammatory cells present (9). In active lesions, inflammatory cells composed primarily of monocytes/ macrophages and T cells with relatively few B cells, together with resident microglia and astrocytes, are found throughout the lesion. In chronic active lesions, myelin removal is evident with numerous foamy macrophages actively ingesting myelin. Resident astrocytes have become reactive and immune cells are found to concentrate at the lesion edge expanding into normal myelinated tissue. Inactive lesions are characterised by the paucity of cells found as well as the formation of the glial scar. Oligodendrocytes are infrequently found in chronic established lesions but are abundant during the early course of the disease or in new active lesions, often associated with remyelination of damaged axons (39; 40). The systematic study of oligodendrocyte loss within multiple MS lesions revealed defined characteristics which when categorised suggested multiple pathogenic mechanisms were at play (41; 42). Whether myelin itself is the target of attack, the myelin forming oligodendrocytes, or a combination of both is not yet known. Similarly, it is not known whether demyelination precipitates axonal damage or axonal insult results in demyelination. What is clear is that there exist a number of different mechanisms in which the myelin/ OD unit and/ or neuronal axon can be damaged by immune cells or their products. Principal among these immune cells are the infiltrating T cells which many believe are the mediators of the disease. Still the focus of most studies and therapeutic strategies, this view of the T cell is perhaps overly simplistic, in light of current opinions regarding MS pathogenesis. That MS is considered to be primarily a T cell mediated disease stems principally from animal studies where the disease EAE can be induced solely from administration of myelin reactive $CD4^+$ $\alpha\beta$ T cells (43; 44). However despite intensive efforts over decades of study, many questions remain unresolved regarding the role of $\alpha\beta$ T cells in MS immunopathogenesis, none more prominent than the required need for MHC restricted antigen presentation. This work focuses on a second T cell subset expressing $\gamma\delta$ T cell receptors and also prominently found within MS plaques (45; 46). $\gamma\delta$ T cells have only recently been studied but their known properties, distinct from those of $\alpha\beta$ T cells, suggest that they may play a critical role in MS

immunopathogenesis. Indeed, if the hypothesis proves true that MS pathogenesis may be tailored for different individuals (41), then γδ T cell effector function(s) may represent the key pathogenic mechanism within a subset of MS patients.

1.4 γδ T cells

With the discovery of the genes encoding TcR γ chains (47) and the subsequent characterisation of a minor T cell subset expressing γδ TcRs (48), researchers have since been trying to elucidate their biological role(s). While initial work centred on drawing parallels to the much more described and understood αβ T cell subset, it is now known that γδ T cells exhibit fundamental differences and may function in roles not previously ascribed to their αβ T cell cousins (49-52). Based on their abundance in both skin and mucosal epithelial relative to lymphoid tissues (53; 54), and their well characterised expansion and reactivity to bacteria (55-59), virus (60-62), parasites (63-65), stress induced proteins/ tumours (66-69), and allergens (70; 71), γδ T cells are thought to play a role in first line defence against invading pathogens and/ or injured/ transformed cells. As such, many consider γδ T cells act as sentinels that bridge the gap between the innate and adaptive immune systems (53). Increasingly however, γδ T cells are implicated in many aspects of an immune/ autoimmune response including initiation of the adaptive response (72); immunomodulation/ immunoregulation of the ensuing response by chemokine (73) and cytokine release (74; 75) or cellular control by cytotoxicity mechanisms (76; 77); direct participation through γδ T cell effector functions (51); induction of oral tolerance (78; 79), and the maintenance of homeostasis (71; 80). Clearly, the aberrant expression (or lack of expression) of γδ T cell effector functions can lead to pathological conditions such as autoimmune disease (81).

1.4.1 γδ T cell phenotype

The human γ gene locus is located on chromosome 7 and is composed of 14 Vγ gene elements, 6 of which are functional, and 5 functional Jγ junctional elements. Based on homology,

the functional Vγ2,3,4,5,8 gene segments are grouped into the Vγl family with the remaining functional Vγ9, the sole member of VγII family (82; 83). The δ gene locus is located within the TcR α locus between the Vα and Jα gene segments on chromosome 14. Vδ1-3 gene elements are used in the majority of γδ T cells, with the remaining fraction composed of δ chains resulting from specific Vα genes rearranging with Dδ-Jδ gene segments (Vδ4-6). Thus the potential diversity for γ and δ genes is limited based on the relatively small number of functional germline gene elements available for recombination. In fact, the potential γδ TcR diversity is estimated to be as high as αβ T cells which possess significantly more Vα and Vβ genes to draw upon. This further diversity is due to the use of repetitive of Dδ segments in tandem, imprecise V-D and D-J joinings, nucleotide trimming, and insertion of random N-nucleotides at the V/D/J joining sites (84). Despite this potential diversity, the majority of circulating γδ T cells coexpress Vγ9 and Vδ2 chains (70-80%) and are collectively referred to as the Vδ2 subtype. Of the remaining circulating γδ T cells, the majority express Vδ1 in combination with various Vγ chains and are referred to as the Vδ1 subtype (~15%). In contrast, tissue γδ T cells have more diverse chain pairings and Vγ-Vδ1 combinations often predominate. Phenotypically, γδ T cells are very similar to αβ T cells in that they both express many of the same cell surface markers, with the notable exception of CD4 and CD8. Although a significant proportion of γδ T cells express CD8 (25-50%), expression is low and restricted to CD8α homodimers (85). Expression of CD4 is rare on circulating γδ T cells but is more common in some organs such as liver (86). In comparison to αβ T cells, γδ T cells express high levels of L-selectin (CD62L) and show greater *in vitro* transendothelial capabilities irrespective of CD45RA and RO isoforms (87; 88). Similarly, recent studies demonstrated that the C-type lectin NKRP1A upregulated on the surface of a subset of γδ T cells, increases their ability to migrate across the vascular endothelium (89). Together, these results suggest that γδ T cells may be advantaged for penetrating the blood-brain barrier and entry into the CNS, even in the absence of inflammation, which is particularly relevant for initiation events. γδ T cells possess an array of NK surface molecules such as CD16, CD56 and the cytotoxicity modulating receptors, CD94, NKG2-A,C,D, CD158a,b, p70, and p140 (90-92) as well as CD95 and CD95L (93).

1.4.2 γδ T cells: Antigen recognition

Although γδ T cells can be restricted in the classical sense by MHC class I and II molecules (94; 95), the majority of γδ T cell interactions bypass this requirement (66; 96; 97). Consistent with this hallmark property, they also lack for the most part, the class I and II adhesion molecules CD8 and CD4 respectively. Another remarkable feature of γδ T cells is that they possess TcRs that more resemble Ig binding sites than classical αβ TcR (98; 99) and they can recognise antigen directly without the processing pathways involved in generating peptide-MHC complexes (97; 100; 101).

One of the most remarkable and at the same time, most confounding property of γδ T cells is the type of antigen that they recognise. Of the relatively few antigens identified, γδ T cells have been shown to recognise antigens as diverse as viral glycoproteins (97), cellular heat shock proteins (102; 103), non-polymorphic class I related molecules such as MICA (67), T10/ T22 gene products (101; 104), and CD1 (105). The most extensive studies of γδ T cell reactivity have been by far those directed towards mycobacteria. The elucidation that γδ T cells recognise small non-peptidic phospholigands in a TcR dependent manner (58; 106-109) has opened up a new era in T cell recognition study and has far reaching implications for many disease states including MS. Besides these revolutionary phospholigands, another non-peptidic class of antigen known as alkylamines have also been recently identified as TcR dependent γδ T cell substrates (110). It remains to be seen whether early studies showing γδ T cell reactivity to molecules such as HSP, class Ib, and CD1 are indeed specific for these substrates or merely to non-peptide molecules presented by them (111). To date, one overriding feature of γδ T cell antigen recognition, has been the almost exclusive response to antigen by the major blood Vγ9Vδ2 subset. This has led to the suggestion that this particular subset responds in a superantigen fashion to Vγ9 gene elements in a manner analogous to Vβ recognition in αβ T cells (106; 112). However, studies demonstrating phospholigand recognition is independent of MHC class II requirements (109) and more recent evidence (113) using TcR chainpair switching and CDR3 mutagenesis argue against

a superantigen effect, at least in a classical sense. These latter studies revealed a dependence on co-expression of both Vγ9 and Vδ2 TcR chains and revealed that disturbances in the CDR3 region of a phospholigand reactive TcR abolished recognition- effects not associated with classic superantigen stimulation. Relatively few examples of antigen recognition by the major tissue subset of γδ T cells (non- Vδ2) have been documented. Within this group is the recognition by $V\delta1^+$ γδ T cells of MICA, a stress related HSP (67; 91; 114), CD1c (105), and lipoprotein (64)- all of which are highly relevant to MS pathogenesis.

1.4.3 Function

1.4.3.1 Cytotoxicity

One of the most potent functions of activated γδ T cells is their promiscuous cytotoxicity towards a number of different cell types including virally infected cells (60; 97), tumour cells of varying lineage (69; 115; 116), and CNS glial cells (117). The recognition of target cells by γδ T cells was not MHC restricted and often target cell lysis was not restricted to the stimulating antigenic ligands (56; 69; 118). Indeed, the overlap between γδ T cell reactivity to mycobacteria and Daudi B cell cytotoxicity has suggested a common recognition of members of the heat shock protein family (66; 119), which has now been extended to CNS glial cells, and in particular oligodendrocytes (120; 121).

γδ T cells have been shown to possess cytotoxicity mediators such as perforin and granzymes (122; 123), FasL (CD95L) expression (93; 124), TNF production (50), and Fc receptors (125) available for antibody dependent cell cytotoxicity (125; 126). How γδ T cells mediate their cytotoxic potentials and regulate them is the subject of much interest, especially in light of their seemingly promiscuous nature. Work carried out during the tenure of this thesis suggests γδ T cells kill tumour and oligodendrocyte targets primarily via release of perforin although the Fas/ FasL pathway is functional and synergistic (127). The seemingly random cytotoxicity displayed by activated γδ T cells has been difficult to reconcile *in vivo* due to their lack

of specificity. However, control may be exerted through NK killer inhibitory (KIR) and killer activating receptors (KAR) possessed by the majority of circulating γδ T cells, which have been shown to modulate γδ T cell cytotoxicity through recognition of MHC gene products (67; 76; 128; 129). Both immunoglobulin-like receptors (p58.1 and 2, p70, p140) and lectin-like receptors (CD94, NKG2A,C,D) with specificity for HLA-A, B, C ligands and for HLA-E and MICA respectively, have been identified (130). The decision to kill their targets depends on the co-ordinated interactions between stimulatory (γδ TcR, KARs) and inhibitory (KIRs) receptors with their ligands (91). The broad pattern recognition by γδ TcR to such ubiquitous antigens such as HSPs (111), tumour ligands (114) and microbial metabolites (i.e. phospholigands) (53) positions them to recognise danger/ stress which is complementary to the self/ non-self recognition of αβ T cells. Disturbances of this delicately co-ordinated γδ T cell killing control, has direct ramifications for autoimmune diseases such as MS.

1.4.3.2 Cytokine release/ Regulation

The increasingly recognised role of γδ T cells as sentinels in the innate immune setting has led to increased speculation that γδ T cells are involved with immune initiation events. The ability to recognise broad spectrum microbial antigens directly, detect damaged or injured cells, their predilection for tissue surveillance coupled with their ability to secrete the full spectrum of cytokines (65; 82) and chemokines (73) released by αβ T cells, lends support to this hypothesis (51; 53). Although γδ T cells more commonly secrete proinflammatory molecules such as IFN-γ and TNF-α, widely believed to exacerbate cell mediated autoimmune diseases such as MS and rheumatoid arthritis (131; 132), they also have the inherent capacity to secrete Th2-like cytokines (IL-4, IL-10) early in the response to allergens (133) and Th2 inducing pathogens (74). As such, γδ T cells can exist phenotypically and functionally as distinct Th1-like and Th2-like subsets (74; 75) . Numerous examples demonstrate that γδ T cells have the capacity to promote B cell Ig production including the production of autoantibodies (134-136). Recently, a γδ T cell type has been found in human liver which appears to be a human homologue of the murine NK1.1 T cells known to influence the ensuing murine immune response by high level production of IL-4 (86). γδ

T cells can even regulate cells of the innate immune system, which in turn sets the scene for the ensuing adaptive response. For instance, γδ T cells have been implicated in activating tissue macrophages to secrete TNF-α and the Th1 inducing cytokine, IL-12 (72). Finally, other than positive or negative regulation by cytokine secretion, γδ T cells are also implicated in modulating the ensuing immune response through cytolytic mechanisms (76; 77).

1.5 γδ T cells and MS

A growing body of evidence now supports a potentially important role for γδ T cells in the pathogenesis of MS. The strongest evidence comes from immunopathological studies of the MS lesion, where upwards to 30% of the T cell infiltrates express the γδ TcR as opposed to normal circulating levels of 3-7% (46). Furthermore, γδ T cells were found at increased frequencies and activation states in CSF relative to blood (137-139) and from patients with recent onset disease (active) but not established/ quiescent disease (140). Infiltrating γδ T cells were found in chronic active plaques as well as in areas of active demyelination and co-localised around one of the putative MS targets, the myelin forming oligodendrocytes (46; 141). Studies identifying the over expression of heat shock proteins in acute lesions (46; 103) and in particular on the surface of oligodendrocytes (103; 141; 142), together with the preferential expression of HSP on oligodendrocytes relative to other glial cells (143), support the hypothesis that γδ T cell recognition of inflammatory/ virally stressed oligodendrocytes may contribute to autoimmune pathogenesis in MS (120). Alternatively, non-polymorphic molecules such as class Ib, MICA, and CD1 found on nearby astrocytes (144) may themselves function as γδ T cell targets (145; 146) or, together with HSPs, they may participate by presenting yet to be identified myelin derived prenylphosphate, alkylamine, or glycolipid antigens belonging to the recently discovered novel class of γδ T cell reactive ligands. As illustrated in a number of *in vitro* and animal model studies (see below), activated γδ T cells possess all the armament needed to inflict injury on key MS targets such as oligodendrocytes, recruit and control other immune cells, or even to limit immune mediated damage (77; 93; 147).

A number of studies analysing the Vγ and Vδ gene usage of γδ T cells in both MS lesions (148) and CSF (137-140), have identified the presence of a limited γδ T cell repertoire. Although no clear distinction was made regarding a MS CSF dominant VγVδ phenotype, there was a general trend for accumulation of $V\delta1^+$ γδ T cells within CSF vs. blood (138; 140). Interestingly, the predominant γδ T cell subtype found in MS lesions (as opposed to CSF) expressed the Vδ2Vγ9 TcR (148; 149) suggesting CSF/ MS lesion differences, which may be reflective of HSP or non-peptidic Vδ2Vγ9 ligand expression within MS lesions. Additional supporting evidence for γδ T cell involvement in MS comes from the TcR sequences found in this restricted set of CNS γδ T cells. Oligoclonal γδ T cell subsets, as judged by identical junctional sequences, were found in a number of studies and suggest these cells responded to local brain derived antigens (137-139). Battistini and colleagues reached similar conclusions and went on to postulate a Vδ2Dδ3Jδ3 MS brain dominant sequence based on the 100% prevalence of this lineage within brain tissue of nine different MS patients (149), however in follow up studies, this transcript was not as universally found (150). In the majority of studies, common Vγ or Vδ sequences were rare between inter-patient samples lending support for the probable lack of a single "MS" antigen and/or patient specific MS pathogenesis (42).

There is significant *in vitro* evidence to support a role for γδ T cells in MS pathogenesis including the finding that γδ T cells represent the most potent cytotoxic mediator known to date for human oligodendrocytes (151). Furthermore, oligodendrocytes were found to selectively expand γδ T cells (121), possibly in relation to their expression of HSP although, other oligodendrocyte ligands were not ruled out (117). During the course of this work, mechanistic studies pointed to the release of perforin as the primary γδ T cell mediator of oligodendrocyte death (127) which is supported by *in vivo* studies showing both increased levels of perforin mRNA in MS patients (152) and the propensity for lytic as opposed to apoptotic oligodendrocyte death (153). Complementing the perforin death pathway (127), activated γδ T cells also express FasL on their surface (93) and are able to kill Fas expressing oligodendrocytes, although classic DNA fragmentation was not detectable (154). γδ T cells also secrete an array of cytokines such as high levels of TNF-α

which was shown to be injurious to oligodendrocytes *in vitro* (155; 156) and resulted in oligodendrocyte death and demyelination in CNS specific TNF-α transgenic animals (157). Oligodendrocyte death and the resulting demyelination was mediated by the interaction of TNF-α and its receptors found to be selectively expressed on oligodendrocytes (158). Recent studies have emphasised the potential importance of autoantibody production and demyelination in MS (159; 160). γδ T cells possess the necessary helper functions to promote B cell Ig production and may have a predilection for generating autoantibody (135; 136) which may participate in ADCC or complement mediated pathology (161).

Indirect support for γδ T cell participation in MS pathogenesis is also found in studies using the EAE animal model of MS. In γδ T cell depleted mice (162; 163) as well as in TcR $\delta^{-/-}$ transgenic mice (164), the severity of EAE induced by adoptive transfer or active immunisation was significantly reduced as compared to non-depleted or wild type animals. Both groups concluded that γδ T cells functioned by augmenting the proinflammatory cytokine profile of the inflammatory infiltrates and/ or modulating the development or influx of the encephalitogenic immune cells into the CNS.

Despite this fast expanding body of evidence implicating γδ T cells in MS pathogenesis, it is clear that their biological role(s) in the CNS (or in the periphery with CNS implications) is not well elucidated and even controversial. This is clearly illustrated with the EAE studies described above as other investigators using similar strategies, have concluded that γδ T cells either do not play a role in EAE disease pathogenesis (165; 166) or even in their absence, EAE disease is exacerbated (167). These conflicting viewpoints are also representative of the inherent problems of using MS animal models to probe a human disease. This is succinctly illustrated by the finding that TNF-α removal in EAE mice for the most part ameliorates disease, while the same treatment in humans exacerbated MS (168). This points to the need to study and understand human γδ T cells, and in particular γδ T cells found in the target tissue, the CNS.

1.6 Human γδ T cell limitations

Despite intense interest, the study of human γδ T cells as they apply to MS, has been slow to develop, owing in large part to their relatively small numbers found in blood (3-7% of T cells) and even smaller numbers in target tissues such as the brain parenchyma or CSF. This paucity of cells, coupled with the need for patient CNS samples poses the dilemma of how one studies human γδ T cells and their relationship with MS pathogenesis. One strategy that has provided much information, although in a retrospective and static manner, is through immunopathological studies on brain autopsy material (9). Otherwise, most studies to date have relied on limited *ex vivo* studies or the propagation of short term γδ T cell lines (117; 121; 127; 151) or γδ T cell clones (77; 137; 138; 147) from the PB or CSF of patients with MS or other neurological diseases. There are several distinct concerns and/ or limitations with each of these strategies. First and foremost, *ex vivo* studies on CNS materials are limited by the few γδ T cells present within a typical lumbar puncture derived CSF sample. It is estimated that in 10 ml of CSF, between 2-20,000 mononuclear cells are present, representing 200-6000 γδ T cells (10-30%) (169). Most *ex vivo* CSF studies are therefore limited to sensitive PCR type applications (139).

Most detailed studies regarding the functional ability of human γδ T cells stems from the analysis of a few γδ T cell clones derived from peripheral and cord blood, as well as the γδ T cell rich gut epithelium, with relatively few examples with brain derived γδ T clones (137; 138). Furthermore, the γδ T cell clones produced for these studies were in large part selected for by known γδ T cell ligands or putative MS antigens, despite the fact that none have been identified for MS γδ T cells. Although the use of γδ T cell clones has contributed significantly to our understanding of this cell type, they do not necessarily represent their original *in vivo* γδ T cell populations or distributions, and extrapolations made to the disease state may be questionable. Indeed, the properties that make them clonable may already be one indication of an anomalous nature. Furthermore, repetitive mitogen stimulation in conjunction with heterologous accessory cells often leads to loss of antigen specificity and modulation of properties such as cytokine secretion profiles, which further compounds their extrapolation to the *in vivo* setting (170).

Several studies have advanced the γδ T cell knowledge base as it pertains to MS, using short term γδ T cells lines (117; 121; 127; 151). Although there remains a possibility that γδ T cell culture can select for clonal variants, short term culture using γδ T cell specific polyclonal stimulation does afford sufficient numbers of cells for study and is believed to be more reflective of the original population (127). This strategy was employed in the initial phase of this work, with efforts made to extend the lifespan of the short term γδ T cell cultures. It was evident from these studies that generating long term CSF and PB derived γδ T cells was problematic beyond a 3-4 week period, possibly due to their inherent apoptotic nature (171; 172). γδ T cells generated in this fashion provided the means to probe their *in vitro* mechanism of killing towards oligodendrocytes (127) but also illuminated some key limitations of this technique. Due to their limited culture lifespan, only 1-2 single attempt experiments can be performed per patient sample before exhaustion of the cell line, with the unlikely chance of re-sampling CSF from the same patient. Within this context, it is impossible to perform repeat experiments, make result based judgements for further experiments, or to perform phenotype/ function correlation studies with any one cell line. Furthermore, PB and CSF γδ T cell growth kinetics were often at variance, often necessitating experimentation at different time points and γδ T cell purity levels. These factors combined with the inherent variability seen among human samples such as disease state, genetic makeup, and age, limits the comprehensive analysis of this cell type and obscures any potential differences that may be present between PB and CSF γδ T cells from either MS or OND controls. In order to take the study of human γδ T cells to the next level, a consistent source of both PB and CSF γδ T cells which reflects their original *in vivo* distributions and properties is required.

Other methods shown to have some success in prolonging the culture life of human αβ T cells, allowing their comprehensive study, are the construction of T cell hybridomas and immortalisation through viral transformation with human T cell leukemia virus type 1 (HTLV-1). Hybridomas are constrained by the same limitations mentioned above for T cell clones, in addition to the commonly encountered genomic instability resulting from the fusion process (170). HTLV-1 infection of T cells is the principal method of transforming T cells to continual growth

(173-175), however, this technique also suffers from some notable limitations. Besides the fact that immortalised T cells shed infectious HTLV-1 particles (173; 175), and their antigenic specificity and cytotoxic activity are usually lost over a few months with the disappearance of the CD3/ TcR complex (176; 177), the technique is only applicable to CD4 positive T cells (178) and would therefore be inappropriate for the majority of γδ T cells. An alternate and novel approach to generate long lived human PB and CSF γδ T cells and clones is to apply a viral transformation technique using *Herpesvirus saimiri* (HVS), shown to have great success in immortalising αβ T cells (179; 180).

1.7 Strategy: *Herpesvirus saimiri* transformations

HVS is a lymphotropic virus of non-human primates which is non-pathogenic in its native host but leads to fulminant polyclonal T cell lymphomas and leukemias in a number of New World monkeys (181). HVS is able to transform human T cells to continued growth without the need for restimulation, while preserving the essential features of the non-transformed parental cells. At the onset of this work, HVS growth transformation was well documented for human $CD4^+$ and $CD8^+$ αβ T cell lines and clones (179; 182; 183), however, successful accounts of HVS induced γδ T cell transformations were lacking. Subsequently, two groups have reported the successful growth transformation of a limited number of human γδ T cell clones derived from cord and peripheral blood (184-186). Furthermore, due to its oncogenic properties, HVS is classified as a Level III pathogen and appropriate facilities and biosafety standards are required (187).

The transforming capability of HVS is mapped to the left end of the 113 Kb genome, contained within a variable region which serves to delineate the various HVS strains (188; 189). Of the various HVS strains, only those belonging to the subgroup C have been able to transform human T cells. Although the exact mechanism for transformation is not presently known, two virally encoded proteins, Stp-C and Tip-C, are required (190). The presence of viral anti-apoptotic homologs of Bcl-2 and FLICE inhibitory proteins (191; 192) along with the virokine IL-17 (193) and a superantigen-like gene product (194), suggested their possible involvement in T cell

transformations, however the majority of viral genes remain silent in human transformed cells with the exception of the Stp/Tip complex (195).

HVS transformed T cell growth is independent of antigen or mitogen stimulation or the need for accessory cells, and is mediated by their mutual activation via CD2:CD58 interactions, leading to IL-2 dependent autocrine growth (196). The analysis of *in vitro* human transformed T cells has revealed they carry multiple non-integrated circular episomes at high copy number (186) which is stable for extended periods of time. In contrast to HTLV-1 viral transformations, at no stage do HVS transformed human T cells produce virion particles, and no reports of HVS infected cells reverting to a productive state have been cited (195). Permanently growing T cell lines or clones (> than 2 yr.) have been obtained from primary or cultured cells of different purity and origins. Reports of successful HVS transformation of αβ T cells derived from adult peripheral blood, cord blood and thymus, synovial membranes, intestinal mucosa, as well as Th1 and Th2 T cells clones have been cited (197). The efficiency of transformation has been reported to be as high as 100% with $CD4^+$ αβ T cells when transformation conditions have been fully optimised (179; 180), but found to be far less efficient with $CD8^+$ T cells, though it remains a viable option (183; 198; 199). Comparative studies between transformed αβ T cells and their parental cells demonstrated that the immortalisation process conserves the phenotypic and functional properties of the parental cells. In all cases, the transformed cells continued to express a stable functionally intact TcR (200; 201) and retained their antigen specific and MHC-restricted proliferation (201-203), cytokine secretion (200; 202), cytotoxic potential via cytotoxic granules (203) or FasL (192), remained sensitive to activation induced apoptosis (192; 204), and could support B cell activation (205; 206). HVS transformed T cells have found applications in the study of signal transduction (185; 201) and apoptosis pathways (191; 207), HIV pathogenesis (198; 208; 209), and in the elucidation of novel cytokine/ receptor complexes (185). HVS transformation is now currently being applied to the study of MS by the establishment of transformed human myelin reactive T cells. These transformed autoreactive cells are the subject of novel therapeutic strategies such as altered peptides, immunomodulatory agents, and T cell vaccination strategies (197).

1.8 Hypothesis

There is ample evidence that the immune system plays a major role in the pathogenesis of MS, whether triggered by environmental agents such as viruses, the aberrant expression of autoreactive lymphocytes, or in all likelihood by a combination of both. In the process of unravelling the complex pathogenesis of the disease, the γδ T cell subset has been identified as a potential key player based on a growing appreciation of their unique properties which differentiates them from other immune cells. With an intrinsic ability to recognise danger signals, γδ T cells are well positioned to initiate, shape, and regulate an immune response.

The working hypothesis of this study is that, by virtue of this unique position spanning both the innate and adaptive immune systems, γδ T cells play a crucial role in initiating, perpetuating, and regulating the immune response against the oligodendrocyte/-myelin unit, thus contributing to the pathogenesis of MS. Specifically, it is predicted that oligodendroglial danger signals such as elevated heat shock protein expression, lack of MHC class I molecules, or the aberrant expression of non-peptide antigens invokes early γδ T cell responses that result in ODC injury. The injury can be a direct result of the cytotoxic potential of γδ T cells or occur secondary to the release of proinflammatory cytokines or chemokines from either the γδ T cells, or via γδ T cell activated resident glial cells. ODC injury could further liberate myelin autoantigens, which would then be available for presentation and interaction with αβ T cells.

1.9 Specific aims

I. To expand and purify γδ T cells derived from the CSF and PB of MS and OND patients and to investigate methods to extend their cell culture lifespan.

II. To immortalise expanded CSF and PB γδ T cells from MS and OND patients by viral transformation with HVS, establishing lines and subsequent clones for further study.

III. To evaluate the immortalisation process by comparing properties of the transformed γδ T cells with their parental non-transformed cell lines.

IV. To extensively characterise the phenotypic and functional properties of the transformed γδ T cell lines and clones and to address any possible phenotype to function correlations.

V. Based on data obtained in the first four aims, to initiate a comparison of the phenotypic and functional differences found between γδ T cells derived from the CSF vs. PB compartments of MS patients.

2 Materials and methods

2.1 Patient Samples

Cerebrospinal fluid (CSF) and peripheral blood (PB) samples were obtained from consenting patients undergoing clinical assessment and diagnostic work-up for nervous system disease (Eastern Ontario Regional MS Clinic). Subsequently, patients were diagnosed with clinically definite MS (Poser criteria- (210)) or other neurological diseases (OND) comprising viral meningitis, benign intracranial hypertension, brain stem tumour, cerebrovascular disease, anxiety disorder, chronic fatigue syndrome, diabetes mellitus, sytemic lupus erythematosus, and Leber's optic neuritis. In some cases, PB samples were obtained from hospital personnel and used as normal controls.

PB and CSF used in comparison studies with HVS transformed γδ T cells, were obtained from a female patient (32 yr) with a two year history of symptoms and subsequently diagnosed with clinically definite relapsing- remitting MS. The patient exhibited a positive MRI, specific oligoclonal banding, elevated IgG index, and was at an EDSS of 1.5 at the time of sampling.

2.2 Materials and reagents

2.2.1 Tissue culture

All materials and reagents, unless otherwise indicated, were obtained from Canadian Life Technologies (Burlington, On). Specific lots of fetal calf serum were heat inactivated (45 min/ 56°C) and screened for supported γδ T cell growth. Complete RPMI-1640 (cRPMI) consisted of RPMI-1640 supplemented with 10% FCS, 2 mM glutamine (Gln), 100 U/ml penicillin G, and 100 ug/ml streptomycin (P/S); complete R/A medium (cR/A) consisted of a mixture of RPMI-1640, AIM-V medium and FCS at a ratio of 45:45:10 respectively supplemented with Gln and P/S as above. Recombinant human interleukin-2 (IL-2) were generous gifts of Chiron Corp. (Emeryville, CA) (Proleukin) and Hoffmann- La Roche (Nutley, NJ) (Ro 23-6019) and were used as low and high activity reagents respectively; recombinant human IL-4 was generously provided by

Schering- Plough (Kennelworth, NJ). Anti-human γδ TcR stimulating monoclonal antibody used in γδ T cell growth was provided to us in ascites form by M.B. Brenner (Harvard University, Boston, MA) (MB γδ TcR mAb) and titrated for optimal γδ T cell growth. Alternatively, commercial γδ TcR mAbs (11F2, Camfolio- BD; TcRδ-1, T Cell Sciences) were employed. Phorbol myristate acetate (PMA), calcium ionophore A23187 (Ca^{2+} ionophore), monensin, brefeldin A, and phytohemagglutinin (PHA) were all obtained from Sigma (St. Louis, MO). Where appropriate, reagents were titrated on a lot by lot basis for their desired effect (i.e. optimal γδ T cell stimulation indices). Trypan blue dye exclusion (0.4% in saline) and microscopic analysis were used to determine cell viability at various stages during cell growth. All plastic formats were of tissue culture (TC) grade and specific types were used consistently throughout this work.

2.2.2 Cell lines and virus

The following table lists the various human cell lines and their sources, used in this work.

Table 1. Cell lines used in this study.

Cell Line	Source	Description
Colo-205	ATCC CCL-222	Colorectal carcinoma
Daudi	MNI (Montreal, QC)	Burkitt's B cell lymphoma
	ATCC CCL-213 (Manassas, VA)	
Jurkat	ATCC TIB-152	T cell leukemia
KG-1 glioma	D.H. Mattson (Rochester, NY) (211)	Oligodendroglioma
KG-1	ATCC CCL-246	Myeloblastic leukemia
OMK	ATCC CRL-1556	Owl monkey kidney cell
TK6	R. Goldstein (Ottawa, ON)	$HPRT^{-/-}$ B cell lymphoma
U937	ATCC CRL-1593.2	Monocytic-like lymphoma

All cell lines were groomed for growth in cRPMI and maintained by weekly splitting of cells (~300K/ml) into fresh media. The adherent cell lines, KG-1 glioma and OMK were first

trypsinized in the following manner: Trypsin/ EDTA (T/E) solution (10 ml, 0.5/ 0.2%) was incubated with PBS rinsed adherent cells at 37°C for 1-2 min, followed by careful removal of the T/E solution. The cells were further incubated (3 min), dislodged by tapping, resuspended in cRPMI, and split accordingly. OMK cells were always split 1:3 based on surface area. Colo-205, which is a partially adherent cell line related to differentiation state, was split by treating supernatant and adherent cells separately, followed by artificially recombining both fractions (300 K cells/ml total) into fresh medium. Cell lines of <26 passages were always used, otherwise new lines were generated from frozen (-180°C) master stocks.

The level III pathogen, *Herpesvirus saimiri* strain C-488 (HVS) was obtained from ATCC (VR-1414) with an approved Health Canada Level III import permit.

2.2.3 Monoclonal antibodies for flow cytometry

A complete listing of the various mAbs used throughout this study, clone information, and source can be found in Appendix I.

2.3 Routine flow cytometry

PB, PB-derived, and CSF-derived cells were routinely stained for relevant surface markers with either fluorochrome conjugated mAbs or non-labelled mAbs combined with secondarily labelled isotype specific mAbs. Routine experiments employed single, double, or triple staining protocols depending on the application, in conjunction with appropriate isotype controls. Briefly, 200-300K cells were suspended in staining buffer (SB; 2% FCS in PBS with 0.1% (w/v) sodium azide) and the cells pre-blocked with non-specific mouse IgG (0.5 μg; 4°C/ 10 min). Cells were then incubated (4°C/ 30min) with optimised amounts of fluorochrome labelled mAbs (generally 0.1-1.5 μg/ 300K cells), washed and suspended in SB for analysis. For HVS infected cells, staining was as above except washings were carried out with PBS, followed by fixation with 2% *p*-formaldehyde in PBS (4°C/ 18 h) to inactivate any virus present. Data acquisition was achieved

using a Coulter EPICS XL flow cytometer (Burlington, ON) with analysis using either the XL System II or WinMDI (Scripps, La Jolla, CA) software packages. Live cell gating was achieved by excluding propidium iodide positive materials (PI; 10% (v/v) final using a 0.1 mg/ml solution; Sigma), while forward scatter- side scatter (FS/ SS) strategies were employed with fixed cells. Typically, 10K events within the gates of interest were acquired.

2.4 Expansion of short term γδ T cell lines from PB and CSF

Twenty four well and 96-well flat bottom plates (Costar; Fisher Scientific, Ottawa, On) were coated with a solution of sheep anti-mouse IgG1 (The Binding Site, Birmingham, Eng.) in PBS (5 μg/ml) and left at 37°C for 30 min. After PBS washing (2x's), residual free plastic was blocked with cRPMI (10 min). Monoclonal antibody MB γδ TcR was added to the wells such that the final dilution of mAb represented a 1/ 500,000 dilution from ascites fluid. The mAb coated wells were incubated at 37°C for periods up to 2 h, during which time patient cell samples were obtained.

MS and non-MS peripheral blood mononuclear cells (PBMCs) were isolated from heparinised venous blood on Ficoll-Hypaque density gradients (Amersham Pharmacia Biotech, Baie d'Urfe, QC) as previously described (127). A small PBMC fraction ($1x10^6$) was irradiated (3000 rads; ^{137}Cs Gamma Cell-40, University of Ottawa) and reserved as autologous feeder cells for CSF γδ T cell expansion. Remaining PBMCs were plated into the γδ TcR mAb coated 24w plates at a concentration of 1-1.5 x 10^6 cells/ml, followed by incubation at 37°C/ 5% CO_2. CSF derived cells were obtained by centrifugation (400g; 4°C) of freshly drawn patient CSF (5-10 ml), followed by resuspension in cRPMI (400 μl) and division into 2-4 γδ TcR mAb coated 96 wells. Freshly irradiated autologous feeder cells (300K/well) were immediately added to the CSF cell suspension followed by incubation (37°C/ 5% CO_2). The following day, 20 U/ml IL-2 (Proleukin) was added to all samples (PB and CSF), and in select instances, 20 U/ml IL-4 was added to CSF wells.

Following a 5 d culture, PB cells were harvested, resuspended in serum free medium (AIM-V) at 1 x 10^6 cells/ml containing 20 U/ml IL-2 and replated in 24w format. CSF wells were refreshed with new medium (cRPMI) containing 20 U/ml IL-2 or IL-2/ IL-4 combination.

PB and CSF derived cells were evaluated on d8 for γδ T cell expansion using flow cytometric techniques. Cells were double stained with mAbs to CD3 (representing total T cells) and to pan γδ TcR (TcR δ1) or pan αβ TcR (WT31) as described (Section 2.3). γδ T cell purity relative to total T cells present was quantified as follows:

$$\% \gamma\delta \text{ T cell} = \frac{\gamma\delta \text{ TcR positive events}}{\text{CD3 positive total events}} \times 100$$

γδ T cell cultures with purities of < 85% were subjected to negative selection through depletion of contaminating αβ T cells by repeated rounds of complement (C') mediated lyses (c.f. Section 2.5.1). Depending upon their numbers and application, purified PB and CSF- derived γδ T cells were immediately used experimentally, frozen for future use, or maintained in culture. Cells maintained in culture were typically restimulated using two strategies: 1) restimulation with high concentrations of IL-2 (50 U/ml) alone; 2) restimulation with optimised amounts of PHA (typically 0.5 μg/ml) and a 2:1 ratio of freshly obtained, irradiated (3000 rads) heterologous feeder cells to T cells. PHA/ feeder stimulated γδ T cell cultures received IL-2 (and IL-4) (20 U/ml) 1 d following PHA treatment. Individual γδ T cell cultures were continuously monitored for purity (flow cytometry) and growth (live/ dead cell counts), with tailored actions administered depending upon the situation (i.e. restimulation, repurification, or debris removal via Ficoll-Hypaque treatment).

2.5 Purification of γδ T cells

2.5.1 Complement mediated lysis of αβ T cells

The concentration of targeting antibody(s) and complement were periodically titrated for optimum efficiency as assessed by flow cytometry. In a typical purification, semi-pure γδ T cell cultures were treated with CD4 (clone S3.5; Caltag, Burlingame, CA) and CD8 (clone 3B5; Caltag) mAbs (1.0 μg each / 3 x 10^6 αβ T cells) in 15 ml conical centrifuge tubes (max. 15 x10^6 total cells/ 0.3 ml/ tube; Corning, Fisher Scientific) for 30 min at 4°C with periodic resuspension. After PBS washing (2x's; 4°C), freshly thawed baby rabbit complement (25μl/ 3 x 10^6 αβ T cells; Cedarlane) was added to the resuspended cells (300 μl total volume) and warmed to 37°C for 30 min with periodic resuspension. Lysed material was removed via a ficoll-hypaque centrifugation, and the effectiveness of the lysis ascertained by vital cell counts and flow cytometry. The procedure was repeated immediately until a satisfactory γδ T cell purity level was achieved (>85%).

2.5.2 Magnetic bead separations

Purification of γδ T cells was also achieved by negative selection using sheep anti-mouse IgG1 conjugated dynabeads (M-450; Dynal Inc., Lake Success, NY) in combination with an αβ TcR mAb (Clone WT31; Becton-Dickinson, San Jose, CA) according to the manufacturer's recommendations. Briefly, washed M-450 beads (40 x 10^6 beads or 3 mg) were incubated with anti- αβ TcR mAb (6 μg mAb; 2 μg mAb/mg bead) with rotation at room temperature (r.t.) in a total volume of 0.15 ml (20 mg bead/ml) for 45 min. After washing, αβ TcR bound beads (25 x 10^6 beads) were added to a mixture of cells (9 x10^6 cells, 50% of which were αβ T cells as determined by flow cytometry) for a final bead to target cell ratio of ~5. After rotation (45 min, r.t.), the beads were separated and gently washed to recover the unbound cells found in the supernatant. The efficiency of the separation was monitored by flow cytometric analysis of supernatant cell fraction.

2.5.3 Fluorescence activated cell sorting

Impure mixtures of γδ T cells were purified both through positive and negative selection techniques using a Coulter EPICS ALTRA cell sorting system, housed in a Level III facility. Cell mixtures were labelled with either fluorescein isothiocyanate (FITC) labelled αβ TcR (negative selection) or γδ TcR (positive selection) mAbs at 4°C, washed, and sorted immediately. Desired cell populations were recovered based on FS/ SS and fluorochrome gating strategies. Cells were collected into fresh cR/A, concentrated, and plated into 96w U- plates (20-50 K/well) at 2x's normal antibiotic concentrations.

2.6 Herpesvirus saimiri propagation

Permissive OMK cells at low passage number were expanded into 175 cm^2 TC flasks (cRPMI) and grown to confluency (2-3 d). At this time, OMK cells were infected with a low multiplicity of infection of HVS (m.o.i. = 0.01) by overlaying the virus in a minimum volume (10 ml). Virus adsorption (2h, 37°C) was followed by replenishment of medium (50 ml). Over the course of 10-14 d, OMK cells were monitored for signs of viral cytopathic effects (CPE) culminating in the complete lysis of the OMK monolayer. Viral supernatants were generated upon centrifugation (400g) of the lysed OMK solution. Early HVS virus lots were aliquoted and frozen (-180°C) serving as future master stocks. HVS stocks were stored at 4°C for periods not exceeding 4 months before new stocks were generated.

2.7 HVS titration

2.7.1 $TCID_{50}$ assay

Serial 10-fold dilutions of HVS stock virus in cRPMI were titred (1.0 ml) in triplicate into 24 well plates containing OMK cells plated 3 d previously. Wells were scored daily for focal rounding

of the OMK cells (early CPE); syncytia formation (moderate CPE); or OMK cell lysis (late CPE) relative to control wells which received no virus. At the end of 14 d, all wells showing any signs of CPE were ranked as HVS positive and the dilution of virus required to infect 50% of the inoculated OMK cells ($TCID_{50}$) was calculated according to the Spearman- Karber method (212):

$$\text{Log TCID50} = \text{Highest log dilution with 100\% CPE} + \frac{1}{2} - \left[\frac{\text{total number of wells with CPE}}{\text{number of wells per dilution}}\right]$$

$$\text{TCID50 ml}^{-1} = \frac{\text{TCID50}}{\text{volume of innoculate (ml)}}$$

2.7.2 Plaque assay

Serial 10-fold dilutions of HVS viral stocks in cRPMI (1.0 ml) were overlaid in triplicate on confluent OMK cells plated 3 d previously in 6 cm Petri plates (Falcon, VWR, London, ON). Incubation (2 h, 37°C) allowing virus adsorption was followed by careful removal of the residual viral supernatant. Methyl cellulose (MC; 2% in cRPMI) was prepared by suspending methyl cellulose (4.0 g; 4000 centipoise, Sigma) in water (100 ml) followed by autoclaving (45 min/ 121°C). To this mixture was added 2x's concentrated cRPMI and the suspension stirred at 4°C until homogeneous. MC (5 ml) was added to each plate and plaques were allowed to develop over the next 14 d (37°C/ 5% CO_2). Plaques were visualised by pipette removal of most of the methyl cellulose, followed by PBS washing (3x; rocking, 1 h), and cell staining with crystal violet (1% in 20:80 EtOH: H_2O; Sigma) for 5 min. Excess stain was removed with water washes, with a final overnight wash with agitation. Non- stained plaques were enumerated and the HVS titre expressed as the number of plaque forming units per ml (pfu/ml) calculated according to:

$$\text{PFU / ml} = \frac{\text{Plaque number}}{\text{Dilution x Volume inoculated}}$$

using plates with at least 25-100 plaques/ plate for statistical purposes.

2.8 Growth transformation of γδ T cells by *Herpesvirus saimiri*

2.8.1 Generation of HVS transformed γδ T cell lines

γδ T cells generated by short term expansion (d8: PB; d12: CSF) with sample purities exceeding 85%, were plated at 1 x 10^6 cells/ ml of cR/A (PB) or cRPMI (CSF) in 24w plates. As actively growing γδ T cells proved optimal for HVS growth transformation, cells were subjected to two concurrent stimulation regimes with simultaneous HVS infection: 1) PHA (0.5 μg/ml) with irradiated freshly obtained heterologous feeders (1 x 10^6 cells/ml) and IL-2 addition the following day (20 U/ml); 2) IL-2 (50 U/ml). All transformation related studies used the high activity IL-2, Ro 23-6019 (Hoffman- La Roche) unless otherwise indicated. Sample wells were infected with 10% (v/v) freshly prepared HVS virus supernatant containing a minimum virus titre of 10^6 pfu/ ml or 10^6 $TCID_{50}$ units/ ml for a period of 7 d. The HVS concentration (10% v/v) was maintained during this period when cells were split for density reasons. Infected cells were harvested, washed and plated in both 96w U-bottom plates (30K/ well) and 24w plates with cR/A (PB) and cRPMI (CSF) containing IL-2 (50 U/ml). During the subsequent weeks, infected cultures underwent bi-weekly media changes (cR/A or cRPMI with 50 U/ml IL-2) with close attention paid to cell maintenance issues such as overcrowding, debris accumulation, and αβ T cell overgrowth. Cells were maintained at densities between 100-400K cells/ 96-U well and 1-2 x 10^6 cells/ 24 well. Accumulated cell debris was periodically removed by ficoll-hypaque centrifugation and contaminating αβ T cells addressed by the methods outlined in Section 2.5. The effect of HVS in promoting γδ T cell growth transformations was only observable once the normal mitotic lifespan of the stimulated γδ T cells was reached (in the order of 30-40 d). Therefore, the criteria used to assess γδ T cell growth transformations were:

a) cells exhibited a blastoid morphology characterised by irregular sizes and shapes.
b) cells exhibited growth rate doubling times of 1-3x's/ week and this was maintained over a minimum period of several months.
c) death of non-infected control cells treated under identical conditions.

Established PB and CSF transformed γδ T cells (*t*-γδ T cells) eventually required only weekly media change with exogenous IL-2 (20 U/ml) addition for continued cell growth.

2.8.2 Generation of HVS transformed γδ T cell clones

HVS *t*-γδ T cell clones were generated through limiting dilution of the parent HVS infected cell line 21 d after simultaneous HVS infection and PHA/ feeder stimulation (Section 2.8.1). Infected γδ T cells were plated into 96w U-bottom plates at concentrations of 50, 10, and 1 cell/well and containing freshly irradiated heterologous feeder cells (10K/well) in either cR/A (PB) or cRPMI (CSF) with 50 U/ml IL-2. Over the subsequent weeks, the plates were scored for growth (microscopically) and individual proliferating wells were selected and split 1:2 into 96 U-bottom wells. After further rounds of splitting, a minimum number (~50K) of each clone was removed and double stained with pan γδ TcR mAb-FITC (clone TcRδ1)/ CD3-PE (clone UCHT-1) and analysed by flow cytometry for clone identification. γδ T cell positive clones were further expanded in 96w U-bottom plates with media renewal twice weekly and transformation assessed using the same criteria as with the parent cell lines (Section 2.8.1).

2.9 Phenotypic characterisation of γδ T cells

Phenotypic characterisations of γδ T cells were performed using a panel of mAbs directed towards relevant cell surface markers and evaluated by flow cytometry. Complete descriptions of the various mAbs used can be found in Appendix I. γδ T cells were subtyped based on their expression of various Vγ and Vδ gene elements using FITC labelled mAbs directed towards Vδ1, Vδ2, Vδ3, Vγ4, and Vγ9 and co-stained with CD3-PE. γδ T cells were characterised for differentiation and cell signalling markers (CD3, CD4, CD8, CD28, CD45RA/RO, CD152, CD154); activation markers (CD25, HLA-DR); adhesion markers (CD2, CD58); apoptosis markers (CD95, CD95L); and the presence of NK markers (CD16, CD56, CD94, p158a,b, NKB1P).

2.10 γδ T cell cytotoxicity studies

2.10.1 ^{51}Cr- Release assay

Target cells (listed in Table 1- Section 2.2.2) were suspended in cRPMI (1 x 10^6 cells/ml) and labelled with 100 μCi of ^{51}Cr (Amersham Pharmacia Biotech) at 37°C for 1 h, followed by washing (PBS x 2). Serial dilutions of freshly ficolled (if necessary) γδ T cells were made in 96w U-bottom plates (100 μl/ well) to give final effector to target (E:T) ratios of 40-1:1 based on the addition of 10K labelled targets (100 μl). Cells were gently pelleted by centrifugation (100g/ 1min) and the plates incubated at 37°C for 5 h. Following incubation, supernatant (100 μl) was transferred to 96w spotwell plates (Canberra Packard, Mississauga, ON), then residual ^{51}Cr released by the addition of 100 μl of a 1% cetrimide (mixed alkyltrimethylammonium bromides; Sigma) solution in water for 30 min. Following transfer of cetrimide treated supernatants (100 μl), the dried spotwell plates were read using a direct count Matrix 9600 β-counter (Canberra-Packard). Percent specific lysis was calculated from the ratios of induced and spontaneous chromium release as follows:

$$\% \text{ Specific lysis} = \left[\frac{\text{Induced (sample)}}{\text{Induced + Residual}} - \frac{\text{Spontaneous (control)}}{\text{Spontaneous + Residual}} \right] X\ 100$$

2.10.2 JAM assay

Target cells (Table 1, Section 2.2.2) in log phase growth were labelled with 2.5 μCi/ml [*methyl*-^{3}H] thymidine (Amersham Pharmacia Biotech) for 18-30 h prior to the assay. Quadruplicate serial dilutions of freshly harvested γδ T cells (75 μl) were prepared in 96w U-bottom plates such that final E:T ratios ranged from 70-0.1:1 based on the addition of 10K targets/ well. Cells were gently pelleted by centrifugation (100g/ 1min) followed by incubation for 4 h at 37°C. Following this incubation period, the 96w plates were harvested onto glass fibre filter mats (Canberra Packard) prior to radiolabel measurement using the Matrix 9600 β-counter. An additional deactivating step was performed on HVS infected cells prior to harvesting and

counting. Following the 4 h incubation, 50 µl/well of a detergent/ chelating solution (0.001% w/v SDS, 0.01% v/v Triton X-100, and 10mM EDTA in Tris HCl pH8) was added and the plates allowed to sit for 30 min followed by storage at -20°C until further analysis. Prior to harvesting and counting, the plates were thawed and surface decontaminated upon exiting the Level III facility. The percent specific lysis was calculated indirectly from the ratio of remaining live targets to targets alone according to:

$$\% \text{ Specific lysis} = \left[\frac{\text{CPM (targets alone) - CPM (experimental)}}{\text{CPM (targets alone)}}\right] X\ 100$$

2.10.3 DiO staining and flow cytometry

Target cells (1 x 10^6/ml) were labelled with 20 µl of a 3 mM solution of DiO (3,3'-dioctadecyloxacarbocyanine perchlorate; Molecular Probes, Eugene, OR) for 1 h at 37°C/ 5% CO_2. Targets were washed (PBS x 2) and then resuspended at 20K cells/ml in cRPMI. Freshly harvested γδ T cells were suspended at 1 x10^6 cells/ml of cRPMI. In a total of 0.5 ml, effectors were serially diluted into individual culture tubes (Falcon, VWR, London, ON) to which were added 10K labelled targets (0.5 ml) thus yielding E:T ratios ranging from 50-0.8:1. Cells were gently pelleted (100g/ 2 min) and incubated (37°C/ 5% CO_2) for 2-4.5 h. Thirty minutes prior to the end of the incubation period, 100 µl of a 0.1 mg/ml solution of PI was added to all tubes. Immediately following the incubation period, the samples were analysed by two parameter flow cytometry with DiO positive cells (live) falling within the FL1 channel and PI positive cells (compromised) within the FL4 channel. Percent specific lysis was calculated by:

$$\% \text{ Specific lysis} = \left[\frac{DiO^+ PI^+}{DiO^+ PI^+ + DiO^+ PI^-}\right] X\ 100$$

2.11 Intracytoplasmic staining

Prior to performing intracytoplasmic cytokine (IC) staining experiments, *t*-γδ T cells were first forced into a quiescent stage by resting the cells in 96w U-bottom plates (50K cells/well) for

48 h in cR/A lacking IL-2. Cell viability was verified (trypan blue) following harvesting and if necessary, debris was removed by ficoll-hypaque centrifugation. In a typical cytokine screening experiment, rested γδ T cells were set up at 1 x 10^6 cells/ ml in 24w plates, to which all wells received either brefeldin A (2 μg/ml) or monensin (2 μM) as protein transport inhibitors. The γδ T cells were polyclonally stimulated with PMA (10 ng/ml) and Ca^{2+} ionophore (0.2 μg/ml), while spontaneous wells (a measure of cytokine background levels) received medium alone. Following a 5 h incubation (37°C/ 5% CO_2), cells were harvested and divided into lots for IC staining. Relevant surface molecule staining and cell fixation were performed at this point as described (Section 2.3). The labelled cells were then washed (2x's) with permeabilisation buffer (PerB; 0.1% saponin (Sigma) in SB) and redistributed into individual staining tubes. For specificity controls, cells were stained with titred amounts of non-fluorochrome labelled cytokine specific mAbs (typically 1-5 μg/ 400K cells/ 50 μl PerB) at 4°C for 30 min. All samples at this stage were treated in an identical fashion as the specificity controls. Optimised concentrations of fluorochrome labelled cytokine specific mAbs and isotype controls were then added in a further 50 μl PerB (typically 0.1-1 μg/ 400K cells) and reincubated for 30 min (4°C). All samples were then washed (2x's) with PerB followed by SB (1x) and resuspended for flow analysis. Negative gates were set on cell populations blocked with the specific cytokine mAbs and a minimum of 10K events for the population of interest were acquired. Cytokine enumeration was represented in two manners:

1) $$\% \text{ Positive cells} = \frac{\#\ \gamma\delta \text{ T cell events above negative gate}}{\text{total \# of } \gamma\delta \text{ T cell events}} \times 100$$

2) $$\text{MCF unit} = (\%\ \gamma\delta \text{ T cells x MCF of events above negative gate}) + (\%\ \gamma\delta \text{ T cells x MCF of events below negative gate})$$

2.12 Antigen recognition studies

2.12.1 Antigens

Cellular antigens consisted of the various cell lines listed in Table 1 (Section 2.2.2) and sonicated heat killed *Chlamydia pneumoniae* elementary bodies (generous gift of S. Sriram, Vanderbilt University, Nashville, TN). Cellular lysates consisted of freshly prepared, multiple rapid freeze/ thaw cycles (4x's) of know cell concentrations, and aliquots were used directly. Protein antigens consisted of human αB crystallin (generous gift of Hans van Noort, TNO Prevention and Health, Leiden, NL), bovine myelin basic protein (MBP; Sigma), and *C.* pneumoniae recombinant outer membrane protein (S. Sriram). Non-peptidic phosphate antigens included dimethylallyl pyrophosphate and isopentenyl pyrophosphate, while trehalose 6,6' dimycolate represented a CD1 restricted antigen (Sigma). Alkylamine antigens consisted of methyl-, ethyl-, propyl-, isopropyl-, butyl-, isobutyl-, secbutyl-, amyl-, and isoamylamine (Aldrich Chemical Co., Milwaukee, WI and Sigma).

2.12.2 Proliferation

Forty eight hours prior to the assay, *t*-γδ T cells were rested as described in Section 2.11. γδ T cells were plated in quadruplicate at 50K cells/well (75 μl) in 96w U-bottom plates. Antigen at various concentrations, positive controls such as IL-2 (50 U/ml) or PHA/ feeders and negative controls such as irrelevant protein or medium alone were added to the γδ T cells such that the total volume per well was 150 μl. Proliferation assays with γδ T cells were performed on a background with and without exogenous IL-2 (1-5 U/ml). Cells were incubated at 37°C/ 5% CO_2 for 2.5 d and pulsed with 2.5 μCi/ml (25 μl) [*methyl*-^{3}H] thymidine during the last 18 h of the incubation. HVS infected γδ T cells were inactivated with 50 μl of lysing buffer (SDS, Triton, EDTA) for 4 h, followed by cell harvesting onto glass fibre filters and enumeration by direct β-counting. Stimulation indices were calculated based on medium controls.

2.12.3 Cytokine production

Forty eight hours prior to the assay, *t*-γδ T cells were rested as described in Section 2.11. *T*-γδ T cells were plated in 96w flat bottom or 24w plates depending upon the magnitude of the experiment. Typically, γδ T cells were plated in ½ of the final well volume and containing 2x's concentrated brefeldin A (4 μg/ml). The remaining ½ volume was composed of spontaneous control (medium), positive control (PMA (20 ng/ml)/ Ca^{2+} ionophore (0.4 μg/ml)), and antigen dilutions. Cellular antigens such as Daudi or U937 were themselves incubated for 2 h in the presence of brefeldin A (2 μg/ml) prior to the assay. Assays were incubated for 6 h at 37°C/ 5% CO_2 and then worked up and analysed as described in Section 2.11.

2.13 Blocking studies

T-γδ T cell cytotoxicity towards target cell lines was evaluated by JAM assays in the presence blocking mAbs. γδ T effector cells were incubated (1 h/ 37°C) with graded amounts of blocking γδ TcR (TcRδ1, Endogen), CD2 (RPA-2.10, Pharmingen) or irrelevant control IgG mAbs and used without prior washing. Similarly, target cells were blocked (1 h/ 37°C) with graded amounts of HLA class I (W6/32, Sigma), CD58 (TS2/9, Endogen), or irrelevant IgG or CD19 (SJ25-C1, Sigma) mAbs and cytotoxicity was determined in a 4 h JAM assay.

3 Results

3.1 Short term γδ T cells

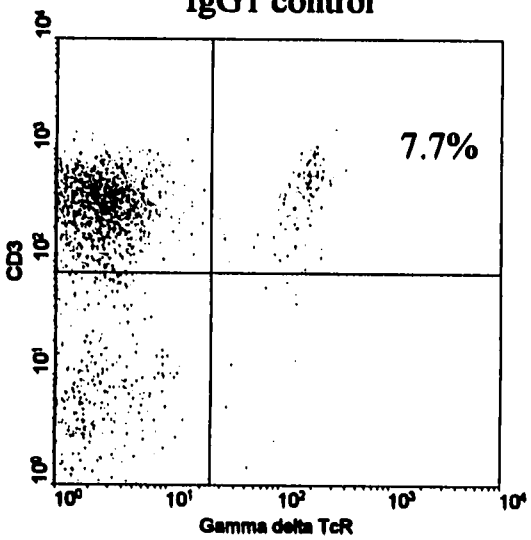
3.1.1 Expansion of human γδ T cells from cerebrospinal fluid and peripheral blood

3.1.1.1 *Ex vivo* stimulation

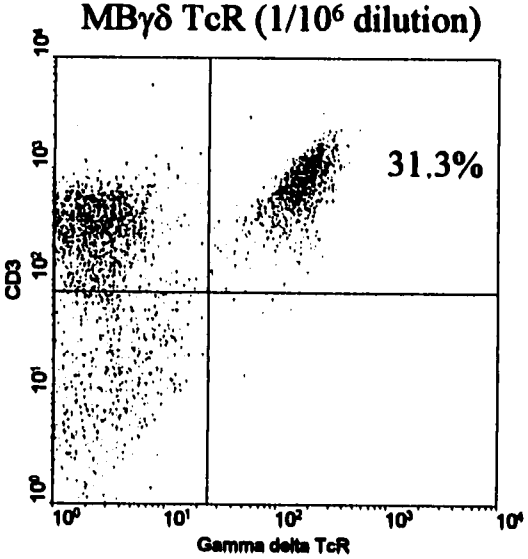
To allow for a complete cross section of CSF and PB derived γδ T cells, it was vital to establish methods that were non-selective. γδ T cells were selectively and polyclonally stimulated from either fresh PBMCs or CSF cells using an immobilised mAb directed to a conserved region of the γδ TcR. Optimal γδ T cell growth was exquisitely sensitive to the source and concentration of the stimulating mAb. Compared to other clones (11F2, Camfolio-BD, TcRδ-1, T Cell Sciences), the most effective stimulating mAb was MB γδ TcR provided to us in ascites form from M.B. Brenner (Harvard University). Figure 1 depicts a titration curve using graded concentrations of MB γδ TcR mAb with γδ T cell growth as the readout. Flow cytometry coupled with cell counts were the most efficient manner to evaluate selective γδ T cell growth since initially small PB γδ T cells numbers (~3-7% of total T cells) hindered assessment by [*methyl*-^{3}H] thymidine incorporation. Optimal mAb stimulation in terms of γδ T cell purity occurred with MB γδ TcR mAb dilutions of 1/200,000- 1/500,000 from ascites (Table 2). Expansion indices indicated that a 1/500,000 dilution of mAb was optimal for maximum γδ T cell numbers. MB γδ TcR concentrations greater than 1/500,000 resulted in poorer expansion indices, primarily as a result of decreased cell viability (i.e. 3.98 vs. 7.5x10^6 cells for 1/200K vs 1/500K dilutions)). As a direct comparison of mAb sources, a second previously optimised γδ TcR mAb (10 ng/ml, 11F2, Camfolio) demonstrated a significantly lower expansion index relative to the 1/500,000 dilution of MB γδ TcR (2.0 vs. 11.1 respectively).

Figure 1. Titration of the stimulating MB γδ TcR monoclonal antibody as assessed by flow cytometry. Freshly isolated PBMCs were stimulated with graded dilutions of immobilised MB γδ TcR mAb ascites fluid as indicated, and γδ T cell expansion assessed by flow cytometry 8 d later. Values shown represent the proportion of γδ T cells relative to total T cells present as described in *Materials and methods*. Non-specific mouse IgG1 served as a nonstimulating control together with an alternate, previously titrated γδ TcR mAb (Camfolio- clone 11F2) as a relative reference agent.

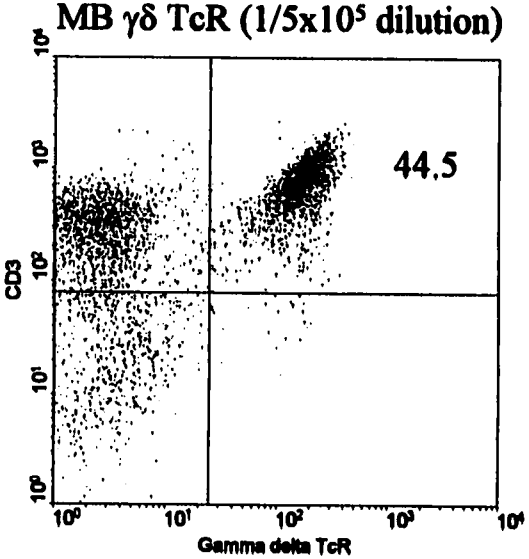
IgG1 control



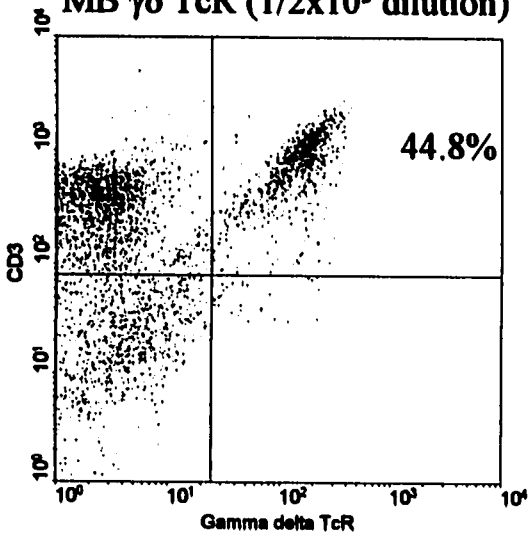
MBγδ TcR (1/10⁶ dilution)



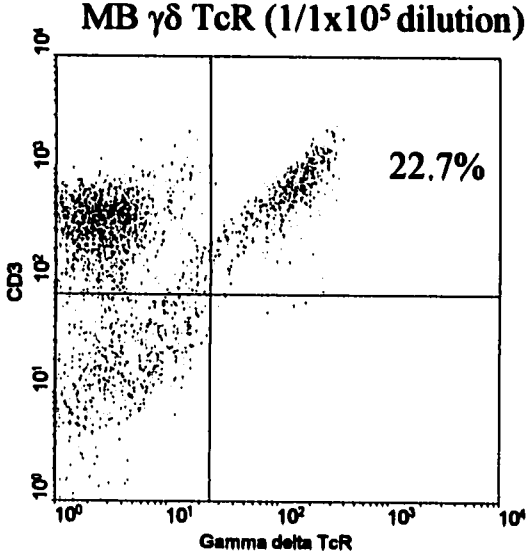
MB γδ TcR (1/5x10⁵ dilution)



MB γδ TcR (1/2x10⁵ dilution)



MB γδ TcR (1/1x10⁵ dilution)



Camfolio 10ng/ml

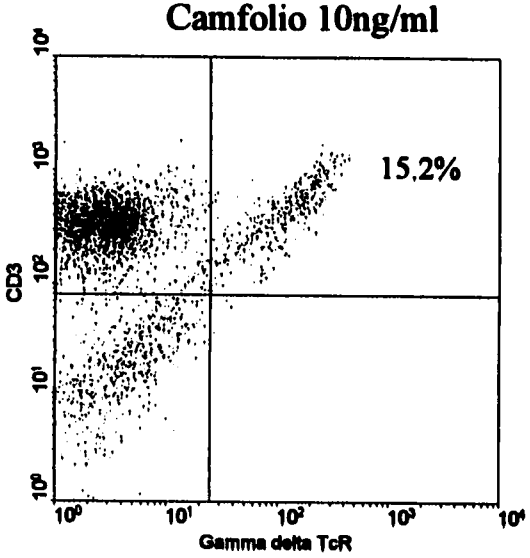


Table 2. Summary of MB γδ TcR mAb titration studies

		mAb dilution					
	Initial	IgG1 (0.5μg/ml)	MB TcR $1x10^{-5}$	MB TcR $2x10^{-5}$	MB TcR $5x10^{-5}$	MB TcR $1x10^{-6}$	Camfolio (10ng/ml)
%γδ T cell	3.0	7.7	22.7	44.8	44.5	31.3	15.2
Cell count ($x10^6$)	10.0	4.90	2.48	3.98	7.50	3.42	3.96
E.I[1]	_	1.26	1.88	6.00	11.1	3.50	2.00

[1] : E.I.= expansion index $\frac{\%\ \gamma\delta\ \text{T cell x cell count}}{\%\ \text{initial}\ \gamma\delta\ \text{T cell x initial cell count}}$

The MB γδ TcR mAb stimulated initial γδ T cell populations in a polyclonal manner (Figure 2). This figure graphically represents the γδ T cell subtype distribution over a cross-section of PB and CSF samples. The PB samples were predominantly Vδ2Vγ9 consistent with the major blood phenotype, but a significant proportion of other Vδ and Vγ subtypes were also detected. The γδ TcR subtype distribution for CSF samples was much more pronounced with significant Vδ1, Vδ3, Vγ4, and Vγ-other contributions.

Overall, these results suggested that optimised anti-γδ TcR stimulation with the mAb, MB γδ TcR, was an efficient manner to stimulate both PB and CSF derived γδ T cells in a polyclonal manner.

3.1.1.2 CSF vs. PB

Expansion and purification of PB or CSF-derived γδ T cells proceeded along two different time lines (Figure 3) due to the relatively small numbers of mononuclear cells found in CSF. Typically, a CSF sample size of ~10 ml contained an estimated 2-20,000 mononuclear cells of which 10-30% (200- 6000 cells) were potentially γδ T cells (169). In contrast, typical PB samples (30 ml) yielded 15-60 x 10^6 PBMCs of which 3-9% were γδ T cells (0.45-5.4 x 10^6 cells). CSF

Figure 2. Cross section of γδ TcR subtypes present after MB γδ TcR stimulation of both blood and cerebrospinal fluid from a series of MS patients. Expanded γδ T cells were phenotyped using Vδ1, Vδ2, Vδ3, Vγ4, and Vγ9 specific mAbs combined with flow cytometry and expressed as a percentage of total γδ T cells present. Vδ-other and Vγ-other represent γδ T cells expressing γ and δ chains not covered by the current VγVδ subtype mAb panel used. ND: not determined for this patient.

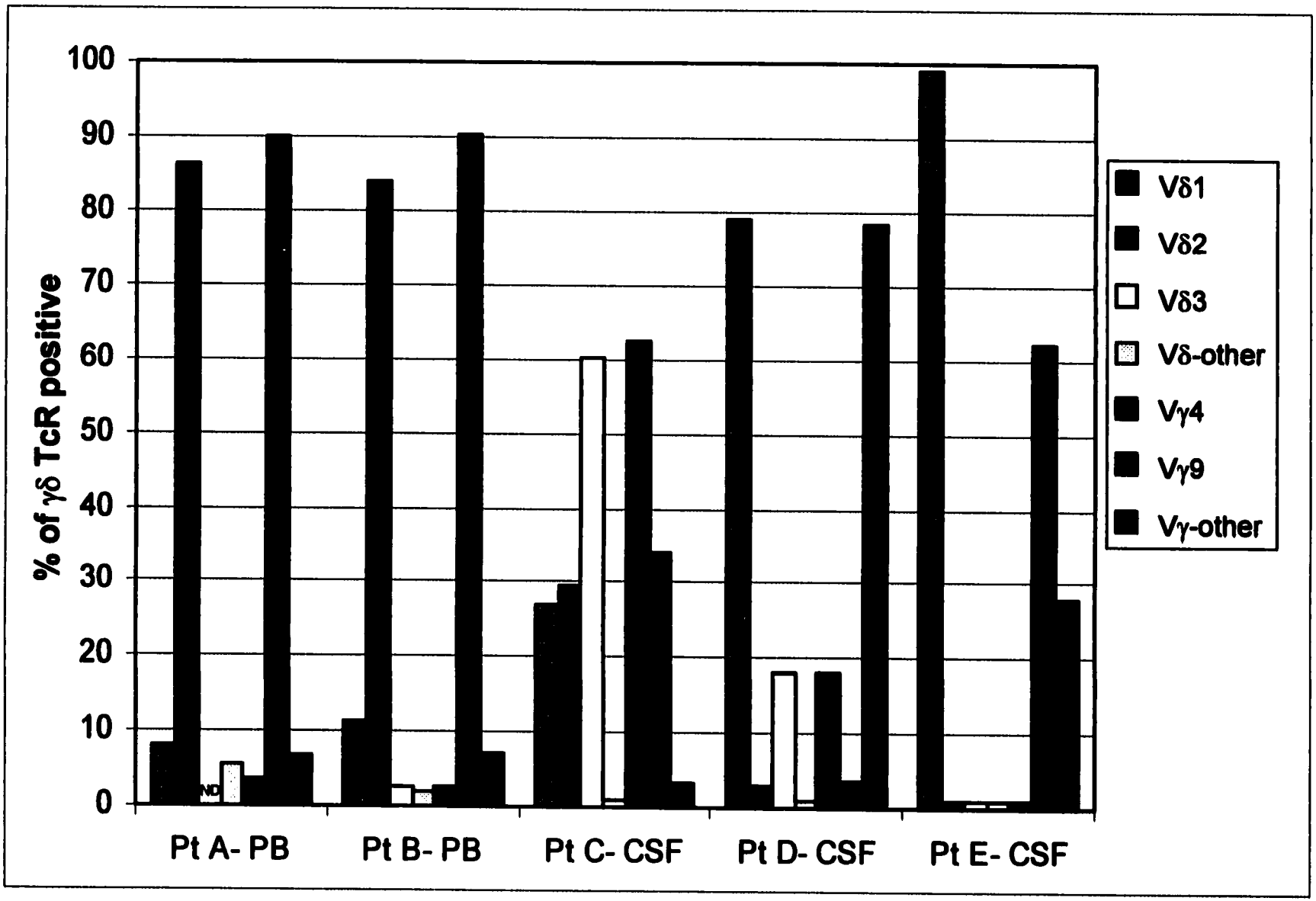
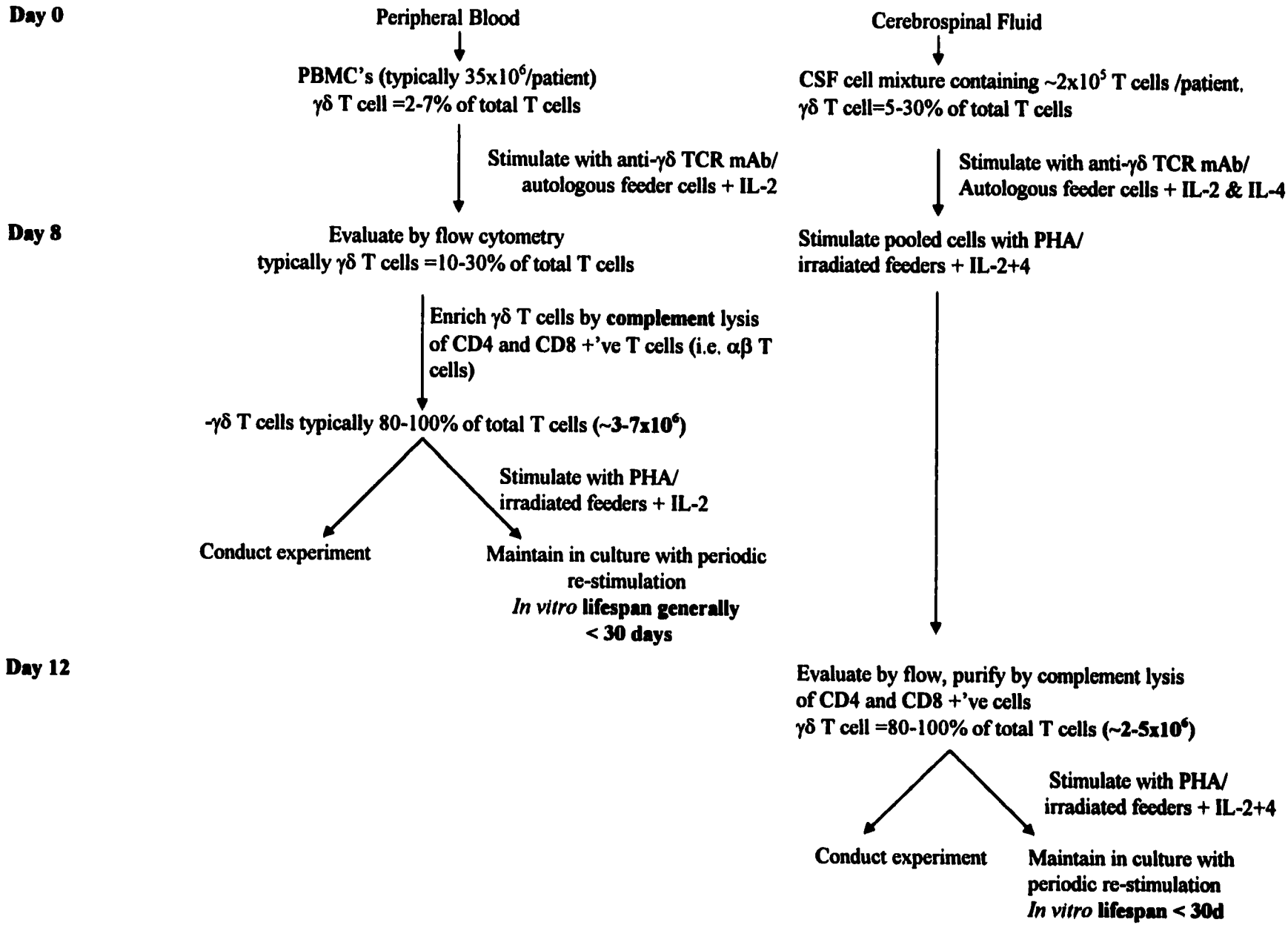

100
90
80
70
60
50
40
30
20
10
0
% of γδ TcR positive
ND
Pt A- PB
Pt B- PB
Pt C- CSF
Pt D- CSF
Pt E- CSF
Vδ1
Vδ2
Vδ3
Vδ-other
Vγ4
Vγ9
Vγ-other

Figure 3. Flow chart depicting PB and CSF γδ T cell expansion and purification. Typical methodology employed to generate short term PB and CSF γδ T cell lines, along with approximate numbers and assessed purities by flow cytometry.

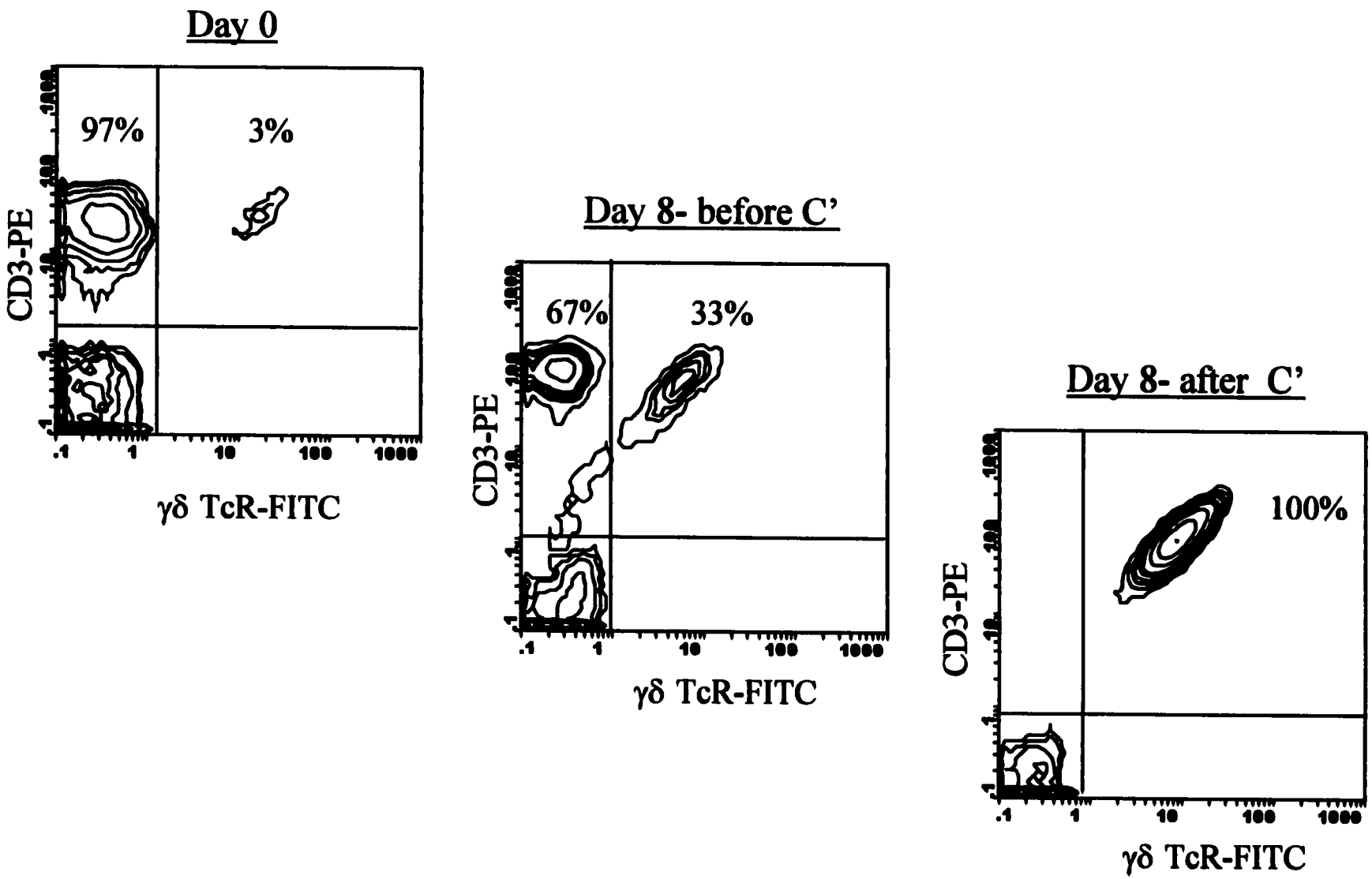

Day 0
Peripheral Blood
PBMC's (typically 35x10^6/patient)
γδ T cell =2-7% of total T cells
Stimulate with anti-γδ TCR mAb/
autologous feeder cells + IL-2
Cerebrospinal Fluid
CSF cell mixture containing ~2x10^5 T cells /patient.
γδ T cell=5-30% of total T cells
Stimulate with anti-γδ TCR mAb/
Autologous feeder cells + IL-2 & IL-4
Day 8
Evaluate by flow cytometry
typically γδ T cells =10-30% of total T cells
Stimulate pooled cells with PHA/
irradiated feeders + IL-2+4
Enrich γδ T cells by complement lysis
of CD4 and CD8 +'ve T cells (i.e. αβ T
cells)
-γδ T cells typically 80-100% of total T cells (~3-7x10^6)
Stimulate with PHA/
irradiated feeders + IL-2
Conduct experiment
Maintain in culture with periodic
re-stimulation
In vitro lifespan generally
< 30 days
Day 12
Evaluate by flow, purify by complement lysis
of CD4 and CD8 +'ve cells
γδ T cell =80-100% of total T cells (~2-5x10^6)
Stimulate with PHA/
irradiated feeders + IL-2+4
Conduct experiment
Maintain in culture with
periodic re-stimulation
In vitro lifespan < 30d

cells were therefore plated into a maximum of 4 x 96U bottom wells containing autologous irradiated PBMCs as a source of feeder cells. Efficiency of PB γδ T cell growth was improved significantly over established conditions (117; 127) by maintaining greater cell densities/ cm^2 of plastic surface using 24w formats. Exogenous IL-2 and IL-4 additions were originally found to be key in supporting efficient CSF γδ T cell growth, however, using conditions fully optimised for γδ T cell growth such as mAb concentration, IL-2 activity, or choice of media, the requirement for exogenous IL-4 has diminished and standard CSF γδ T cell growth relies on exogenous IL-2 only. The timing for growth factor addition was also critical since simultaneous stimulation and IL-2 addition invariably resulted in poor to no γδ T cell growth or death of established γδ T cell lines. PB γδ T cell growth was enhanced by AIM-V medium because contaminating αβ T cells failed to thrive in this medium. Interestingly, CSF γδ T cells also failed to thrive in AIM-V and required traditional cRPMI.

3.1.1.3 γδ T cell purification

γδ T cell cultures were routinely screened by flow cytometry on d8 (PB) and d12 (CSF) using two or three colour fluorescence with mAbs directed to CD3, the γδ TcR, and to the αβ TcR. γδ T cell purity was determined by the ratio of γδ TcR positive cells to total CD3 positive cells (non-γδ TcR + γδ TcR positive cells). Comparison to the percentage of αβ TcR positive cells present provided a secondary means to assess γδ T cell purity. γδ T cells were routinely purified from contaminating αβ T cells via negative selection techniques. αβ T cells were targeted using mAbs to CD4 and CD8 and lysed in the presence of baby rabbit complement (C'). Numerous attempts to target αβ T cells with a panel of αβ TcR specific mAbs were unsuccessful and resulted in the outgrowth of residual αβ T cells. Following C' mediated lysis, samples were reanalysed for γδ T cell purity and subjected to further rounds of C' if purity levels were found to be less than 85%. Figure 4 depicts the flow profile of a PB γδ T cell expansion before and after C' mediated lysis of the contaminating αβ T cells. As can be seen, γδ T cell purities of >90% were achievable with this technique. As with virtually all facets of γδ T cell growth, the efficiency and yield resulting from C' mediated lysis was highly dependent on optimised amounts of targeting

Figure 4. Typical expansion and purification of PB γδ T cells monitored over time. PB cells were doubly stained with γδ TcR and CD3 mAbs and analysed by FACS at three time points: d0- initial, d8- pre-complement mediated purification, and d8- post-complement mediated purification. The proportion of γδ T cells relative to total T cells is indicated in the upper right-hand quadrants.


Day 0
97%
3%
CD3-PE
γδ TcR-FITC
Day 8- before C'
67%
33%
CD3-PE
γδ TcR-FITC
Day 8- after C'
100%
CD3-PE
γδ TcR-FITC

mAbs, the amount of C' per αβ T cell number, and the lot of complement used. This was critical due to the low level expression of CD4 and CD8 on a fraction of γδ T cells, contributing to poorer γδ T cell yields and a potential loss of these subsets. Growth conditions were often such that highly pure fractions of γδ T cells were recovered without the need for C' mediated purification and whenever possible, these cells were chosen for further study.

Taken together, these results showed that starting from relatively small numbers of PB and CSF γδ T cells in complex cell mixtures, γδ T cells can be selectively grown and purified to a high degree. Typically, starting from as little as 200 CSF γδ T cells, > 10 $x10^6$ γδ T cells with purities in excess of 80% were generated over an ensuing 10-14 d culture period, at which point they served as a source for further studies.

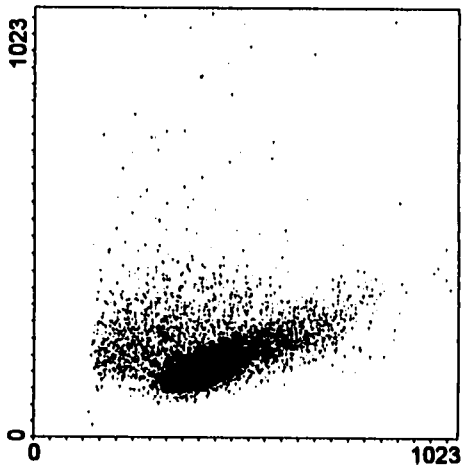
3.1.2 Studies directed towards long term γδ T cell growth

PB and CSF γδ T cells derived as described above were sufficient for initial studies, but a major objective of this work was to generate long lived supplies of MS CSF and PB γδ T cells for further study. To achieve this, studies were conducted to lengthen culture lifespans of the purified γδ T cells. PHA / heterologous irradiated feeder stimulation of γδ T cells yielded mixed results and varied from sample to sample. Both PB and CSF γδ T cells responded favourably to PHA/ feeder stimulation but this treatment often resulted in widespread γδ T cell death in 24-48 h as illustrated by flow cytometry prior to and after stimulation (Figure 5). FS/ SS profiles dramatically changed from a blast pattern (upper left panel) to a granular profile typical of necrotic cells (lower left panel). For these reasons, other stimulation conditions were investigated and are summarised in Table 3.

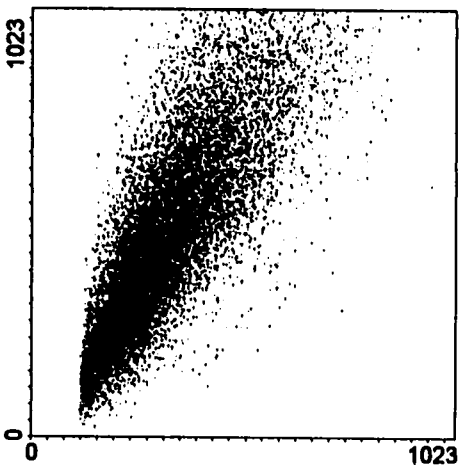
Figure 5. Effect of PHA/ feeder stimulation on pure γδ T cell populations. Day 8 γδ T cell blasts with a purity in excess of 90% (Day 8: panels A and B) were restimulated with PHA/ heterologous feeders and reassessed by FACS analysis 2 d later (Day10: panels A and B). Panel A illustrates the FS/SS profiles prior to and post stimulation and panel B depicts γδ TcR positive cells found in the upper right quadrants.

Panel A

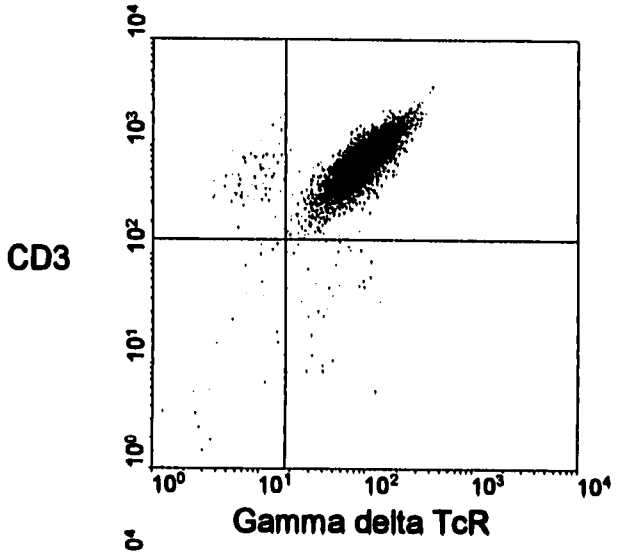
Day 8



Day 10



Panel B



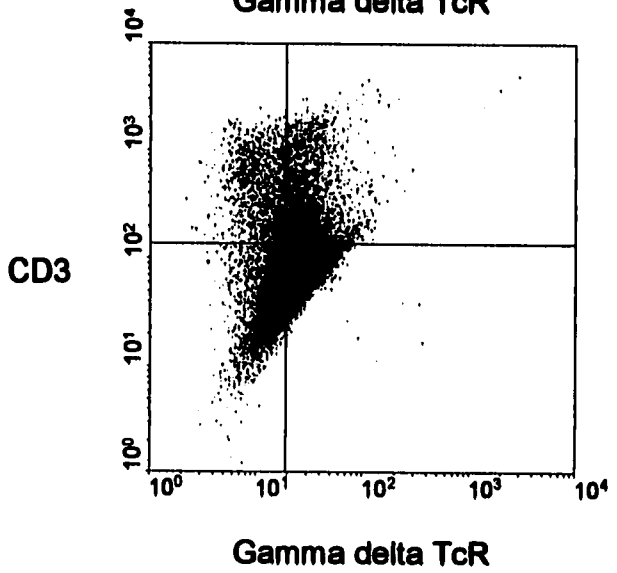


Table 3. Summary of secondary γδ T cell stimulation conditions

PHA/ IL-2	CD3/ CD28
PHA/ heterologous feeders	IL-2 (20 or 50 U/ml) / heterol. feeders
PHA/ TK6 B cell feeders	IL-2 (20 or 50 U/ml)/ TK6 B cell feeders
MB γδ TcR mAb/ heterologous feeders	IL-2 (50 U/ml)
MB γδ TcR mAb/ IL-2	IL-2 (20 U/ml)
MB γδ TcR mAb/ CD28	

Figure 6 compared various stimulation regimes on a d8 PB γδ T cell sample (>85% γδ T cell) as measured by cell expansion indices (E.I.) determined 6d later. From this experiment, PHA/ feeder stimulation was markedly superior to stimulation with the MB γδ TcR mAb in combination with feeders or costimulatory CD28 (E.I. 8.5 vs. 4.8 or 3.3 respectively). The effect of CD3/ CD28 stimulation was relatively low (~2.9x's initial cell numbers) and behaved in an analogous manner to MB γδ TcR/ CD28 stimulation. γδ T cells did not respond to stimulation via TcR in the presence of a high concentration of IL-2.

Alternatively to PHA/ feeder stimulation, PB γδ T cells at the post C' stage often responded well to high concentrations of IL-2 (50 U/ml), although the response was not a protracted one. For example, PHA/ feeder stimulation was compared against IL-2 treatment on highly pure (>80%) d8 PB γδ T cells from four patient samples (Table 4). In all cases, PHA/ feeder treatment resulted in virtually complete cell death 4d post stimulation. IL-2 at 50 U/ml in the same period, resulted in good expansion for 2/4 samples (E.I. 3.1 and 4.5) and marginal expansion with the remaining samples (E.I. 1.4 and 1.2). Significantly, IL-2 selectively stimulated γδ T cell expansion only judging from d14 purity levels (>95%). Despite similar characteristics and purities, this variable response to stimuli was typical for γδ T cell growth in general. Due to their small numbers, CSF γδ T cells were exclusively stimulated on d8-9 with PHA/ feeders cells, and therefore PHA induced death was a constant, unpredictable, and limiting factor for their growth.

Figure 6. The effect of secondary stimulation regimes on expanded γδ T cells. Day 8 γδ T cells (>85% pure) were restimulated with combinations of PHA/ heterologous feeders, γδ TcR mAb, CD3, CD28, and IL-2 as indicated. Six days later, viable cells were enumerated and the expansion indices were calculated as the ratio of d14 viable cells/ d8 viable cells. Data shown represent the mean ± SD of triplicate cultures.

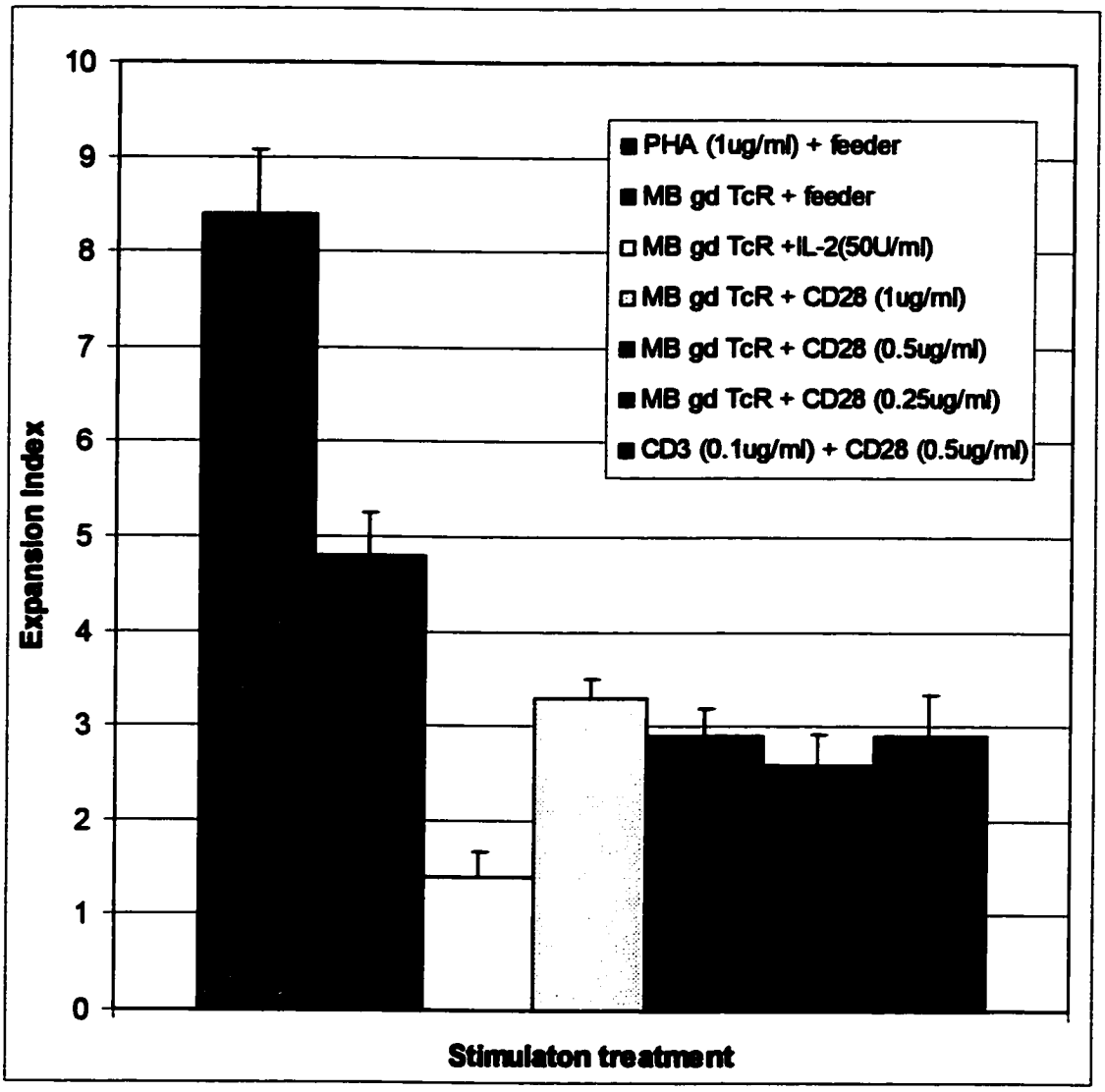

10
9
8
7
6
5
4
3
2
1
0
Expansion Index
PHA (1ug/ml) + feeder
MB gd TcR + feeder
MB gd TcR +IL-2(50U/ml)
MB gd TcR + CD28 (1ug/ml)
MB gd TcR + CD28 (0.5ug/ml)
MB gd TcR + CD28 (0.25ug/ml)
CD3 (0.1ug/ml) + CD28 (0.5ug/ml)
Stimulaton treatment

Table 4. Comparison of PHA/ heterologous feeder stimulation with IL-2 stimulation on four PB γδ T cell patient sample. Four d8 PB γδ T cell samples of high purity (>85% by FACS) were treated with either PHA/ heterologous feeders or IL-2 (50 U/ml) as indicated and viable cells enumerated and assessed by FACS 6d later. [1] E.I: expansion index = % γδ T cells x cell number/ initial % γδ T cells x cell number.

	Day 8			Day 14		
Patient	Cell number	%γδ T cell	Stimulation Condition	Cell number	E.I.[1]	% γδ T cell
TA	$5.5x10^6$	95%	IL-2 (50U/ml)	$16.7x10^6$	3.1	>99%
	$5.5x10^6$		PHA/ feeder + IL-2 (20U/ml) on d9	66K	0.01	
VW	$5.25x10^6$	83%	IL-2 (50U/ml)	$20.0x10^6$	4.5	98%
	$5.25x10^6$		PHA/ feeder + IL-2 (20U/ml) on d9	300K	0.07	
LR	$1.8x10^6$	79%	IL-2 (50U/ml)	$2.12x10^6$	1.4	96%
	$1.8x10^6$		PHA/ feeder + IL-2 (20U/ml) on d9	75K	0.05	
LE	$2x10^6$	75%	IL-2 (50U/ml)	$1.96x10^6$	1.2	95%
	$2x10^6$		PHA/ feeder + IL-2 (20U/ml) on d9	170K	0.11	

Interestingly, a correlation between successful PHA/ feeder stimulation and γδ T cell purity was observed. PHA/ feeder stimulation on samples with significant numbers of contaminating αβ T cells generally promoted good γδ T cell expansion but at the expense of decreasing purity due to stimulated αβ T cell growth. Alternatively, PHA/ feeder stimulation generally led to the rapid and complete cell death of highly pure γδ T cell samples.

The effects of secondary stimulation with PHA or IL-2 often promoted limited γδ T cell cycling, but the effect was not protracted. Repetitive stimulation regimes such as those outlined in Table 5, were attempted to further extend their *in vitro* life. Highly pure samples of γδ T cells (98% on d8), were again refractory to PHA/ feeder stimulation compared to IL-2 (50 U/ml) or IL-2 (20 U/ml) treatments (E.I: 0.92 vs. 10 vs. 3.8 respectively on day 12). Following restimulation as outlined in Table 5, cells originally stimulated with PHA on d8 were moribund and could not be revived regardless of treatment. Cells stimulated on d8 with either 50 or 20 U/ml IL-2 followed by PHA/ feeder stimulation on d12 showed modest expansions of 2.1 and 2.5 respectively. Cells stimulated on d8 with IL-2 and then restimulated on d12 with IL-2 showed modest expansions ranging from 1.2- 2.5. A third round of stimulation was performed on d15 and then evaluated 13d later. At this point, all cells regardless of stimulation condition, contracted in numbers and morphologically were small, unhealthy and moribund. The lone treatment exception (d8: IL-2 (20 U/ml) d12: IL-2 (50 U/ml) d15: IL-2 (50 U/ml)) resulted in a modest expansion (E.I. = 1.3) although morphologically, the cells were characteristically small resting cells.

The results from these long term studies indicated that despite using different methods to maintain the γδ T cells in cell cycle, there existed a finite proliferative stage and consequently cell culture lifespans of only ~20- 40 d. This however, resulted in increased γδ T cell numbers available for study; typically giving rise to 30-80 $x10^6$ pure γδ T cells over this period. Evidence suggested that IL-2 (50 U/ml) stimulation was most effective with highly pure γδ T cell populations, while PHA/ feeder treatment functioned best on semi-pure γδ T cell samples. Under optimal conditions, γδ T cells were induced to cycle for up to ~20-25d followed by a rest/ maintenance phase, then a gradual loss of cell numbers.

Table 5. Effects of repetitive stimulation cycles on γδ T cell growth. γδ T cells stimulated on d0 with MB γδ TcR were restimulated on d8 with either PHA/ feeder and IL-2 at either 50 or 20 U/ml after cell enumeration and FACS analysis. On d12, viable cells were re-enumerated and FACS analysed followed by splitting and restimulation in a similar manner. Further rounds of analysis and restimulation were performed on d15 and d26. [1]E.I: expansion index = (% γδ T cells x cell number) ÷ (initial % γδ T cells x initial cell number); [2] PF: PHA/ heterologous feeders

Day	Stimulation	E.I.[1]	Restimulation
d8	MB γδ TcR (d0)	21.1	PHA/ feeder
			IL-2 (50U/ml)
			IL-2 (20U/ml)
d12	PHA/ feeder (d8)	0.92	IL-2 (50U/ml)
			IL-2 (20U/ml)
	IL-2 (50U/ml) (d8)	10.0	PHA/ feeder
			IL-2 (20U/ml)
			IL-2 (50U/ml)
	IL-2 (20U/ml) (d8)	3.83	PHA/ feeder
			IL-2 (50U/ml)
			IL-2 (20U/ml)
d15	PF[2]/ IL-2 (50U/ml) (d12)	0.29	Not continued
	PF/ IL-2 (20U/ml) (d12)	0.25	Not continued

Day	Stimulation	E.I.	Restimulation
d15	IL-2 (50U/ml)/ PF (d12)	2.1	IL-2 (50U/ml)
	IL-2 (50)/ IL-2 (50) (d12)	1.3	IL-2(50U/ml)
	IL-2 (50)/ IL-2 (20) (d12)	1.2	IL-2(50U/ml)
	IL-2 (20U/ml)/ PF (d12)	2.5	IL-2 (50U/ml)
	IL-2 (20U/ml)/ IL-2 (50U/ml) (d12)	2.5	IL-2 (50U/ml)
	IL-2 (20U/ml)/ IL-2 (20U/ml) (d12)	1.6	IL-2 (50U/ml)
d26	IL-2 (50)/ PF/ IL-2 (50) (d15)	0.28	Small and unhealthy
	IL-2 (50)/ IL-2(50)/ IL-2(50) (d15)	0.41	Small and unhealthy
	IL-2 (50)/ IL-2(20)/ IL-2(50) (d15)	0.36	Small and unhealthy
	IL-2 (20)/ PF/ IL-2 (50U/ml) (d15)	0.45	Small and unhealthy
	IL-2 (20)/ IL-2(50)/ IL-2(50) (d15)	1.3	Small
	IL-2 (20)/ IL-2(20)/ IL-2(50) (d15)	0.18	Small and unhealthy

3.1.3 Distribution of Vδ1 and Vδ2 γδ T cells in PB and CSF

In combination with flow cytometry, specific mAbs directed towards the two major γδ T cell subtypes, Vδ1 and Vδ2, were used to phenotype a series of PB and CSF derived short term γδ T cell lines derived from both MS and OND patients (Figure 7). There was notable variability within each subtype distribution for the samples analysed such that no significant differences were found between CSF vs. PB or MS vs. OND patients. There was however, a trend for increased proportions of Vδ1 γδ T cells within both the PB and CSF compartments relative to OND patients.

3.1.4 γδ T cell cytotoxicity towards various cell lines

γδ T cells derived from the CSF and PB of MS and OND patients were assessed for their cytotoxic potentials towards a series of target cell lines. Using a standard 5 h ^{51}Cr- release assay, the cytotoxicities of d8-d18 PB and CSF γδ T cells towards the HSP expressing B lymphomas (Daudi and RPMI 8226) (121), the monocytic U937, and the oligodendroglioma- like KG-1 were compared and tabulated in Table 6. Comparison of either the PB or CSF compartments from MS patients indicated no significant cytotoxicity differences towards the various cell targets, due in large part to the variability associated with the individual assays. Within the CSF compartment, there was a significant elevation in cytotoxicity potentials of OND vs. MS derived γδ T cells towards Daudi (31.4 vs. 15.5, p= 0.002) and RPMI 8226 (25.0 vs. 9.4, p= 0.025) B cells. Otherwise, γδ T cells generated from either compartment or disease state, showed no killing preference towards the various target cell lines. The exception to this was found in the PB compartment where MS and OND γδ T cells had trends towards lower killing of the oligodendroglioma, KG-1. This trend was not seen within the CSF compartment, where MS and OND γδ T cells possessed KG-1 killing potentials that were on par with the various other cell lines.

Overall, these results indicated that short term PB or CSF derived γδ T cells utilised an efficient means of killing directed towards a series of seemingly unrelated cell targets. That no

Figure 7. γδ T cell subsets in PB and CSF. Proportions of Vδ1 and Vδ2 expressing γδ T cells from the PB (MS: *n* =14; OND: *n* = 8) and CSF (MS: *n* = 9; OND: *n* = 6) of MS and OND patients, as determined by FACS analysis with Vδ1 and Vδ2 specific mAbs. Data shown represent the means ± SEM.

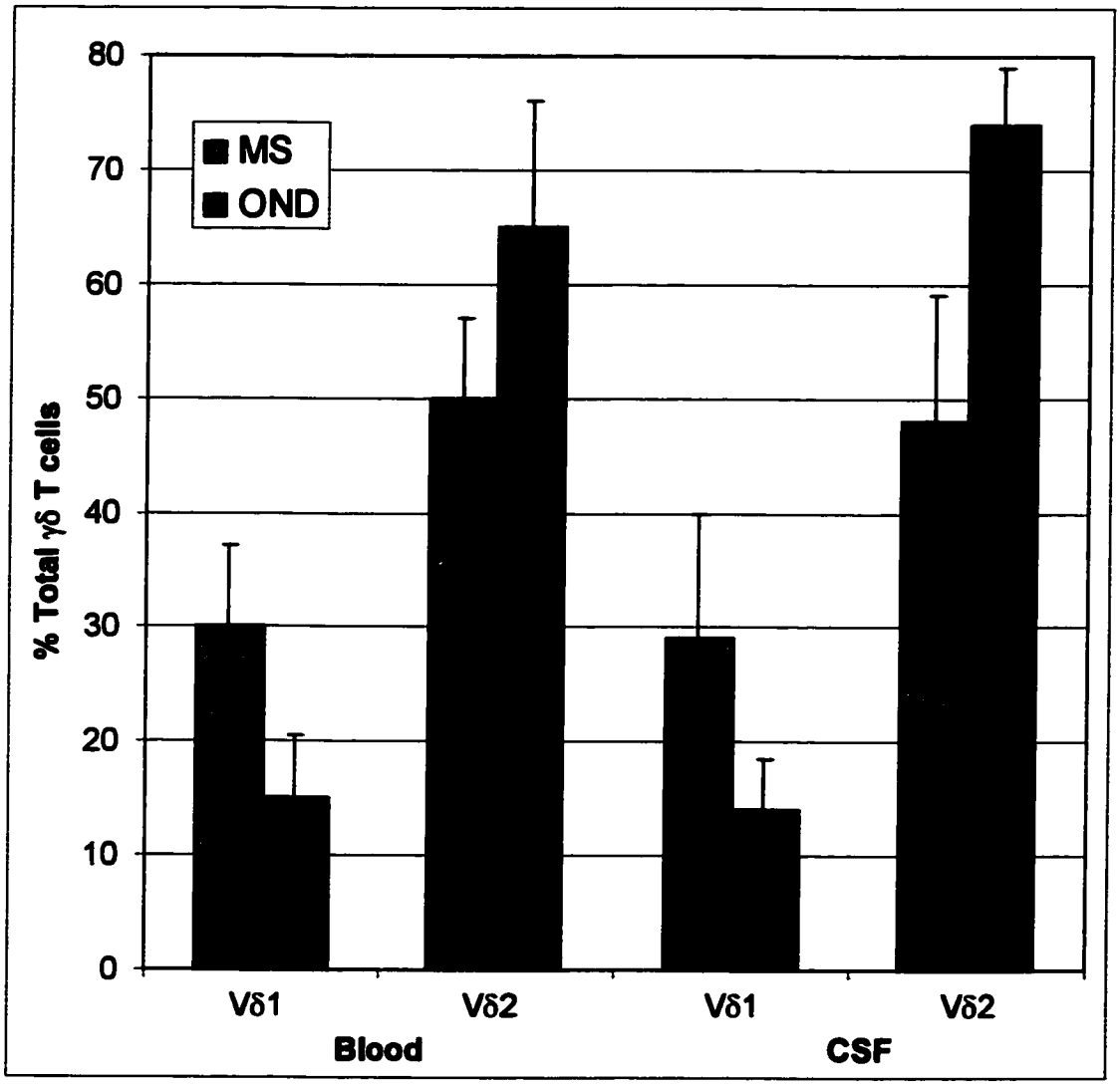

80
70
60
50
40
30
20
10
0
% Total γδ T cells
MS
OND
Vδ1
Vδ2
Vδ1
Vδ2
Blood
CSF

Table 6. Cytotoxicity comparison of PB and CSF derived γδ T cell lines from patients with MS and OND. Cytotoxic potentials of d8-d15 PB and CSF γδ T cell lines from MS and OND patients were evaluated against ^{51}Cr pulsed tumour cell targets in a standard 5 h ^{51}Cr-release assay. Values shown represent mean cytotoxicities at a 20:1 E:T ratio ± SEM. Statistical significance was assessed using an unpaired Student's *t*-test ($p< 0.05$) as indicated. NS = not significant ($p> 0.05$); n = number of individuals.

Target cell	PB					CSF				
	MS	*n*	OND	*n*	*p*	MS	*n*	OND	*n*	*p*
Daudi	21.3 ± 2.4	16	23.4 ± 4.3	12	NS	15.5 ± 3	15	31.4 ± 3.6	11	0.002
RPMI 8226	17.6 ± 2.7	7	19.7 ± 5.9	6	NS	9.4 ± 2.2	5	25.0 ± 5.2	5	0.025
U937	22.7 ± 2.9	16	18.2 ± 4	11	NS	15.6 ± 2.9	13	20.4 ± 3.9	9	NS
KG-1 glioma	13.3 ± 4	12	7.5 ± 1.7	7	NS	15.3 ± 2.7	10	21.1 ± 6.7	6	NS

significant cytotoxicity differences were found between PB and CSF illustrated the difficulty in comparing heterologous γδ T cell populations on a backdrop of human, disease state, and culture variability.

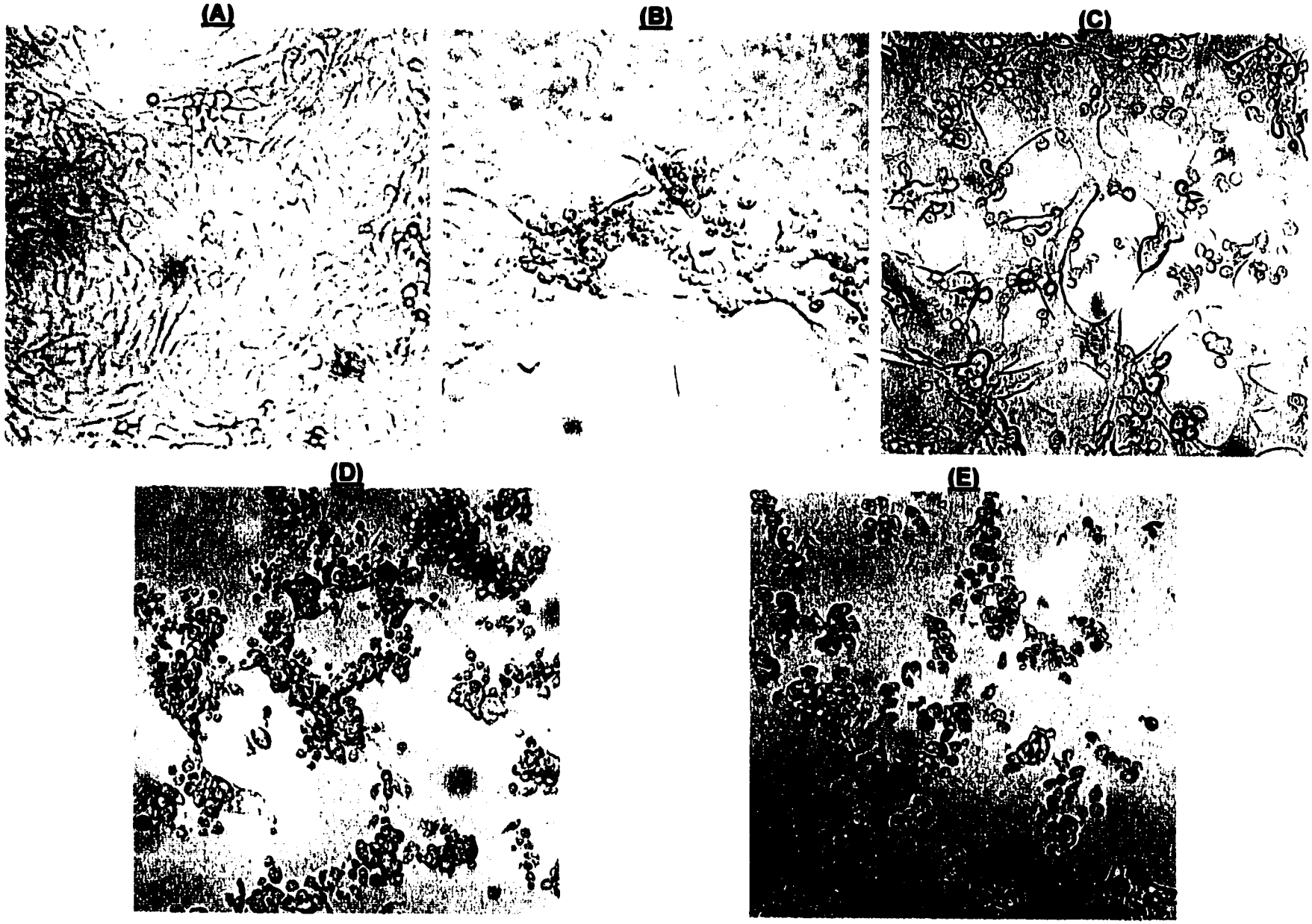
3.2 Long term γδ T cells: HVS growth induced transformation

Studies with short term γδ T cells identified a number of critical limitations that impeded a comprehensive investigation of how these cells may participate in MS pathogenesis. Principal among these limitations was the high degree of variability encountered from patient to patient coupled with the relatively small numbers and short lifespans of expanded γδ T cells. For these reasons, a *de novo* technique to immortalise human γδ T cells using *Herpesvirus saimiri* (HVS) was developed.

3.2.1 HVS growth and titration

Herpesvirus saimiri strain C-488 was selected among the known isolates of HVS due to its efficient ability in transforming human αβ T cells to continuous growth. In order to test this virus strain for growth transforming properties in γδ T cells, the virus sample obtained from ATCC was first expanded into virus master stocks by propagation in permissive owl monkey kidney cells (OMK). Initial HVS stocks were generated by infecting the adherent OMK cells with an unknown multiplicity of infection (m.o.i.) and in subsequent virus lots, by infecting OMK cells at an m.o.i. of 0.01. This latter consideration was important in order to minimise the expansion of HVS variants with altered transforming properties (181). Within 2-3 d of infection, OMK cells (Figure 8-a) morphologically presented with the initial stages of the viral cytopathic effect (CPE) characterised by focal rounding and cluster formation of the adherent cell layer (Figure 8-b). Fusion of infected OMK cells to form cytoplasmic networks (Figure 8-c), followed by their disintegration into cell clusters often seen as giant cells (Figure 8-d), characterised the half way point of the virally induced CPE. At approximately 10-14 d post HVS infection, the OMK monolayer was completely

Figure 8. Cytopathic effect of *Herpesvirus saimiri* on owl monkey kidney cells. Confluent OMK cells (Panel A) were infected with HVS at an m.o.i. = 0.01. At approximately 3-5 d post infection, the earliest signs of viral CPE were evident and characterised by focal rounding and clustering of detached OMK cells (Panel B). CPE progression resulted in fusion of infected OMK cells to form cytoplasmic networks (Panel C), followed by their disintegration into cell clusters often seen as giant cells (Panel D). Complete CPE was observed 10-14 d post infection and was characterised by small granular OMK cells and cellular debris (Panel E). (Original magnification: x100)


(A)
(B)
(C)
(D)
(E)

degenerated leaving behind small, refractile OMK cells and OMK cell debris (Figure 8-e). The resulting virus stock was used as either cell free supernatant or debris ladened stocks with no apparent differences in their associated viral titres (see below). Virus stocks maintained at 4°C for several months, suffered from a gradual loss in titre which became significant *vis à vis* transformation efficiency, when titres dropped to below 10^6 $TCID_{50}$ units/ml.

The virus titres were estimated by limiting dilution on permissive OMK cells using two methods: 1) calculation of the virus dilution capable of infecting 50% of the test OMK wells ($TCID_{50}$ assay); and 2) quantitatively determining the number of plaque forming units/ ml of virus stock using immobilised virus in a plaque forming assay (Figure 9). Comparison of the two assays with different virus stocks (Table 7), consistently yielded virus titres with the same order of magnitude, however $TCID_{50}$ values were more often determined due to the relative simplicity and time saving features of the assay. Studies with αβ T cells have demonstrated the need for high titred HVS for efficient growth transformation and this was achieved by infecting increasing numbers of OMK cells (Table 7). Using confluent OMK cells in 175 cm^2 TC flasks (~15-17 x 10^6 cells) infected in a total volume of 50 ml, HVS titres of >10^6 $TCID_{50}$ /ml were consistently achieved. Virus supernatants with or without OMK cell debris produced equivalent viral titres, indicating no significant contribution of cell associated virus.

In summary, methods were established to consistently produce and characterise high titred HVS virus stocks which provided the foundation for γδ T cell transformation studies.

3.2.2 HVS induced growth transformation of PB and CSF γδ T cells

At the onset of this study, the only reported instances of successful HVS induced growth transformations occurred with αβ T cells, however, a major objective of this work was to be able to apply this technique *de novo* to transform γδ T cells. Subsequently, over 50 transformation experiments have been conducted, establishing methodologies to successfully immortalise human γδ T cells derived from blood and CSF. The range of parameters investigated was extensive due to the lack of precedence in this field and they are summarised in Table 8. These methodological studies have identified a number of critical key elements for the successful

Figure 9. Estimation of *Herpesvirus saimiri* titre using a plaque forming assay. Serial dilutions of HVS virus stock were plated onto confluent OMK cells in Petri plates. After adsorption, OMK cells were overlaid with methylcellulose in RPMI and viral plaques allowed to develop over the course of 14 d. Plaques were visualised by OMK staining with crystal violet and enumerated. The illustration as shown was the result of HVS dilutions ranging from 10^{-3}- 10^{-7} together with a medium control. The virus titre (PFU/ml) = plaque number/ virus dilution x volume of inoculate.

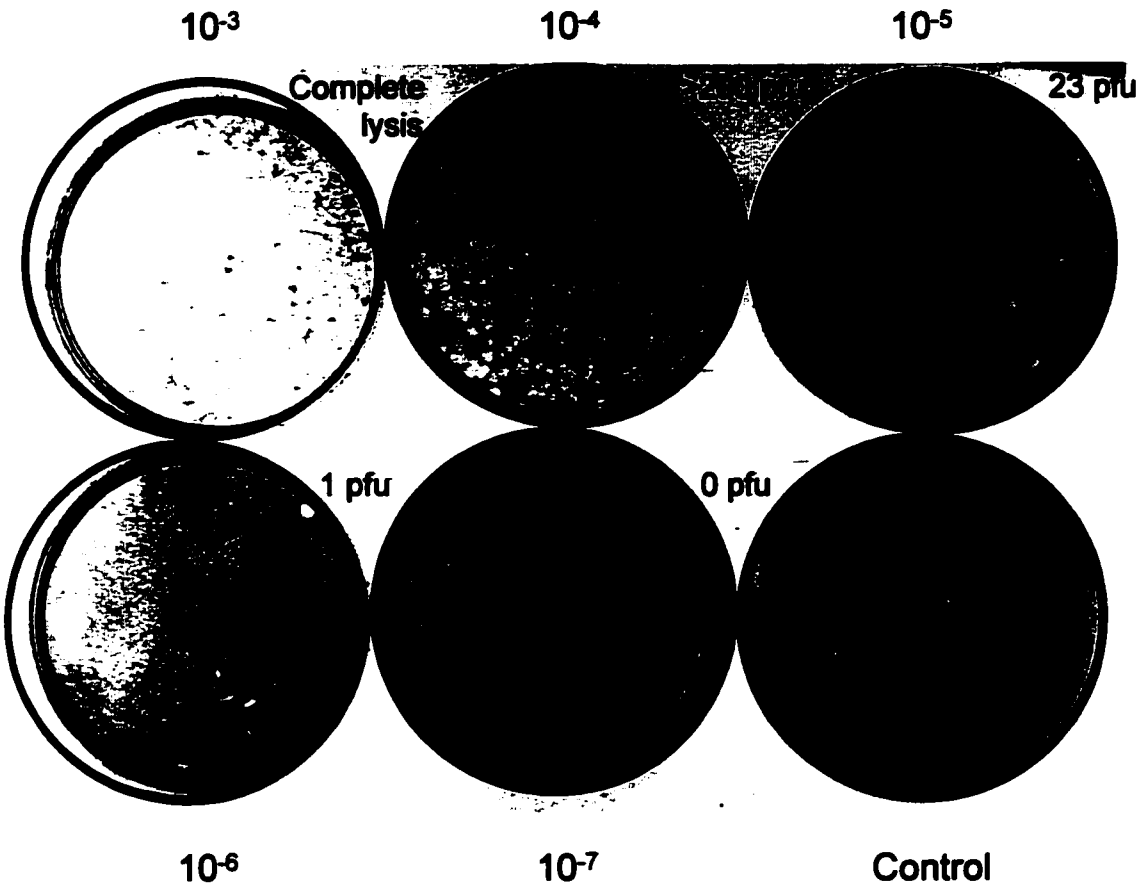

10-3
10-4
10-5
Complete lysis
23 pfu
1 pfu
0 pfu
10-6
10-7
Control

Table 7. Viral titres of HVS stock solutions estimated by $TCID_{50}$ and plaque forming assays. $TCID_{50}$/ ml titres were estimated according to the Spearman- Karber method as described in *Materials and methods*. DF: debris free supernatant; ND: not determined.

Virus stock	$TCID_{50}$/ ml	PFU/ ml
HVS-ST1	6.3×10^3	ND
HVS-ST2	3.2×10^4	ND
HVS-ST3	6.3×10^4	4.7×10^4
HVS-ST4	1.3×10^6	2.6×10^6
HVS-ST4 DF	2.1×10^6	3.4×10^6
HVS-ST5	1.5×10^6	1.0×10^6
HVS-ST6	6.8×10^6	ND
HVS-ST6 DF	1.5×10^7	ND
HVS-ST7 DF	3.3×10^7	ND

Table 8. A summary of the parameters investigated in the development of a γδ T cell immortalisation technique using HVS.

- Culture age of short term γδ T cell lines (d0 to d25 and frozen γδ T cell stocks) prior to HVS infection
- γδ T cell purity at time of infection ranging from ~10- >98%
- γδ T cell densities at time of infection ranging from single cell to 10^7 bulk infection
- HVS multiplicity of infection ranging from 10^2- 10^6 pfu/ ml
- Effect of stimulation regimens prior to, during, or post HVS infection (none, PHA/ feeder, MB γδ TcR, or IL-2)
- Effect of different growth media, supplements, and combinations thereof
- Effect of exogenous growth factors- type, activity, and amount
- Timing of cell culture maintenance regimes such as medium replacement frequency, splitting of cultures, removal of debris, etc.
- Effect of culture plastic format ranging from 96w U-bottom plates to Petri plates to culture tubes
- Optimal γδ T cell densities for protracted and continued growth
- γδ T cell purification strategies on HVS infected cultures
- HVS stabilities at 4°C and –180°C over time
- Permissiveness of CSF vs PB γδ T cells to HVS infection

immortalisation of γδ T cells, the results of which are listed in Table 9. During this period, the majority of the transformation experiments failed to yield the desired immortalised γδ T cells however, the information garnered provided the foundation for techniques which have now established numerous PB and CSF transformed γδ T cell (*t*-γδ T cell) lines and clones (Tables 10 and 11). The culture age of the *t*-γδ T cells range from 3 months to over 2.5 years, far exceeding the maximum 30-40 d periods obtained in long term non-transformed γδ T cell culture studies (Section 3.1.2).

3.2.3 HVS transformation of PB and CSF γδ T cells: transformation conditions

An absolute requirement to successfully generate *t*-γδ T cells was the use of healthy initial γδ T cell populations in log phase growth and of high purity. This invariably meant using γδ T cells that were expanded for short periods (8-14 d) rather than prolonged culture periods where cell morphology and numbers indicated a large proportion of cells were resting and/ or expiring. Contrary to work with αβ T cells (179; 180), it was found that simultaneous PHA/ feeder stimulation and HVS infection provided the most consistent means for transforming γδ T cells, but like short term studies, this result was offset by the individual nature of each γδ T cell patient sample. Transformed γδ T cells were also obtained when HVS infection occurred with initial cell populations that were in active growth due to treatment with IL-2 at elevated concentrations (50 U/ml). A high initial γδ T cell purity level relative to other non-γδ TcR $CD3^+$ populations was critical to the generation of long lived *t*-γδ T cell lines and the efficiency of the technique was indirectly proportional to the numbers of contaminating non-γδ TcR $CD3^+$ cells (primarily αβ T cells) present at the time of infection. This was a direct consequence of an apparent greater susceptibility of αβ T cells to HVS infection and an overwhelming overgrowth of these cells. In an effort to circumvent the selection of possible dominant γδ T cell subpopulations predominating in expanded cultures, attempts were made to directly transform *ex vivo* CSF cells but were never successful. Significantly, *ex vivo* CSF cells that were concurrently stimulated with the MB γδ TcR mAb and infected with HVS were successfully transformed to continuous growth. Taken

Table 9. Summary of the results of HVS infectivity studies on γδ T cells.

- Multiple growth transformed CSF and PB derived γδ T cell lines and clones obtained primarily from MS patient samples were achieved. Transformed cell culture lifespans range from 3 months to > 2.5 years with an associated cell doubling period of 1-3 cycles per week.
- Successful HVS induced transformations critically required healthy, actively growing initial γδ T cell populations.
- Successful HVS induced transformations critically required high titred, fresh stocks of virus.
- Close cell contact was critically essential for HVS induced proliferative growth: 96w U-bottom plates were optimum for maximal growth.
- PB derived γδ T cells required an AIM-V/ RPMI-1640/ FCS (cR/A) mixture (45:45:10) for optimum HVS induced transformation while CSF γδ T cells required initial cRPMI medium followed by transfer to cR/A once cells failed to thrive.
- HVS induced transformation of γδ T cells required high activity recombinant IL-2 at 50 IU/ml as a source of exogenous growth factor.
- The generation of long lived transformed γδ T cell lines was critically dependent on highly pure initial γδ T cell populations and constant removal of contaminating transformed αβ T cells.
- Successful HVS induced transformations required careful and constant cell maintenance during the critical initial infection period.
- HVS induced transformation of γδ T cells was successful with short term PB and CSF expanded cultures as well as with *ex vivo* stimulated CSF cells.

Table 10. Summary of HVS growth transformed γδ T cell lines.

Table 11. Summary of HVS growth transformed γδ T cell clones.

Table 10. HVS growth transformed γδ T cell lines

Sample	Origin	Culture life	Status
RPC-10	PB	158d	αβ/ γδ T cell mix- frozen
RPC-17	PB	113d	Supply exhausted
RPC-19	PB	351d	αβ/ γδ T cell mix- frozen
	CSF	158d	Supply exhausted
RPC-23	PB	71d	Supply exhausted
RPC-27	PB	92d	αβ/ γδ T cell mix- frozen
	CSF	153d	αβ/ γδ T cell mix- frozen
RPC-30	PB	121d	Supply exhausted
RPC-34	PB	122d	αβ/ γδ T cell mix- frozen
RPC-38	PB	380d	Supply exhausted
	CSF	99d	Frozen
RPC-44	PB	150d	αβ/ γδ T cell mix- frozen
RPC-45	PB	175d	αβ/ γδ T cell mix- frozen
	CSF	336d	αβ/ γδ T cell mix- frozen
RPC-47	PB	169d	Active- pure γδ T cells
	CSF	182d	Active- pure γδ T cells
RPC-48	*Ex vivo* CSF	85d	Active- αβ/ γδ T cell mix

Table 11. HVS growth transformed γδ T cell clones

Sample	Clone	Origin	Culture Life	Status
RPC-10	10-3, 10-5, 10-8, 50-2, 50-3	PB	223d- 2.5yr	Frozen
RPC-19	50-21	PB	246d	Supply exhausted
RPC-40	40-3, 40-6, 40-7, 40-10	PB	80- 148d	Supply exhausted
RPC-44	44-3, 44-16, 44-27, 44-40, 44-42	PB	107- 365d	Frozen
RPC-46	46-1 to 46-26	CSF	174d	Frozen
RPC-47	47-1 to 47-31	PB	162d	Active

together, these results suggested that γδ T cell susceptibility to HVS infection/ transformation required the γδ T cell population to be in an activated, proliferative state relative to some degree of contaminating αβ T cells, in order to maximise the likelihood of obtaining long lived *t*-γδ T cells.

γδ T cell transformations were critically dependent on the quality and amount of HVS virus. It was imperative that fresh virus stocks with titres in excess of 1×10^6 $TCID_{50}$ /ml were used as the infecting agent. In terms of the amount of virus used during infection, this represented in excess of 1×10^5 pfu/ ml of γδ T cell culture. From studies evaluating the decay of HVS virus titre over time, it was found that there was a log loss over 2-3 months at 4°C. Frozen stocks of HVS when unthawed, similarly exhibited an approximate 1 log loss in titre. Consequently, loss of 1 log in titre necessitated γδ T cell infection with ~10^4 pfu of HVS/ ml of culture, and at or below this level, all attempts failed to generate transformed cells. There were also similar findings when HVS stocks were generated from other than sub-master virus stocks, presumably because a significant proportion of the virus had diminished capacity to efficiently transform human γδ T cells. Taken together, these results indicated that a minimum threshold of active virus existed and needed to be surpassed in order to effectively transform human γδ T cells to continuous growth.

Post infection studies revealed further key determinants towards generating immortalised γδ T cells with cell density and tissue culture format prominent among these factors. Cells infected and maintained at densities where close contact was not achieved proliferated poorly and eventually succumbed. Many transformation attempts proved conclusively that only 96w U-bottom tissue culture plates provided the appropriate configuration necessary to grow infected γδ T cells. It was found that this format was essential both during the initial phase when normal mitotic events preceded the effects of HVS transformation, as well as when the transformed cells began to proliferate rapidly. When *t*-γδ T cells were plated at initial densities of 20,000 cells per 96 U-bottom well, they rapidly expanded to >200,000 cells/ well. Following harvesting and replating at initial levels, this proliferative cycle afforded large numbers of cells over extended periods. In stark contrast, using other plastic formats such as flat bottom well plates and tissue

culture flasks or tubes, *t*-γδ T cells underwent an initial burst of proliferation which rapidly tailed off to levels where expansion was minimal and cell numbers were maintained over prolonged periods. The morphological differences of these cells relative to those maintained in 96w U plates were striking. Cells were typically small with irregular shapes and generally on a background of cell debris while 96w U bottom derived cells were large, lustrous, and with little debris. Transformed cells exhibiting poor expansion and irregular morphologies could be rescued by replating in 96w U bottom format, re-establishing increased proliferative rates and blast-like morphologies.

Based on strategies used in HVS transformation of αβ T cells (179; 180), different medium formulations were tested to ascertain optimal transformation of γδ T cells. From these studies, it was determined that a mixture of RPMI-1640 and the serum free medium AIM-V with FCS (45:45:10) (cR/A) was optimal and indeed the only medium formulation which produced *t*-γδ T cells. Interestingly, CSF derived γδ T cells performed poorly in cR/A and preferred standard cRPMI, but the effect was transient (~30 d) and eventually required the use of cR/A for long term growth. It was also determined that in the critical initial stages of transformation, cells required frequent medium changes (2x's/ week) and constant monitoring and maintenance otherwise cell viability was terminal. Infected γδ T cells characteristically underwent periods of normal growth, followed by a levelling off and eventual decline of cell numbers before transformation effects were observed and this did not occur before 4-6 weeks. The source and activity of recombinant IL-2 used throughout the transformation process was also a key determining factor. γδ T cell transformations were successful only when high activity rIL-2 (Hoffman-La Roche) was used. Other sources of IL-2 (e.g. Chiron) which was standard in our lab, performed poorly in transformation experiments despite appropriate titration profiles.

3.2.4 HVS transformation of PB and CSF γδ T cells: transformation results

γδ T cells subjected to HVS infection strategies underwent normal mitotic cycling for several weeks however, this alone did not represent a transformed phenotype. HVS transformed cells characteristically exhibit blast morphologies and this represented one of three criteria used

to signal growth transformation. Figure 10 illustrates this feature with a PB derived γδ T cell line at 65 d post infection. Note the numerous irregular shaped cells that varied over a wide spectrum of cell sizes, which is characteristic of activated, blastoid cell types. The second criterion used to assess transformation was an increase in the proliferative rate of the infected cells and maintenance of this rate over several months without further antigen or mitogen stimulation. As an example, the established PB derived *t*-γδ T cell clone 10-10-3, starting from 50 cells expanded to 87 x 10^6 cells in a period of 60 d representing 1 cell cycle every ~ 2.8 d. The sole requirement for growth was an absolute dependence on exogenous IL-2. The final criterion was based on the behaviour of non-infected γδ T cells that were treated in identical manners to their infected counterparts. Typically, non-infected controls behaved as in short term studies (Section 3.1.2), undergoing initial rounds of division (large blasts), followed by a resting phase (small), and culminating in death. This is represented graphically in Figure 11, with 1 x 10^6 PB γδ T cells infected or not infected with HVS on d0. By d43, infected cells had expanded 51.2 x's their original number while non-infected controls only 8.3 x's. These non-infected control cells represented residual cells from the initial clonal burst since they were small and samples contained significant debris relative to the large, debris-free infected cultures. By d53, non-infected cultures had contracted to 1/25 their original number while the infected γδ T cells had expanded to 116 x's their original number.

The efficiency of HVS transformation of γδ T cells was difficult to estimate largely due to the multitude of parameters varied in order to successfully achieve γδ T cell transformation. However, using the most recent transformation experiments, transformation efficiencies have approached a 100% success rate. This figure was based on using several million actively growing, vital γδ T cells (PB or CSF), fresh high titre HVS, and all other optimised parameters discussed above. It was also contingent upon controlling the expansion of contaminating *t*-αβ T cells which represented the major limitation of this technique. αβ T cells were found to be more efficiently transformed by HVS, growth transformation effects occurred earlier, and cell doubling times were shorter relative to γδ T cells. These enhanced characteristics of αβ T cells led to their

Figure 10. Blastoid morphology of HVS growth transformed γδ T cells. Illustrated is an HVS growth transformed PB γδ T cell clone (d125) within a 96-U bottom well, one day following cell splitting. Note the variety of cell sizes and irregular shapes characteristic of blast cells. (Original magnification: x100)

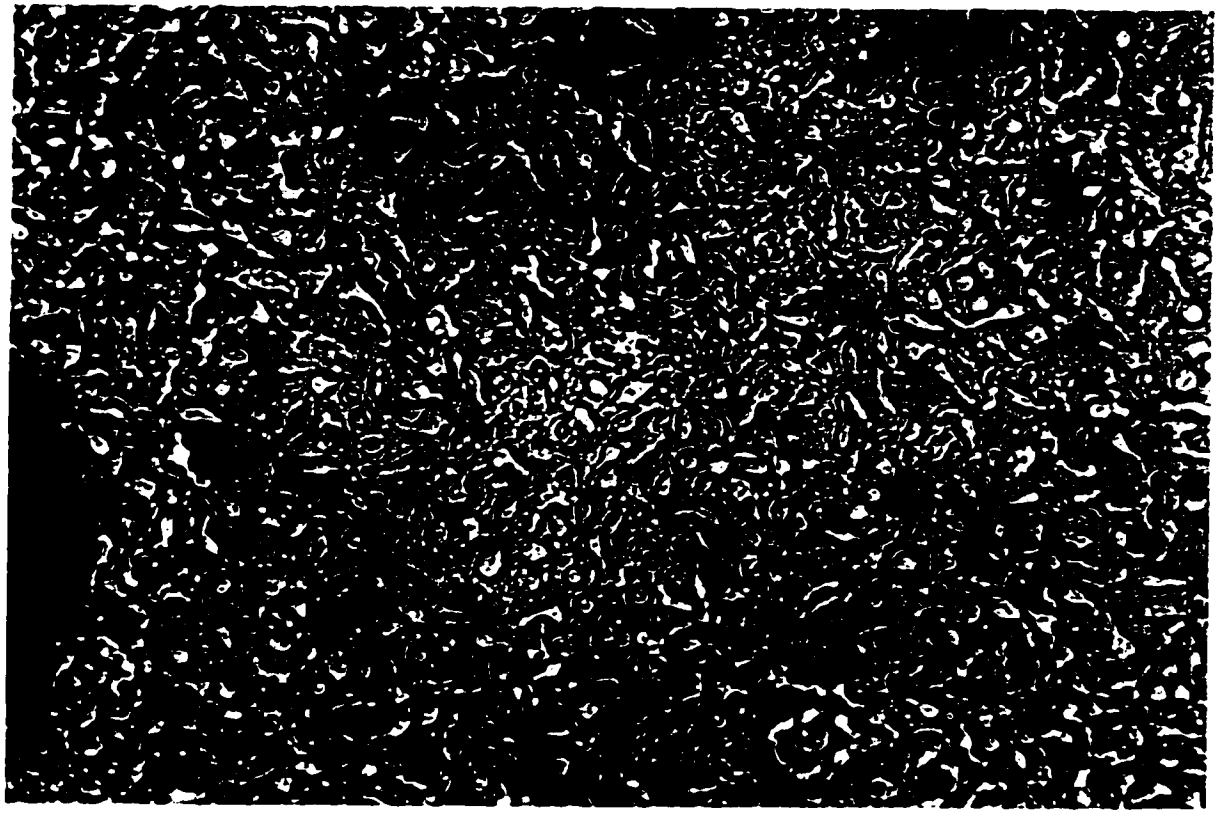
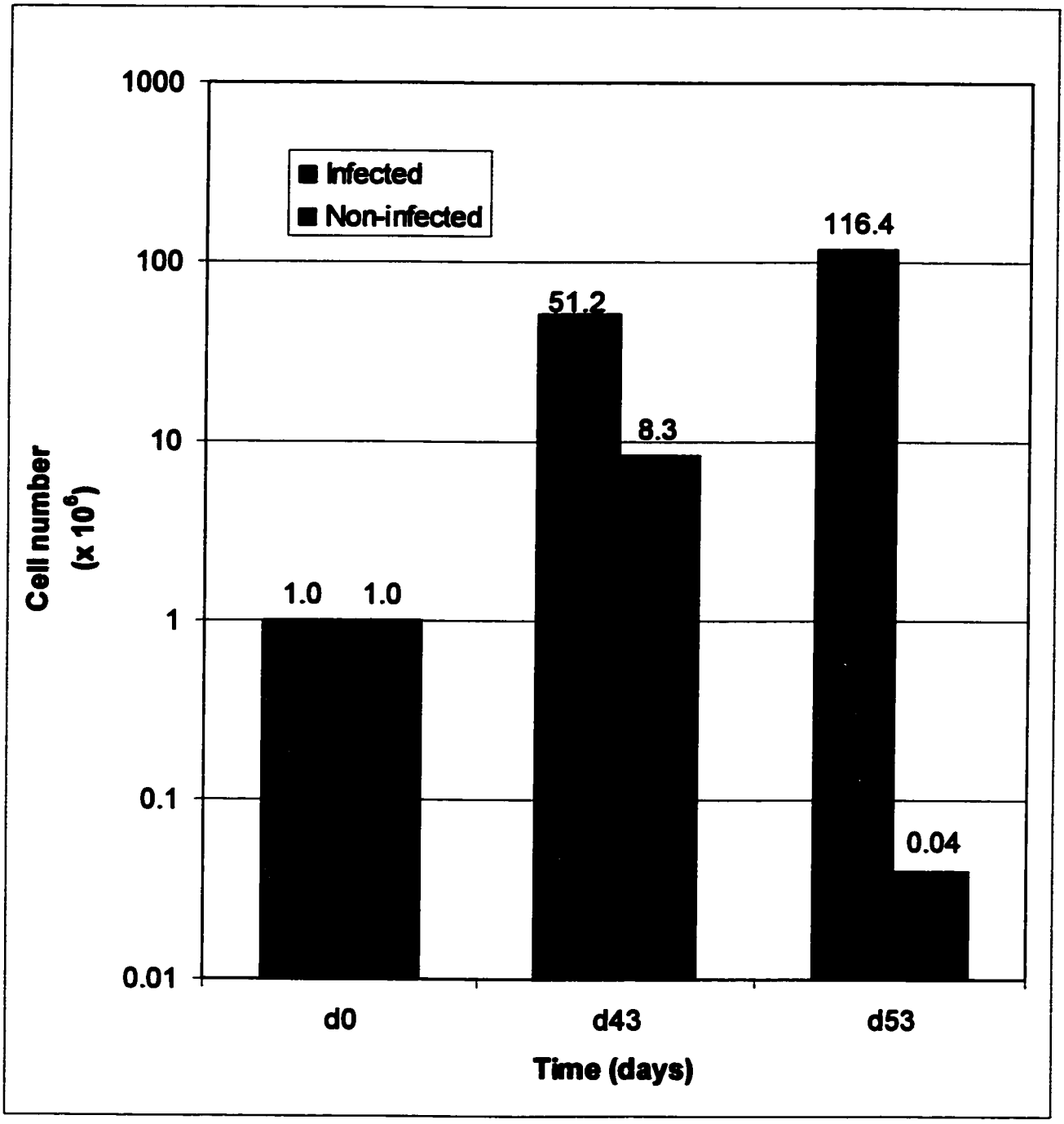


Figure 11. Comparison of the growth profiles of HVS infected γδ T cells vs non-infected controls. Normalised values of PB derived γδ T cells were simultaneously stimulated with PHA/ heterologous feeders and infected with 10^5 PFU HVS or medium controls. Infected and non-infected γδ T cell growth was monitored over time by assessing γδ T cell purity by FACS analysis and enumeration of viable cells. Values above the curve represent the expansion index calculated by: (# γδ T cells x % purity) ÷ (# initial γδ T cells x % purity).


1000
100
10
1
0.1
0.01
Infected
Non-infected
Cell number
(x 10^6)
1.0
1.0
51.2
8.3
116.4
0.04
d0
d43
d53
Time (days)

overgrowth in infected γδ T cells cultures and ultimately led to γδ T cell death if not controlled. To address this issue, a series of negative selection purification strategies were pursued. Magnetic beads together with specific mAb directed to cell surface antigen performed inadequately. Using specific αβ TcR mAbs, purities were never achieved that exceeded 90% which it was found, was not a sufficient level. Compounding the purity issue, residual αβ T cells subjected to multivalent magnetic beads, quickly increased in numbers erasing and decreasing the net benefit of the initial purification. A number of attempts were made to separate *t*-αβ T cells from *t*-γδ T cells using negative selection by fluorescence activated cell sorting. This technique, while yielding higher percentages of purification, suffered from the limitations imposed by Level III pathogens and the technical requirements of the FACS to achieve high purity and yield. In most instances, >95% purities were achieved but at the expense of poor yields. Out of six FACS experiments, no pure *t*-γδ T cell line was obtained. A single positive selection experiment was performed but the results of using γδ TcR mAb as the selecting agent on the purified *t*-γδ T cells was inconclusive and this avenue warrants further investigation. Standard complement mediated lyses suffered from the limitations of the targeting mAbs. Using a series of C' fixing αβ TcR mAbs, similar results were achieved as with the above magnetic bead technique. Using CD4 and CD8 as the targeting mAbs was the most effective manner to negatively select for *t*-γδ T cells, although this was not the preferred manner due to the expression of both of these labels on a small percentage of γδ T cells. In order to maintain *t*-γδ T cell lines for any length of time, cultures had to be maintained in a state of high *t*-γδ T cell purity relative to *t*-αβ T cells and monitored frequently. For example, a 78% pure PB sample was purified by C' to >99.5% but over the course of the following 22 d, the purity fell to 96.4%. Repurification led to the re-establishment of a high level of purity (99.7%) which again subsequently dropped to 89.8% over the ensuing 14d. Clearly as the percentage of the exponentially growing contaminating *t*-αβ T cells increased, the more problematic it became to maintain cultures in a state of high γδ T cell purity. The most efficient manner found to circumvent this behaviour and for generating long term γδ T cell cultures, consisted of cloning out cells from the transformed cell lines.

3.2.5 Transformed γδ T cells clones

A series of *t*-γδ T cell clones were generated by limiting dilution of HVS infected γδ T cell lines at both early and late stages of infection and are summarised in Table 11. The purpose of generating *t*-γδ T cell clones was three-fold: 1) to attempt to correlate individual γδ T cell phenotypes with function such as cytotoxicity and cytokine expression; 2) to provide a source of defined and renewable materials for mechanistic studies; and 3) as an alternative γδ T cell purification strategy alluded to above. All of the requirements outlined in Section 3.2.3 also applied to the generation of *t*-γδ T cell clones. Transformed γδ T cell clones from both PB and CSF were obtained using classical limiting dilution techniques with modifications to the numbers of cells plated per well and the lack of need for further antigen or mitogen stimulation. Initially, 10, 1 and 0.1 γδ T cells per 96-U well containing 10K irradiated heterologous feeders were plated out, however the number of resulting clones were small. Using 50 and 10 cells/ well increased the frequency of clones generated in the short term to approximately 30 and 5 clones per 96w plate respectively. However, if cloning was performed during the initial phase of γδ T cell transformation, many of the resultant clones did not survive, presumably because transformation did not occur. Surviving *t*-γδ T cell clones did not follow Poisson kinetics because in a large proportion of cloned wells, rather than proliferate, they appeared to be non-specifically activated by, and cytolytic towards the feeder cell layer. This was characterised by the disappearance of the feeder cell layer and was clearly distinguished by comparison to feeder cells plated alone. Within these wells, several large cell types emerged with numbers closely resembling the original number of γδ T cells plated (Figure 12). In an attempt to identify these cells, wells displaying these features were combined and analysed by flow cytometry. Forward scatter/ side scatter profiles indicated the cells were large and very granular characteristic of cytotoxic cells (Figure 13). The cells were identified as γδ T cells when co-stained with the pan leukocyte marker CD45. All attempts to stimulate these large effector cells into cell cycle failed and they eventually died. This cytolytic event was minimised, and hence proliferation encouraged, by extending the period between the initial PHA/feeder stimulation and cloning of cells. That the resulting *t*-γδ T cell

Figure 12. Macroscopic cell formation observed during cloning of HVS growth transformed γδ T cell lines. HVS infected PB γδ T cells were plated at 50 cells/ well containing 10K irradiated heterologous feeder cells 7 d post infection. Depicted is the morphological profile of a single 96-U well illustrating the formation of a large, granular cell type (black arrows) concurrent with the degeneration of the feeder cell layer. For reference, characteristic cell appearances for large blast cells (white arrow) and small resting lymphocytes (small arrow) are also illustrated. (Original magnification: x100)

Figure 13. Flow cytometric profile of the macroscopic cell type observed during *t*-γδ T cell cloning. Wells containing the large granular cell type with an associated loss of the feeder cell layer were combined and doubly stained with γδ TcR and CD45 mAbs and analysed by FACS. Panel A depicts the granularity and size profile (SS/ FS) of the macroscopic cells and Panel B identifies the population as γδ TcR and CD45 positive (upper right quadrant).

Figure 12

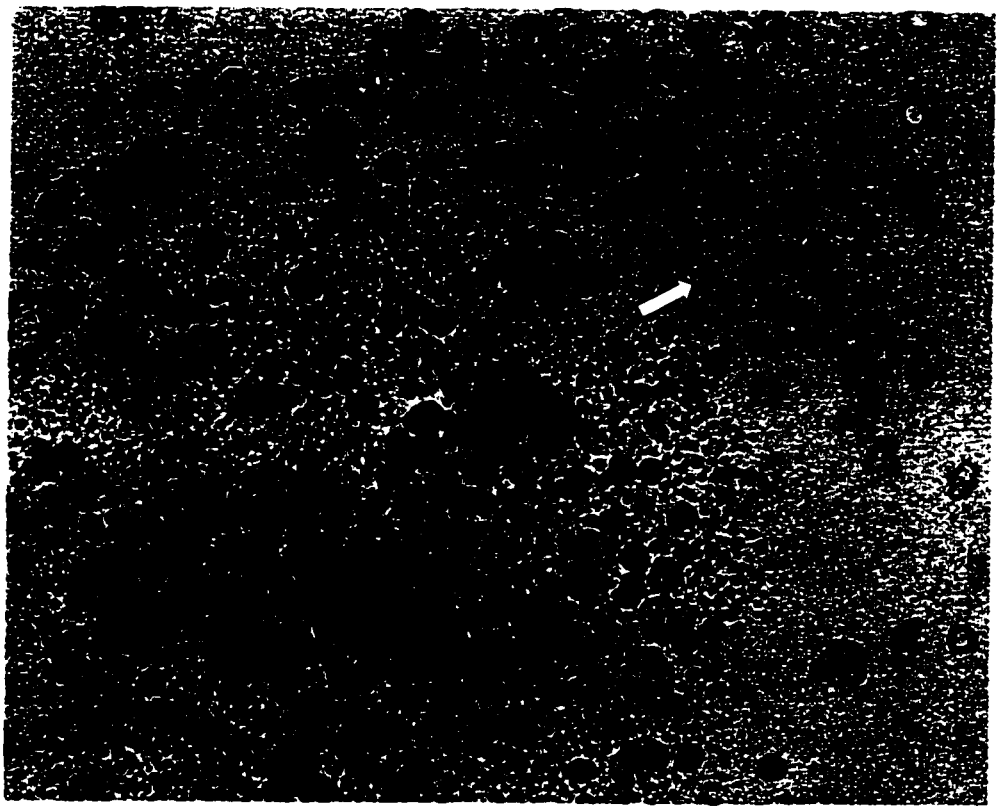
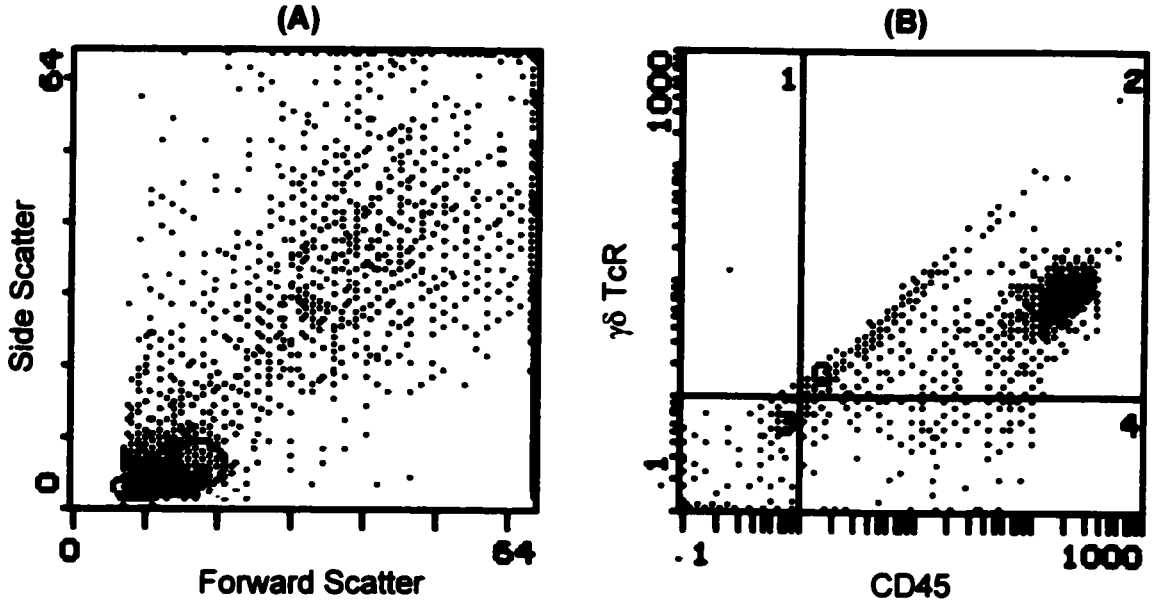


Figure 13



clones were indeed monoclonal was not conclusively established since sub-cloning was difficult for the same reasons. However, microscopic analyses at early junctures in the cloning process revealed the majority of growing wells contained single growing clusters as seen in Figure 14. Furthermore, flow cytometric analysis of γδ T cell receptor subtypes (c.f. Section 3.4.1) revealed the presence of only one subtype, although this does not preclude the possibility that multiple clones utilised the same Vγ and Vδ chain pairing . Of note, the frequency of cloned *t*-αβ T cells vs. *t*-γδ T cells recovered when plated at 50 cells/ well, far exceeded expected numbers based on initial αβ T cell contaminants. This suggested that of the cells plated, a significant proportion of γδ T cells went on to display non-proliferative terminal effector functions thus enriching the *t*-αβ T cell percentages recovered.

In summary, methods were established to successfully transform numerous PB and CSF derived γδ T cells (Table 10) and γδ T cell clones (Table 11) to IL-2 dependent continual growth. These immortalised cells, existing in culture for periods up to 2.5 years, provided a pipeline of γδ T cells to further investigate their potential role(s) in the pathogenesis of MS. However, for these investigations to be meaningful, the question of how HVS induced growth transformations may have affected the phenotypic and/ or functional characteristics of the transformed cells was first addressed.

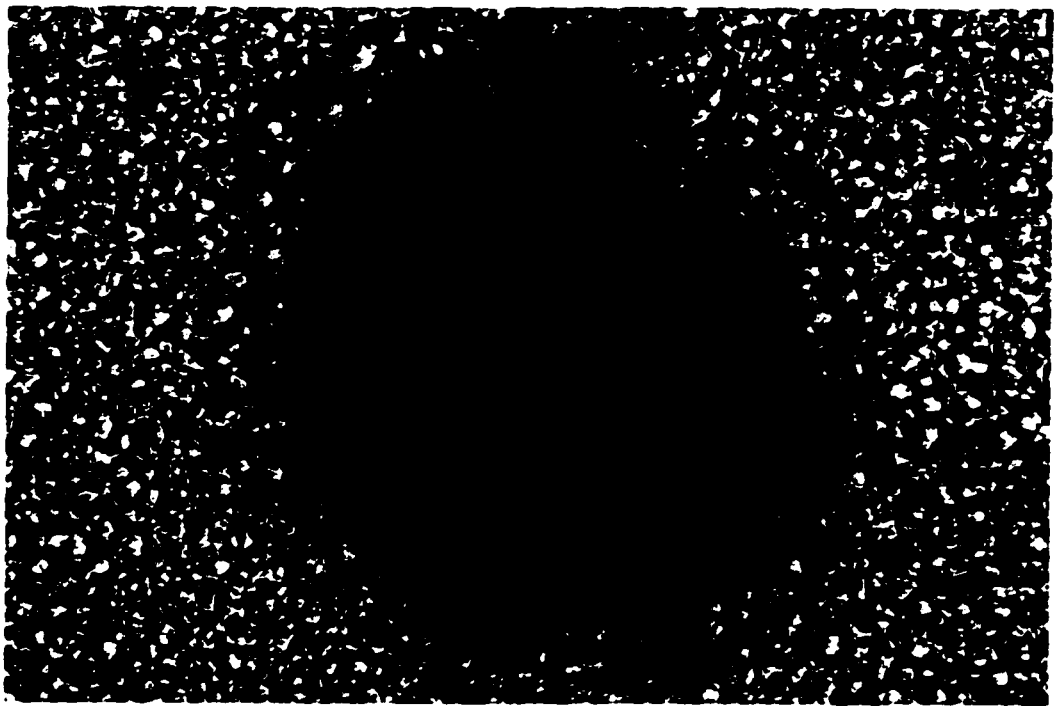
3.3 HVS induced transformation related changes

Ultimately the end goal of this work was to study *t*-γδ T cells, especially CSF derived, in relation to their potential role(s) in MS pathogenesis. Central to this goal was the requirement that *t*-γδ T cells possessed most of the attributes of their parental cultured cells which in turn reflected the *in vivo* situation. To address this issue, a series of PB and CSF *t*-γδ T cell lines and clones were phenotypically and functionally compared to their parent cell lines in order to assess for transformation related differences.

Figure 14. Illustration depicting the presence of single growing *t*-γδ T cell clones. Two illustrations demonstrating the characteristic formation of single clone clusters present within individual 96-U wells when *t*-γδ T cells were initially plated at 50 cells/ well. (Original magnification: x100)

(A)

(B)



3.3.1 Phenotypic comparisons

Transformed γδ T cell lines were phenotypically compared to their parental γδ T cell lines using flow cytometry and a panel of mAbs directed to relevant cell surface molecules. Except for small differences, cell surface molecule expression was highly conserved between HVS infected PB or CSF γδ T cell lines and their parental lines as seen in Table 12 or depicted in Figure 15. γδ T cell subtype distribution (Vδ1:Vδ2) within PB and CSF of the *t*-γδ T cells were relatively consistent with their parental lines, however there appeared to be a small amplification of the Vδ1 subtype within the HVS infected populations.

3.3.2 Intracytoplasmic cytokine comparisons

A) Technique

Due to their suspected role in immunomodulation, it was of great interest to determine the cytokine profiles of both non- and transformed γδ T cells. This was accomplished primarily using a new technique to detect intracytoplasmic cytokine profiles (ICP) in the presence of a protein transport inhibitor, coupled with analysis by flow cytometry. Meaningful interpretation of the experimental results was contingent upon the use of optimised amounts of cytokine specific mAbs and reliable negative controls to ensure specific detection of intracytoplasmic cytokine. Cytokine specificity was conclusively determined through cytokine blocking experiments using identical non-labelled cytokine mAb prior to staining with fluorochrome labelled mAb. The use of non-labelled mAb also served as an intrinsic negative control and allowed for an accurate gating strategy. Illustrating these points, fresh PBMCs were stimulated for 18 h with PHA in the presence of monensin as protein transport inhibitor. In a separate experiment (Figure 16), comparison of monensin vs. brefeldin A to inhibit cytokine transport, showed no significant differences between the two reagents and were used interchangeably. Stimulated PBMCs were stained with graded amounts of the representative Th1 and Th2 cytokines, IFN-γ and IL-4

Table 12. Phenotypic comparison of surface molecule expression between *t*-γδ T cell lines and their parental non-transformed lines. FACS analysis and two colour fluorescence were used to compare established PB and CSF *t*-γδ T cell lines (>90 d) with their non-transformed parental cells derived from MS patients. Percentages are relative to total γδ T cells present. N.D: not determined. Vδ1, Vδ2, Vγ4, and Vγ9 refer to TcR variable chain specific mAbs together with relevant surface specific mAbs as described in Appendix 1.

Surface Marker

Patient			$V_\delta 1$	$V_\delta 2$	$V_\gamma 4$	$V_\gamma 9$	CD4	CD8	CD16	CD25
A	Parental	PB	4.0%	83.0%	3.0%	97.0%	N.D.	N.D.	0.0%	22.0%
	Infected	PB	4.5%	82.0%	5.0%	95.0%	N.D.	N.D.	0.0%	33.0%
B	Parental	PB	8.2%	86.2%	3.5%	89.8%	3.8%	17.4%	0.0%	1.8%
	Infected	PB	16.9%	69.8%	8.9%	79.0%	1.1%	15.7%	0.0%	12.9%
C	Parental	PB	2.1%	93.7%	3.3%	97.0%	1.0%	8.0%	0.0%	0.2%
	Infected	PB	3.5%	95.2%	3.5%	96.7%	0.0%	13.8%	0.0%	4.5%
D	Parental	CSF	1.0%	94.5%	0.4%	99.0%	0.0%	11.4%	0.0%	0.6%
	Infected	CSF	0.9%	98.2%	1.1%	98.8%	0.0%	17.0%	0.0%	0.7%

Surface Marker

Patient			CD28	CD40L	CD45RA	CD45RO	CD56	CD94	CD95	HLA-DR	HLA-ABC
A	Parental	PB	34.0%	N.D.	0.0%	100.0%	40.0%	N.D.	88.0%	100.0%	100.0%
	Infected	PB	35.0%	N.D.	0.0%	100.0%	32.0%	N.D.	91.0%	100.0%	100.0%
B	Parental	PB	26.7%	37.4%	2.7%	76.3%	76.4%	65.1%	100.0%	28.3%	100.0%
	Infected	PB	25.4%	44.1%	3.0%	79.0%	86.1%	52.3%	100.0%	33.5%	100.0%
C	Parental	PB	28.1%	2.3%	0.0%	66.5%	79.3%	44.2%	100.0%	6.1%	100.0%
	Infected	PB	16.9%	16.4%	0.0%	96.0%	93.1%	49.7%	100.0%	22.0%	100.0%
D	Parental	CSF	25.7%	7.4%	0.0%	75.5%	71.3%	39.1%	100.0%	3.3%	100.0%
	Infected	CSF	27.5%	19.1%	0.0%	92.0%	83.9%	42.1%	100.0%	5.5%	100.0%

Figure 15. Graphical illustration comparing the surface molecule expression of an HVS infected PB γδ T cell line with its non-infected parental cell line. FACS analysis and two colour fluorescence were used to compare an established PB *t*-γδ T cell line (d93) with its non-transformed parental line. Percentages are relative to total γδ T cells present. Vδ1, Vδ2, Vγ4, and Vγ9 refer to TcR variable chain specific mAbs together with relevant surface specific mAbs as described in Appendix 1.

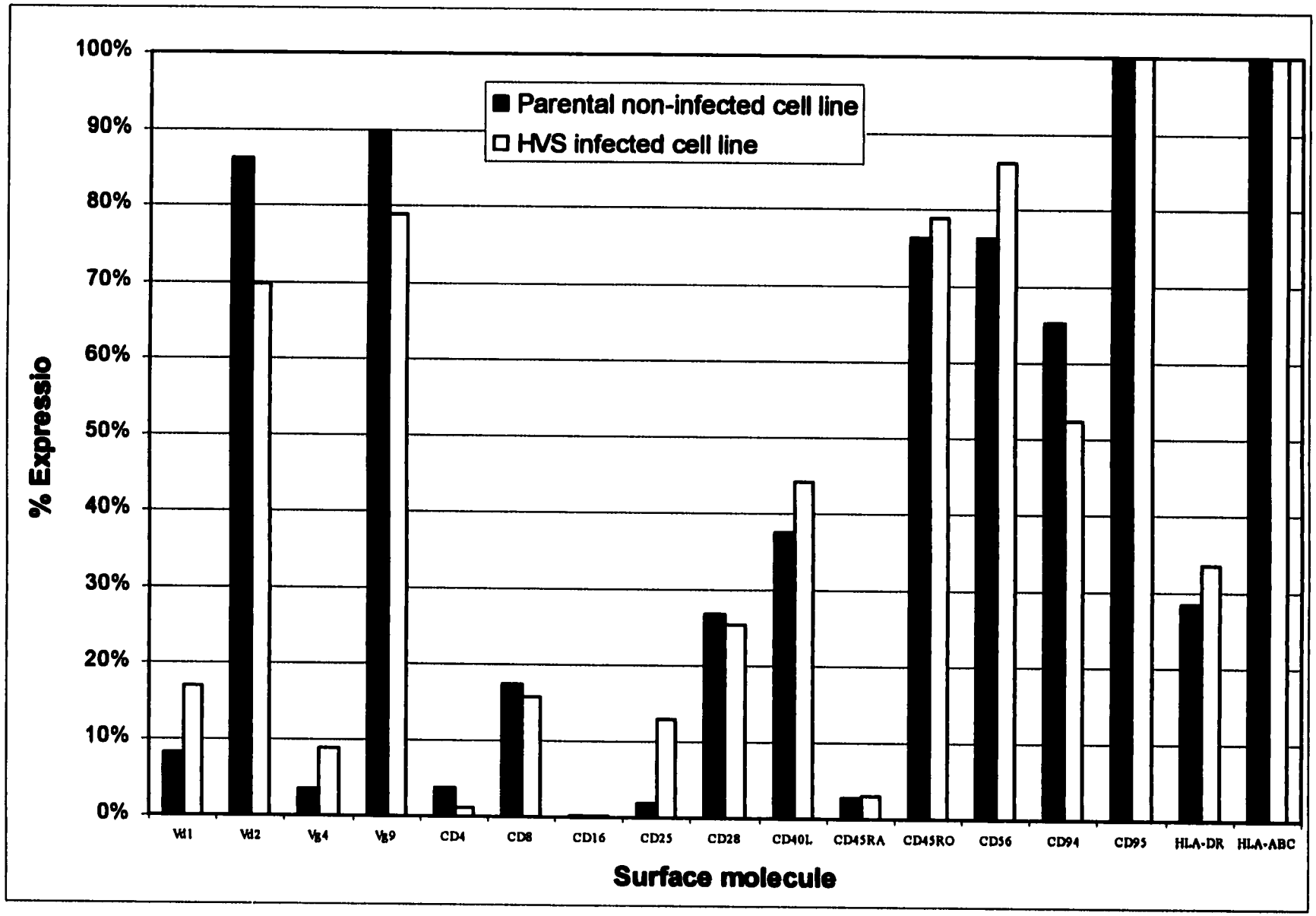

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% Expressio
Parental non-infected cell line
HVS infected cell line
Vd1
Vd2
Vg4
Vg9
CD4
CD8
CD16
CD25
CD28
CD40L
CD45RA
CD45RO
CD56
CD94
CD95
HLA-DR
HLA-ABC
Surface molecule

Figure 16. Comparison of Brefeldin A and monensin as protein transport inhibitors for intracytoplasmic cytokine staining. Transformed clone 10-10-3 ($1x10^6$ cells/ml) was rested for 24 h in IL-2 free medium prior to stimulation with PMA (50 ng/ml)/ Ca^{2+} ionophore (1 μg/ml) in the presence of either Brefeldin A (2 μg/ml) or monensin (2 μM) for 6 h. Following surface staining with γδ TcR mAb and fixation, permeabilised cells were stained with fluorochrome labelled IFN-γ (0.5 μg) or IL-4 (0.25 μg). Control cells were first treated with either non-labelled IFN-γ (5μg) or IL-4 (10 μg), followed by the respective fluorochrome labelled mAbs. Fluorescence profiles were obtained through FACS analysis and the % cytokine positive cells relative to total γδ T cells calculated as described in *Materials and methods*.

Figure 17. Titration of cytokine specific mAbs for use in intracytoplasmic cytokine profile studies. Freshly obtained PBMCs were stimulated with PHA (1μg/ml) for 18 h in the presence of monensin (2 μM), followed by surface staining with αβ TcR mAb and fixation. Graded amounts of fluorochrome labelled IFN-γ and IL-4 as indicated were added to permeabilised cells. Blocking controls consisted of prestaining cells with unlabelled cytokine mAb (5 μg) followed by labelled antibodies. Fluorescence profiles were obtained through FACS analysis and the % cytokine positive cells relative to total αβ T cells calculated as described in *Materials and methods*.

Figure 16.

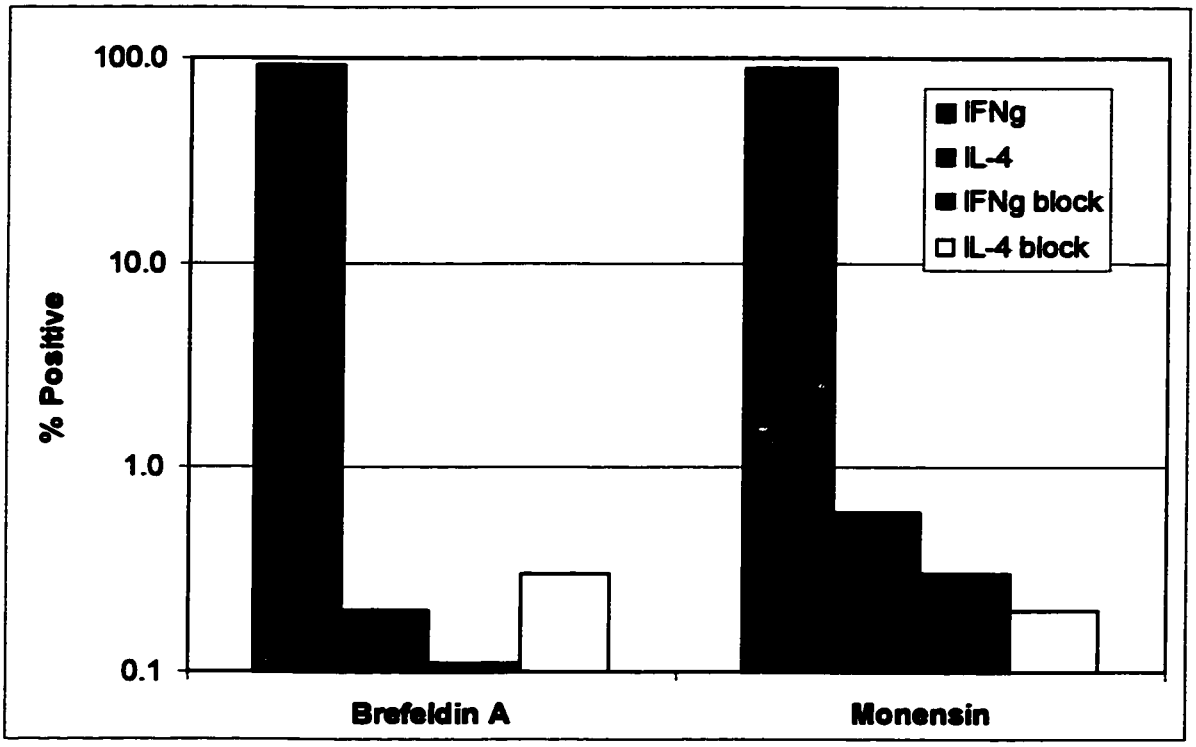
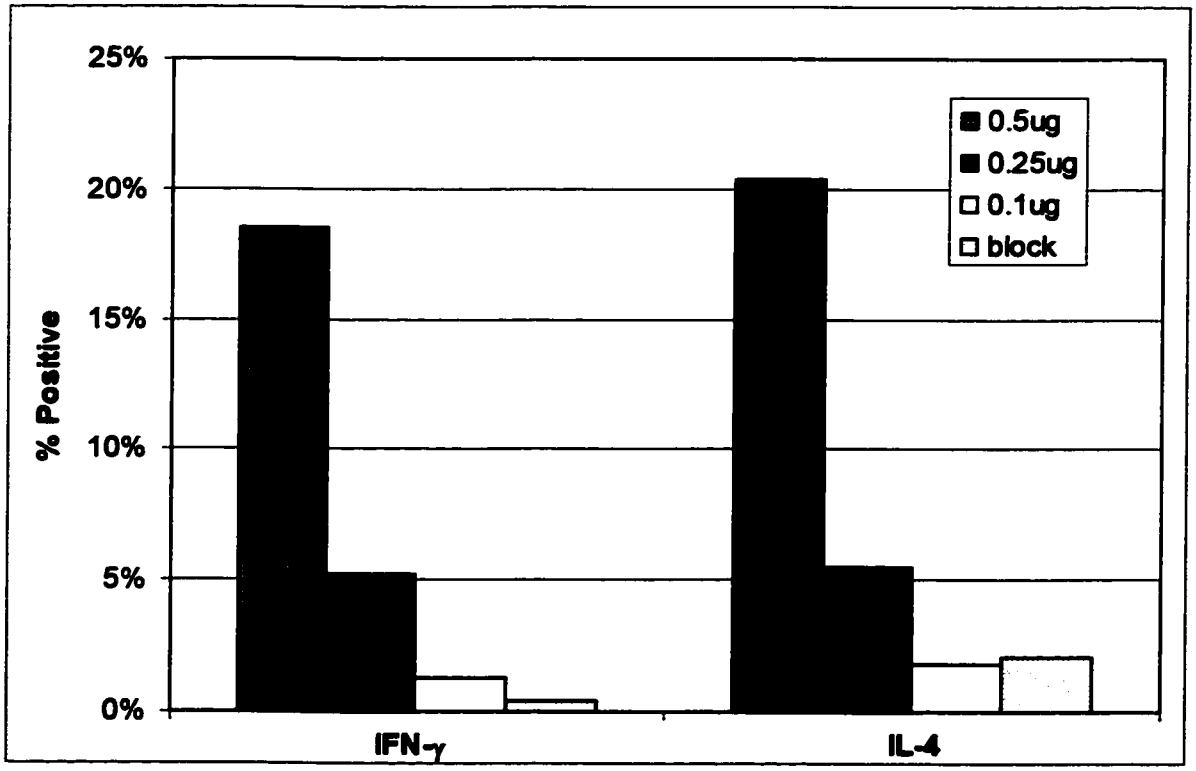


Figure 17.



respectively and the results depicted in Figure 17. IFN-γ and IL-4 signals exhibited typical titration profiles and pre-incubation with non-labelled IFN-γ or IL-4 virtually blocked all cytokine signal. Setting the cut-off threshold on the negative "blocked" signal, ensured that cells above this threshold were positive. Increasing the amount of cytokine increased the amount of non-specific signal and was reflected by an increased difficulty in blocking it. Transformed γδ T cells required an initial resting step prior to stimulation and cytokine detection since they are by definition in an activated state and continuously generate basal levels of cytokine. Resting involved culturing *t*-γδ T cells in IL-2 free medium for 48 h prior to the assay. Shorter rest periods contributed to higher spontaneous production of cytokine while longer periods resulted in poor recovery of viable *t*-γδ T cells.

B) Parental vs. transformed γδ T cells

Using ICP analyses, parental γδ T cell lines (2 PB, 1 CSF) were compared to their transformed progenies and a representative experiment is illustrated in Figure 18. All samples behaved in a similar manner displaying a predominant proinflammatory cytokine profile upon PMA/ Ca^{2+} ionophore stimulation, with the majority of cells staining for intracellular IFN-γ and TNF-α and showed little to no staining for the Th2-like cytokine IL-4. Parental short term γδ T cell IC profiles were virtually indistinguishable from their transformed progenies (IFN-γ: 51.7 vs. 40%; TNF-α: 66.9 vs. 62.5%; IL-4: 1.4 vs. 0.8%) and cytokine specificity was assured by the loss of signal with blocking controls. The lone CSF sample analysed in this manner behaved somewhat differently relative to the two PB samples (Figure 19). Although IFN-γ positivity was still predominant in both CSF parental and *t*-γδ T cells (87% vs. 96%), TNF-α positivity was reduced and significant levels of IL-10 positive cells were detected within the parental CSF line relative to the transformed line (TNF-α: 44.6 vs. 78%; IL-10: 46 vs. 4%) (Figure 19-A). However, comparison of mean channel fluorescence (MCF) values, which is a relative indicator of cytokine concentration per cell, showed that both IFN-γ and TNF-α levels were markedly reduced in the parental short term line relative to the transformed line and despite IL-10 positivity, the MCF level was only marginally elevated over controls (Figure 19-B). This difference in IC profiles between

Figure 18. Intracytoplasmic cytokine profile comparison between a PB *t*-γδ T cell line and its parental non-transformed line. Both the growth transformed and parental PB γδ T cell lines were rested in IL-2 free medium for 24 h prior to stimulation with PMA (10 ng/ml)/ Ca^{2+} ionophore (0.2 μg/ml) in the presence of Brefeldin A (2 μg/ml) for 6 h. Following γδ TcR mAb surface staining and fixation, permeabilised cells were treated with optimal amounts of fluorochrome labelled IFN-γ, TNF-α, and IL-4. Blocking controls consisted of cells treated with unlabelled cytokine specific mAbs prior to treatment with the labelled antibody. Cytokine profiles were generated by FACS analysis and the percent cytokine positive γδ T cells indicated in the upper right quadrants.

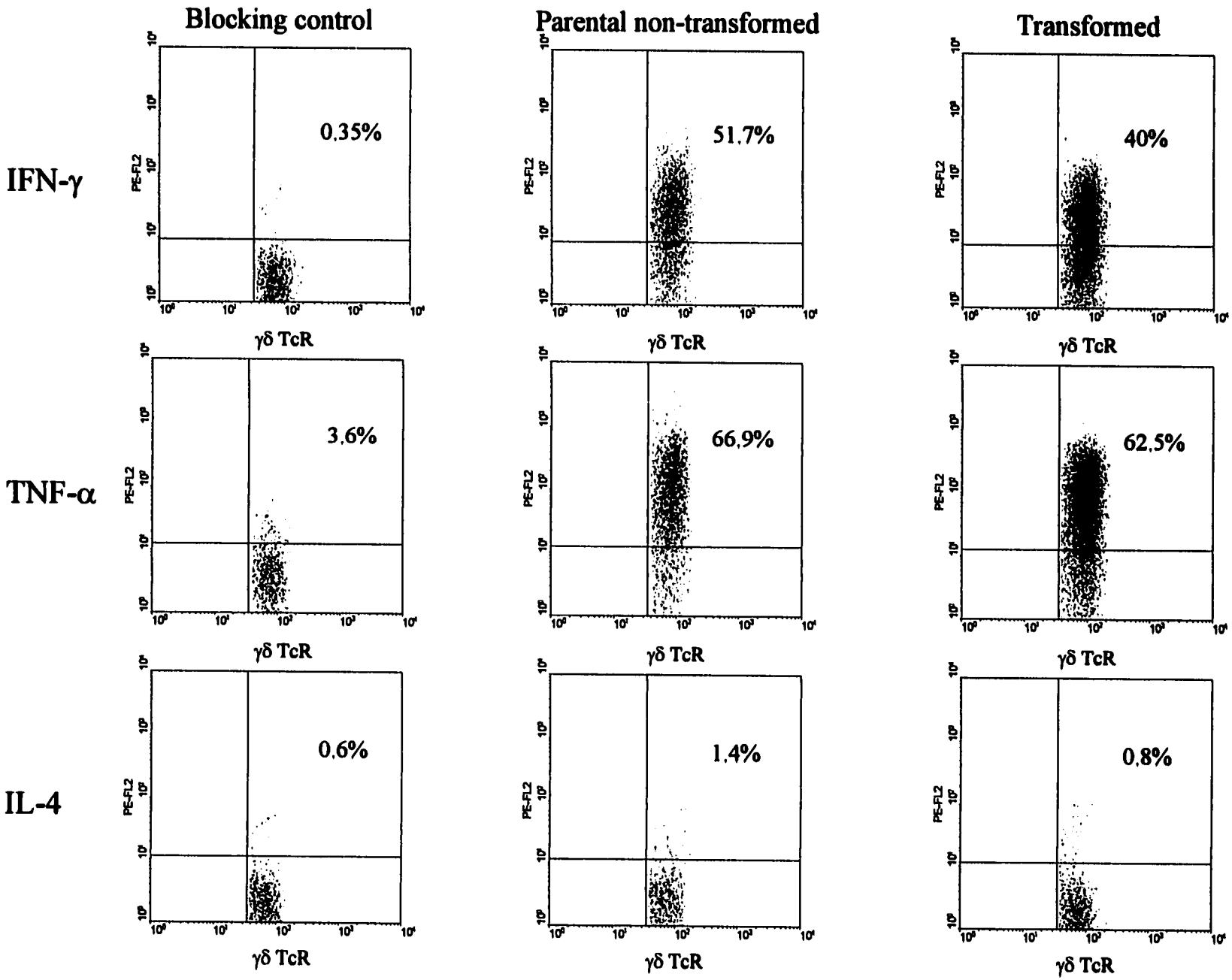
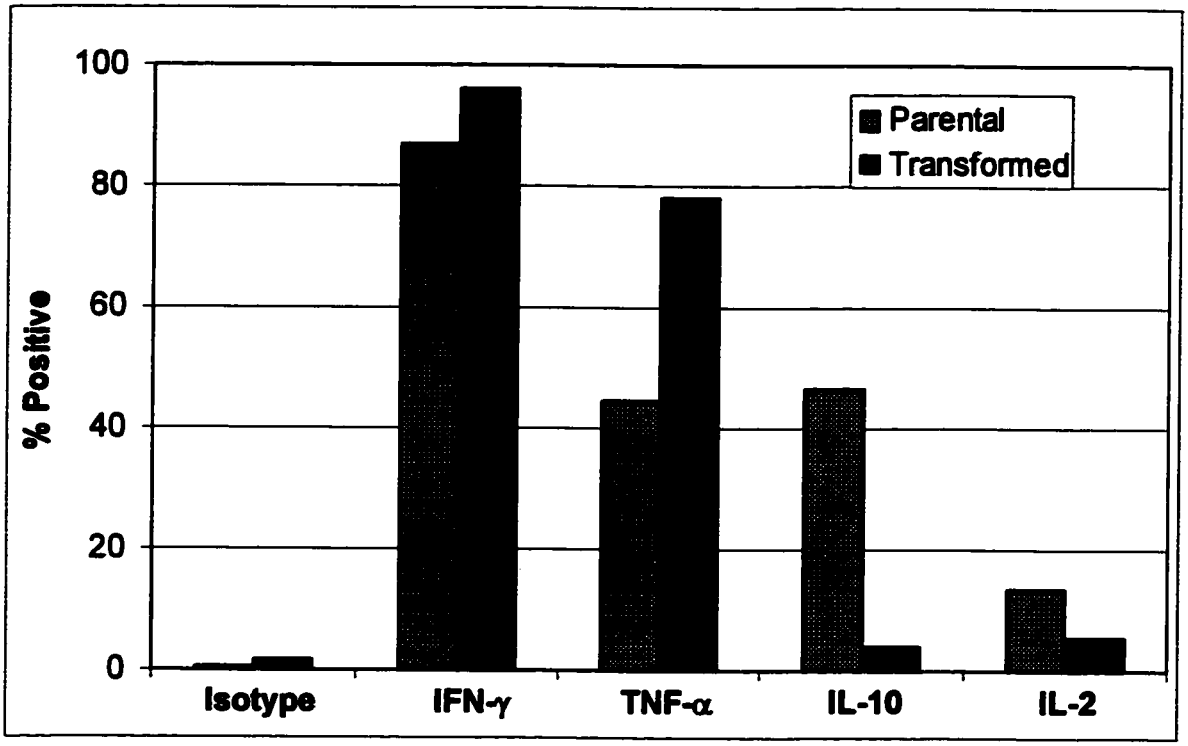
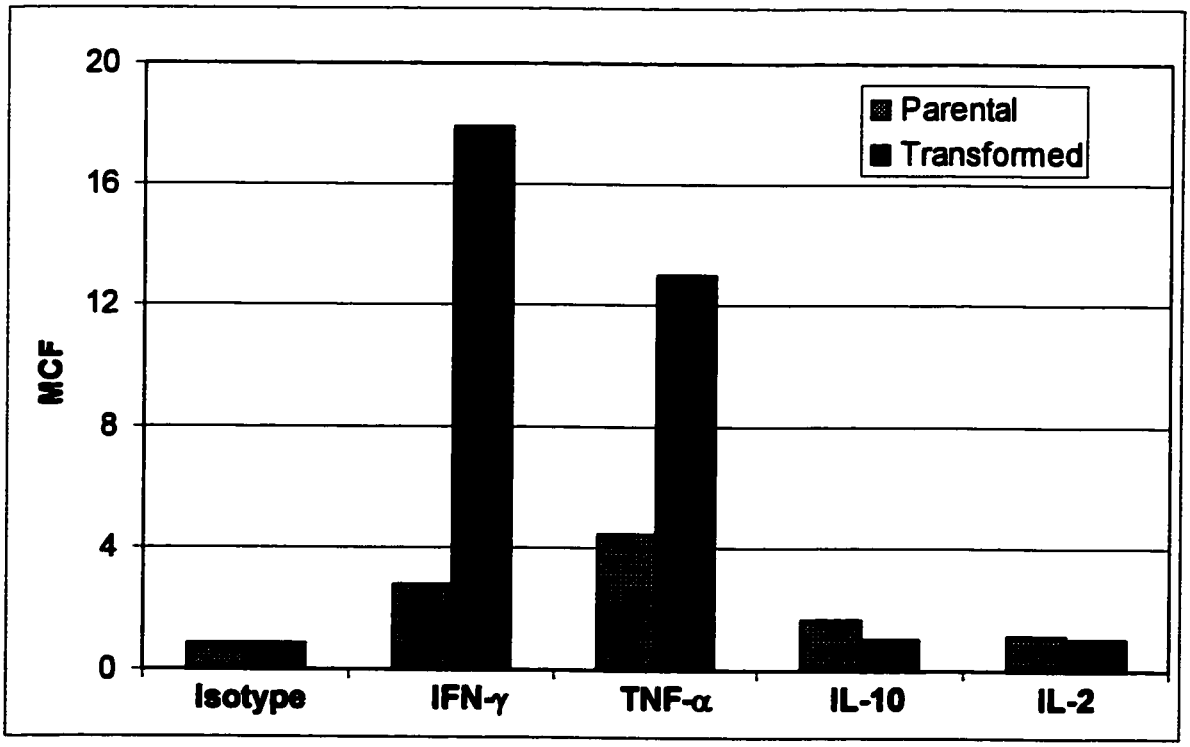

Blocking control
Parental non-transformed
Transformed
IFN-γ
TNF-α
IL-4
0.35%
51.7%
40%
3.6%
66.9%
62.5%
0.6%
1.4%
0.8%
PE-FL2
γδ TcR

Figure 19. Intracytoplasmic cytokine profile comparison between a CSF *t*-γδ T cell line and its parental short term line. Both the growth transformed and parental CSF γδ T cell lines were rested in IL-2 free medium for 24 h prior to stimulation with PMA (10 ng/ml)/ Ca^{2+} ionophore (0.2 μg/ml) in the presence of Brefeldin A (2 μg/ml) for 6 h. Following γδ TcR mAb surface staining and fixation, permeabilised cells were treated with optimal amounts of fluorochrome labelled IFN-γ, TNF-α, IL-10, and IL-2 followed by FACS analysis. Negative controls consisted of cells treated with non-specific fluorochrome labelled isotype mAb. Cytokine responses were analysed either as the % of γδ T cells positive for cytokine (Panel A) or as the relative change in cytokine levels (Panel B), calculated as described in *Materials and methods*.

(A)


100
80
60
40
20
0
% Positive
Parental
Transformed
Isotype
IFN-γ
TNF-α
IL-10
IL-2

(B)


20
16
12
8
4
0
MCF
Parental
Transformed
Isotype
IFN-γ
TNF-α
IL-10
IL-2

short term and transformed CSF may be attributed to initial CSF γδ T cell culture in the presence of IL-4, a standard protocol originally used in CSF γδ T cell expansion.

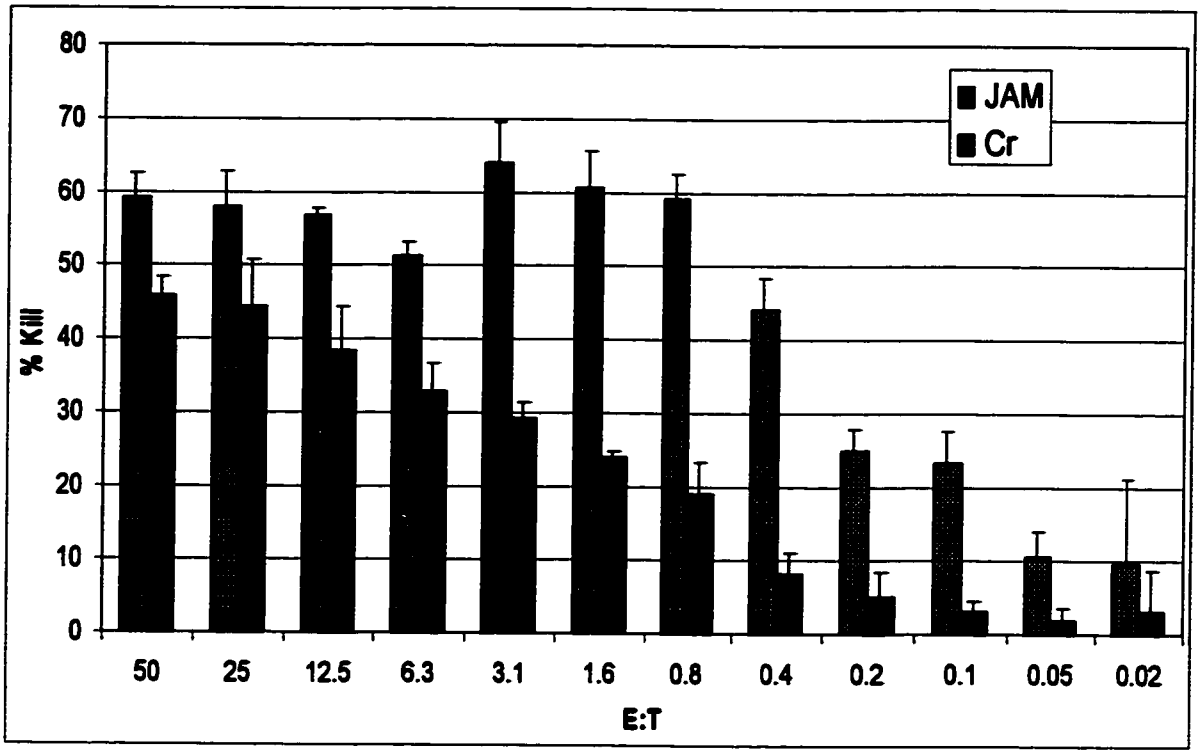
3.3.3 Cytotoxic potential comparisons

A) JAM cytotoxicity assay

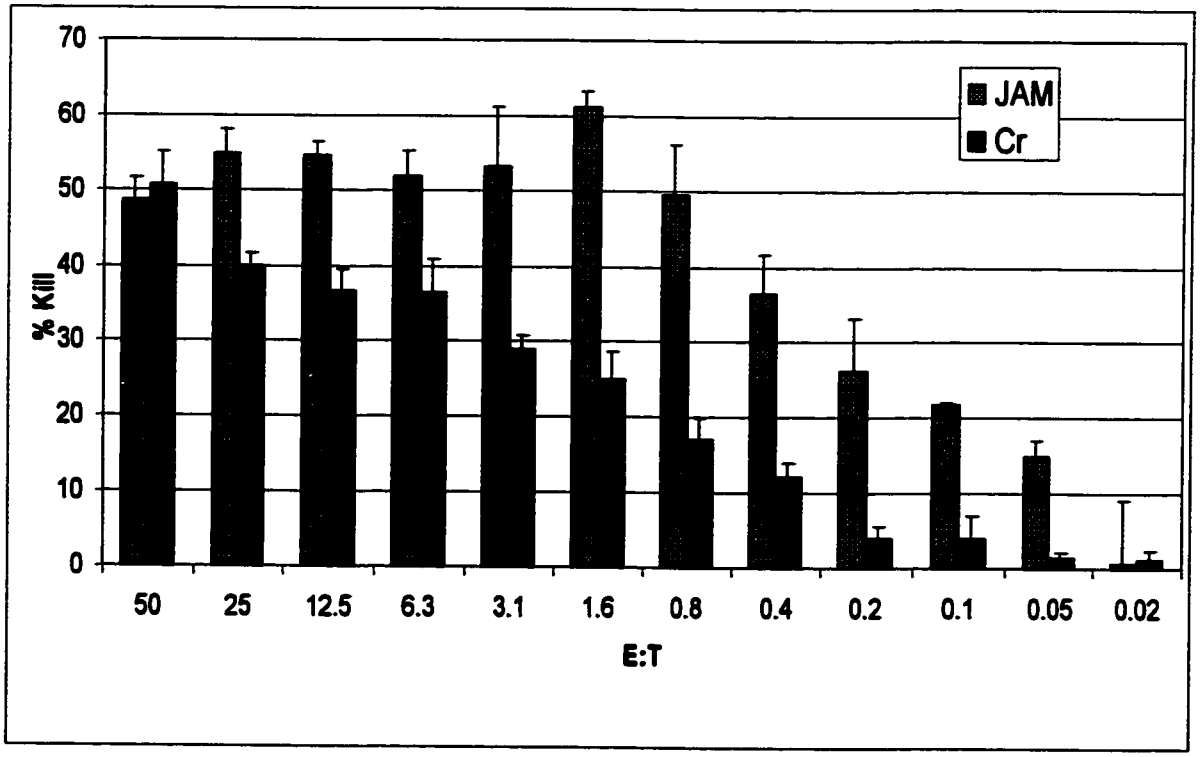
A primary focus in our lab has been the study of γδ T cell cytotoxicity towards relevant target cells. Cytotoxic potentials were traditionally measured using the gold standard ^{51}Chromium release assay, however this assay was not amenable due to Level III considerations. An alternate JAM cytotoxicity assay (213) was adopted and adapted to evaluate *t*-γδ T cell cytotoxicity. This assay is based on measuring cell survival by detecting incorporated [*methyl*-^{3}H] thymidine into DNA as opposed to cell death by chromium release. Cells that have undergone a lethal hit, quickly fragment cellular DNA, and only full length DNA (survival) is captured upon cell harvesting. Alternatively, a vital membrane dye technique coupled with flow cytometry was also evaluated (214). To validate the use of either assay for cytotoxicity analysis, side by side comparisons were performed and compared to the standard ^{51}Cr- release assay. Figure 20 depicts the results of a direct comparison of the JAM and ^{51}Cr-release assays using either a PB *t*-γδ T cell clone (Panel A) or a CSF *t*-γδ T cell line (Panel B) as effectors (E) and the monocytic cell line, U937, as targets (T). The JAM assay used a 4 h incubation period as opposed to 5 h for the ^{51}Cr-release assay. Clearly from Figure 20-A and B, the JAM assay behaved similarly to the ^{51}Cr-release results in that cytotoxicity displayed a typical titration drop-off as the E:T ratio was reduced. However, it is also clear that the JAM assay was markedly more sensitive than the ^{51}Cr-release assay exhibiting greater maximal levels of kill (i.e. Fig. 20-A: 64 vs. 45%) and significantly greater levels of killing at the far end of the E:T spectrum where ^{51}Cr-release values were close to background. Typically for all JAM assays and represented in Figure 20-A and B, target killing plateaued at higher E:T ratios before falling off whereas for the ^{51}Cr-release assay, this was rarely observed because effector cell numbers and cell density issues precluded using higher E:T ratios. In other validation experiments using either 5K targets or 3 h assays, the JAM assay

Figure 20. Comparison of the JAM vs the ^{51}Cr-release assay to assess *t*-γδ T cell induced cytotoxicity of U937 monocyte targets. JAM assay: Serial dilutions of either a PB *t*-γδ T cell clone (Panel A) or a CSF *t*-γδ T cell line (Panel B) were combined with U937 targets (10K) previously labelled with *methyl* ^{3}[H]- thymidine in overnight cultures. After a 4 h incubation, full length DNA was harvested onto glass fibre filters and incorporated radiolabel quantified. The level of kill was calculated based on targets treated alone as described in *Materials and methods*. Data shown represents the mean ± SD for quadruplicate wells. ^{51}Cr-release assay: Serial dilutions of either a PB *t*-γδ T cell clone (Panel A) or a CSF *t*-γδ T cell line (Panel B) were combined with ^{51}Cr loaded U937 targets (10K) and incubated for 5 h. Supernatants containing spontaneous or induced ^{51}Cr-release were removed followed by cetrimide lysis for maximal ^{51}Cr detection. The level of kill was calculated based on targets treated alone as described in *Materials and methods*. Data shown represents the mean ± SD for triplicate wells. E:T: effector to target ratio.

(A)


80
70
60
50
40
30
20
10
0
% Kill
JAM
Cr
50
25
12.5
6.3
3.1
1.6
0.8
0.4
0.2
0.1
0.05
0.02
E:T

(B)


70
60
50
40
30
20
10
0
% Kill
JAM
Cr
50
25
12.5
6.3
3.1
1.6
0.8
0.4
0.2
0.1
0.05
0.02
E:T

consistently yielded somewhat lower but significant levels of γδ T cell target killing (data not shown). The significance of this increased sensitivity was the ability to use 50-100 fold fewer effector cells in the JAM assay to achieve similar levels of killing in the ^{51}Cr-release assay. Taken together, the JAM assay furnished cytotoxicity data on a variety of cell lines in a highly reproducible and sensitive manner which outperformed the standard ^{51}Cr-release assay in terms of functionality, numbers of required effectors, and assay time.

A flow cytometric cytotoxicity assay was also evaluated due to its overall simplicity and reported short assay times. This assay was based on incorporation of the vital dye, 3,3'-dioctadecylcyloxacarbocyanine (DiO) into the cell membrane of live target cells. Changes in cell membrane permeability after receiving a lethal hit allowed propidium iodide (PI) staining of nuclear material separating "killed" from live populations (DiO^+, PI^-). Figure 21 compared the DiO flow assay at two time points with a 4.5 h JAM assay using CSF derived γδ T cells directed towards U937 cells. Despite reported 2 h incubation times for optimal NK killing of target cells (214), it was found that this resulted in low levels of γδ T cell induced target killing (11.8% maximum), although the pattern of killing followed a dose response curve. Increasing the incubation time to 4.5 h significantly increased the levels of kill obtained (26% maximum) but these levels did not compare to levels of induced kill measured by the JAM assay (78.8 vs. 16.4% kill at E:T of 12.5:1) in the same time frame. Due to the lower sensitivities obtained with the DiO vs. the JAM assay, the assay was deemed not acceptable for routine cytotoxicity evaluation despite its ease of use.

B) Parental vs. transformed cells

Direct comparisons of parental and t-γδ T cell cytotoxicity were limited by the availability of sufficient short term γδ T cells enabling cytotoxicity studies and at the same time, furnishing sufficient cells for transformation studies. One such example is illustrated in Figure 22 comparing a short term CSF γδ T cell line with either its transformed CSF line or a derived *t*-γδ T cell clone in a JAM assay. Both the *t*-γδ T cell line and clone displayed virtually identical cytotoxicity towards U937 target cells while the parental cytotoxicity profile dropped off at a steeper rate as the E:T

Figure 21. Comparison of the DiO flow cytometry assay vs the JAM assay to assess γδ T cell induced target killing. DiO assay: serial dilutions of a short term CSF γδ T cell line were combined with U937 monocyte targets previously treated with the membrane dye, DiO, for 1 h. One hour prior to the end of either a 2.5 h or 4.5 h incubation period, PI was added. Data was acquired via FACS analysis with DiO^+ and PI^- cells characteristic of intact cells and DiO^+ and PI^+ cells characteristic of compromised cells. The % kill was calculated as described in *Materials and methods*. JAM assay: Serial dilutions of the CSF γδ T cell line were combined with U937 targets (10K) previously labelled with *methyl* ^{3}[H]- thymidine in overnight cultures. After a 4 h incubation, full length DNA was harvested onto glass fibre filters and incorporated radiolabel quantified. The level of kill was calculated based on targets treated alone as described in *Materials and methods*.

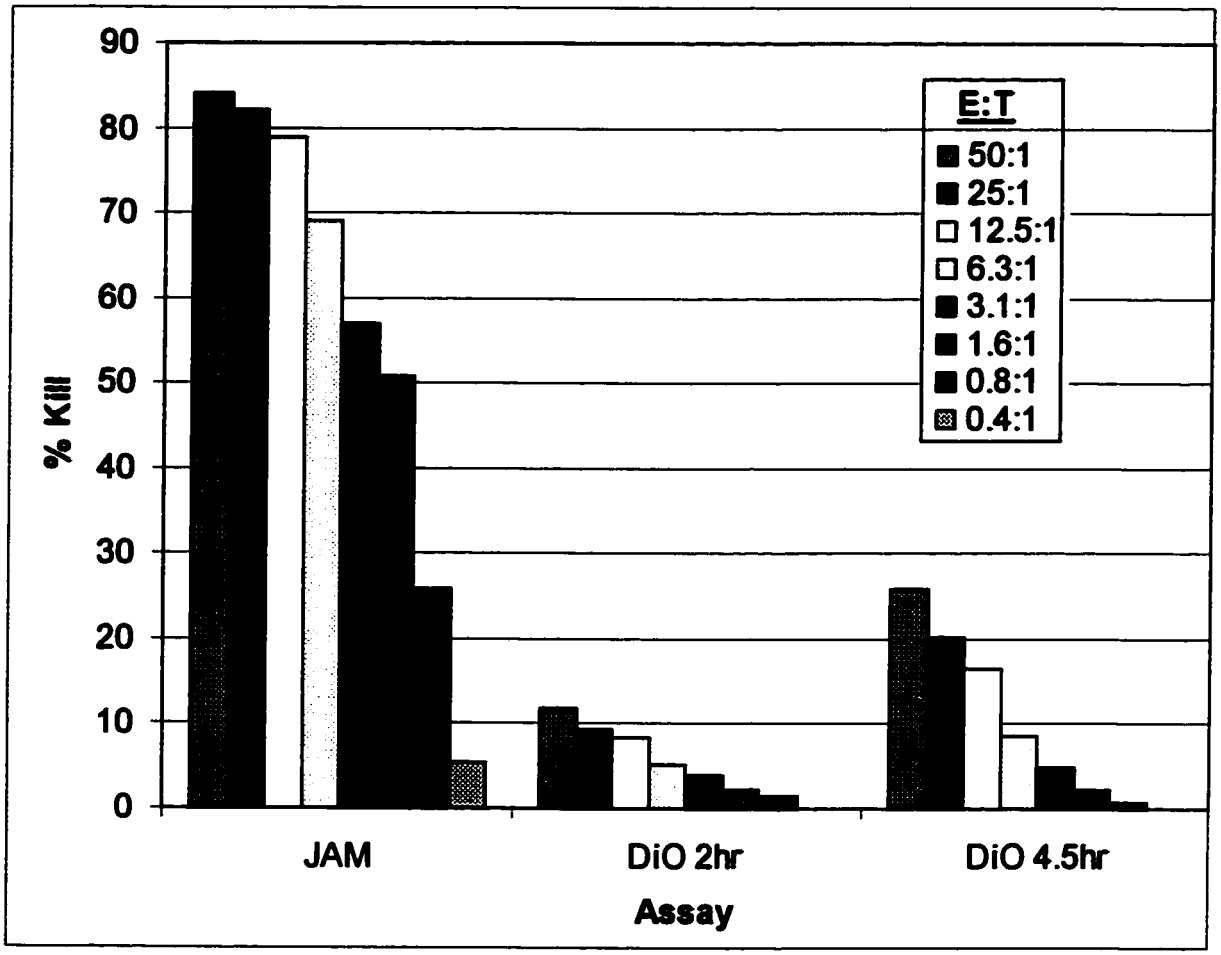
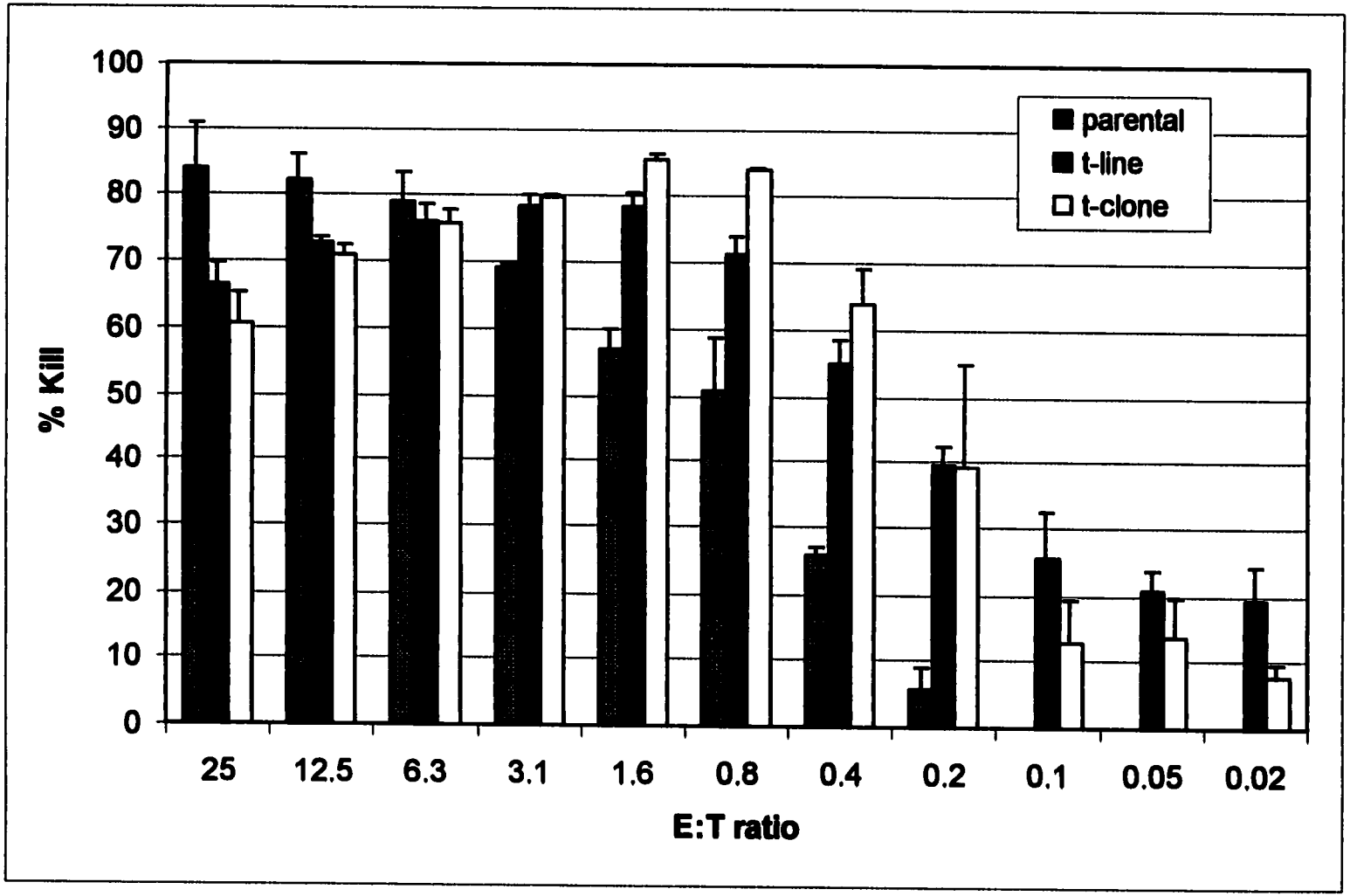

90
80
70
60
50
40
30
20
10
0
% Kill
E:T
50:1
25:1
12.5:1
6.3:1
3.1:1
1.6:1
0.8:1
0.4:1
JAM
DiO 2hr
DiO 4.5hr
Assay

Figure 22. Cytotoxicity comparisons between a CSF *t*-γδ T cell line, a derived *t*-γδ T cell clone, and their parental CSF non-transformed line. A CSF *t*-γδ T cell line, a representative clone derived from this line, and the parental non-transformed CSF line were evaluated in a standard 4 h JAM cytotoxicity assay against U937 target cells. The % kill was calculated based on target cells treated alone and calculated as in *Materials and methods*. Data as shown represents the mean ± SD for quadruplicate wells.


100
90
80
70
60
50
40
30
20
10
0
% Kill
parental
t-line
t-clone
25
12.5
6.3
3.1
1.6
0.8
0.4
0.2
0.1
0.05
0.02
E:T ratio

was lowered (i.e. 25.9 vs. 55 and 64% kill for the parental vs. *t*-line and clone respectively at an E:T= 0.4). The decreased cytotoxic potential of the parental line was not surprising considering that the γδ T cell sample purity was 90% relative to the pure transformed line and clone. Additionally, the 23 d culture life of the parental line on the day of the assay was normally associated with time periods when a significant proportion of cells were not thriving, as opposed to the actively proliferating *t*-γδ T cells. These results, together with similar cytotoxicity profiles observed between *t*-γδ T cells and non-parental short term lines, suggested the transformation process impacted marginally on γδ T cell cytotoxicity.

Overall, studies comparing parental γδ T cells and their transformed progenies in terms of cell surface marker expression, PMA/Ca^{2+} ionophore induced intracytoplasmic cytokine profiles, and cytotoxic potentials towards target cells, all suggested that the HVS transformation technique induced little if any transformation related effects. The significance of this result suggests that further investigations using MS derived *t*-γδ T cells are directly relevant to their *ex vivo* counterparts and their *in vitro* behaviours may accurately reflect aspects of their potential role in MS pathogenesis.

3.4 Phenotypic and functional properties

With an established source of long lived γδ T cells derived principally from MS PB and to a lesser extent CSF, the stage was set to continue and expand on studies initiated with short term γδ T cells and their relationship to MS pathogenesis. Central to these investigations was the continued study of γδ T cell cytotoxicity towards relevant targets, but also γδ T cell cytokine profiles and their potential for immunoregulation, and γδ T cell antigen recognition events. A comprehensive phenotypic analysis of the immortalised γδ T cell lines and importantly, the clones derived from these lines was performed to give insight on which form of γδ T cell may potentially be relevant to MS pathogenesis and how specifically they may accomplish that role.

3.4.1 MS transformed γδ T cells: Phenotypes

Phenotypic characterisation of a number of *t*-γδ T cell lines was performed by evaluation of surface molecule expression using flow cytometry and the results summarised in Table 13. A survey of the data indicated a number of general patterns but also illuminated some important differences. γδ TcR usage, which allowed grouping into subtype categories, was performed with a panel of available mAbs specific to Vγ and Vδ gene expression. PB transformed cells all reflected the normal PB γδ subtype distribution with the vast majority of cells displaying a Vδ2Vγ9 TcR. Of the remaining cells, approximately 3-17% belonged to the Vδ1 subtype family also reflecting ratios normally found within PB. In instances where applicable, the newly available Vδ3 specific mAb detected only small numbers of PB *t*-γδ T cells displaying this TcR chain. A survey of γδ TcR chain composition in transformed CSF cells indicated a much more dynamic distribution of subtypes. As with short term CSF lines (Section 3.1.1.1, Figure 2), γδ subtype distribution was found to be patient dependent and ranged from almost exclusively Vδ2Vγ9 (Vδ2 subtype) to exclusively Vδ1 expressing subtypes to mixtures including significant proportions expressing the Vδ3 gene segment. Importantly, typing for Vγ gene expression revealed both Vγ4 and Vγ9 positive cells but a significant proportion were found to express other Vγ gene elements not identifiable with the current repertoire of Vγ specific mAbs. In general, the *t*-γδ T cells expressed little CD4, although two CSF samples had low but significant populations of $CD4^+$ γδ T cells. *T*-γδ T cells lines had variable expression of CD8 ranging from none to >50% of γδ T cells, with this latter originating within a CSF sample transformed *ex vivo*. All *t*-γδ T cells were of the memory phenotype and displayed the activation marker HLA-DR but expression of the high affinity IL-2 receptor, CD25, was variably high to low. A proportion of the *t*-γδ T cells consistently displayed CD40L (CD154) suggesting their potential ability as helper T cells. Transformed γδ T cells constitutively expressed the apoptosis marker CD95 (Fas) but several attempts using the mAb NOK-1 failed to detect conclusively the presence of FasL. This was attributed to the short half life of FasL on the cell surface prior to cleavage by metalloproteinases rather than lack of FasL altogether, as others have demonstrated the expression of FasL on activated γδ T cells (93).

Table 13. Phenotypic characterisation of HVS growth transformed γδ T cell lines. Surface molecule expression was evaluated for PB and CSF *t*-γδ T cell lines using two colour fluorescence with a series of specific mAbs (Appendix A) and FACS analysis. Percentages listed are relative to all γδ T cells present. [1]: CSF *ex vivo* = an *ex vivo* CSF sample simultaneously HVS infected and MB γδ TcR stimulated.

Surface Marker

Patient		Vδ1	Vδ2	Vδ3	Vγ4	Vγ9	CD4	CD8	CD16	CD25
A	PB	4.5%	82.0%	N.D.	5.0%	95.0%	N.D.	N.D.	0.0%	33.0%
B	PB	16.9%	69.8%	N.D.	8.9%	79.0%	1.1%	15.7%	0.0%	12.9%
C	PB	6.0%	93.4%	N.D.	8.0%	95.9%	0.0%	11.6%	0.0%	4.2%
D	PB	3.0%	99.0%	N.D.	3.8%	98.0%	0.0%	0.0%	N.D.	N.D.
E	PB	4.0%	93.3%	N.D.	6.7%	97.0%	N.D.	22.8%	0.0%	3.3%
F	PB	13.7%	82.8%	3.2%	3.8%	92.7%	0.0%	6.4%	0.0%	7.0%
G	CSF	0.9%	98.2%	N.D.	1.1%	98.8%	0.0%	17.0%	0.0%	0.7%
H	CSF	18.1%	68.1%	N.D.	10.0%	72.5%	8.1%	15.8%	0.0%	51.3%
I	CSF	100.0%	0.0%	0.0%	0.0%	100.0%	0.0%	9.6%	0.0%	42.9%
J	CSF *ex vivo*[1]	79.1%	3.1%	18.0%	0.0%	3.6%	7.6%	54.3%	N.D.	N.D.

Patient		CD28	CD40L	CD45RA	CD45RO	CD56	CD94	CD95	CD152	HLA-DR	HLA-ABC
A	PB	35.0%	N.D.	0.0%	100.0%	32.0%	N.D.	91.0%	N.D.	100.0%	100.0%
B	PB	25.4%	44.1%	3.0%	79.0%	86.1%	52.3%	100.0%	N.D.	33.5%	100.0%
C	PB	17.3%	15.2%	0.0%	100.0%	93.4%	48.2%	100.0%	N.D.	22.3%	100.0%
D	PB	N.D.	N.D.	N.D.	N.D.	N.D.	62.3%	N.D.	N.D.	N.D.	N.D.
E	PB	5.6%	34.1%	0.0%	54.0%	85.7%	64.2%	67.2%	N.D.	7.0%	65.0%
F	PB	6.2%	61.7%	0.0%	69.0%	80.0%	22.0%	100.0%	0.0%	73.7%	100.0%
G	CSF	27.5%	19.1%	0.0%	100.0%	83.9%	42.1%	100.0%	N.D.	5.6%	100.0%
H	CSF	59.6%	31.7%	0.0%	95.0%	24.8%	81.2%	89.7%	N.D.	100.0%	100.0%
I	CSF	0.0%	22.7%	0.0%	100.0%	88.9%	49.1%	100.0%	0.0%	90.2%	100.0%
J	CSF *ex vivo*	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Lastly, *t*-γδ T cells displayed numerous NK cell markers such as N-CAM (CD56), and the killer inhibitory receptor (KIR) CD94, but interestingly none of the lines investigated possessed the FcRIII, CD16. This was consistent with results using short term γδ T cells where CD16 was rarely found, but was in contrast to many reports claiming constitutive CD16 expression on circulating γδ T cells (125).

Characterisation of the *t*-γδ T cell lines pointed out a number of interesting phenotypes but the identification of phenotypic subsets was hampered by the polyclonal nature of the lines. For this reason, *t*-γδ T cell clones were generated and similarly evaluated resulting in the identification of a number of unique and somewhat surprising phenotypes (Table 14). Analysing the Vγ and Vδ TcR gene usage identified a number of clones with the expected Vδ2 and Vδ1 phenotype (major and minor populations as described above), but also several *t*-γδ T clones expressing either Vδ3 or Vδ other (Vδ4 or 5) TcR variable regions. Similarly, Vγ9 and Vγ4 gene expression were frequently found but significant levels of non Vγ4 and Vγ9 cells were also observed. γδ TcR analysis revealed non-traditional chain pairing such as Vγ9Vδ1, Vγ9Vδ3, Vγ9non-Vδ1,2,3, and Vγ4non-Vδ1,Vδ2,Vδ3. Clone 44-16 uniquely displayed a Vδ1 expressed δ chain in conjunction with both Vγ4 and Vγ9 expressed γ chains. Expression of both Vγ chains on a single clone was verified by co-staining cells with either Vγ4 or Vγ9 in conjunction with CD3. All $CD3^+$ cells doubly fluoresced with both Vγ4 and Vγ9 mAbs, rather than an expected fraction if two clones were present. Clone 44-40 was also unique due to its abundant expression of CD4 in conjunction with modest expression of CD40L suggestive of T helper abilities. As a corollary to this observation, along with multiple CD8 expressing *t*-γδ T cell clones, this indicated that despite targeting CD4 and CD8 to remove contaminating αβ T cells, it was still possible to recover $CD4^+$ and $CD8^+$ *t*-γδ T cells. The 44 and 47 series of *t*-γδ T cell clones similarly displayed levels of CD40L ranging from high to none and would also be useful in T helper studies. *T*-γδ T cell clones were also identified with variably high to low expression of CD8 allowing for investigations of MHC class I restriction in terms of cytotoxicity. Screening both *t*-γδ T cell lines and clones for the activation antigen CD152 (CTLA-4) failed in all cases to detect this surface molecule. Since CD152 is

Table 14. Phenotypic characterisation of HVS growth transformed γδ T cell clones. Surface molecule expression was evaluated for PB and CSF *t*-γδ T cell clones using two colour fluorescence with a series of specific mAbs (Appendix A) and FACS analysis. Percentages listed are relative to all γδ T cells present. [1]: CD94 clone HP3D9. [2]: CD94 clone HP3B1. N.D: not determined.

Clone	Origin	Vδ1	Vδ2	Vδ3	Vγ4	Vγ9	Vδ1 (TCS1)	Vδ2-Vγ9 (TiγA)
10-10-3	PB	0.0%	0.0%	0.0%	100.0%	0.0%	0.0%	0.0%
10-10-5		100.0%	0.0%	0.0%	40.0%	0.0%	100.0%	0.0%
10-10-8		100.0%	0.0%	N.D.	N.D.	N.D.	N.D.	N.D.
10-50-2		100.0%	0.0%	0.0%	0.0%	0.0%	N.D.	N.D.
10-50-3		100.0%	0.0%	N.D.	N.D.	N.D.	N.D.	N.D.
44-16	PB	100.0%	0.0%	0.0%	100.0%	100.0%	100.0%	100.0%
44-27		0.0%	0.0%	0.0%	15.0%	100.0%	0.0%	100.0%
44-40		100.0%	0.0%	0.0%	100.0%	0.0%	100.0%	0.0%
44-42		0.0%	0.0%	100.0%	0.0%	100.0%	0.0%	100.0%
46-7	CSF	100.0%	0.0%	0.0%	0.0%	100.0%	N.D.	N.D.
46-14		100.0%	0.0%	0.0%	0.0%	100.0%	N.D.	N.D.
46-21		100.0%	0.0%	0.0%	0.0%	100.0%	N.D.	N.D.
46-26		100.0%	0.0%	0.0%	0.0%	100.0%	N.D.	N.D.
47-5	PB	0.0%	100.0%	0.0%	0.0%	100.0%	N.D.	N.D.
47-6		0.0%	0.0%	0.0%	0.0%	100.0%	N.D.	N.D.
47-7		0.0%	100.0%	0.0%	0.0%	100.0%	N.D.	N.D.
47-26		0.0%	100.0%	0.0%	0.0%	100.0%	N.D.	N.D.

Clone	Origin	CD2	CD4	CD8	CD16	CD25	CD28	CD40L
10-10-3	PB	100.0%	0.0%	19.3%	0.0%	70.3%	0.0%	N.D.
10-10-5		N.D.	0.0%	57.8%	0.0%	88.0%	0.0%	N.D.
10-10-8		N.D.	0.0%	60.0%	0.0%	POS	0.0%	N.D.
10-50-2		N.D.	0.0%	68.7%	100.0%	75.0%	0.0%	N.D.
10-50-3		N.D.	0.0%	90.0%	N.D.	N.D.	N.D.	N.D.
44-16	PB	100.0%	16.0%	33.5%	0.0%	15.2%	30.2%	0.0%
44-27		100.0%	0.0%	19.0%	0.0%	29.5%	0.0%	78.0%
44-40		100.0%	100.0%	0.0%	0.0%	100.0%	0.0%	35.0%
44-42		100.0%	3.0%	16.4%	0.0%	4.0%	20.0%	0.0%
46-7	CSF	N.D.	N.D.	0.0%	N.D.	N.D.	N.D.	0.0%
46-14		N.D.	N.D.	0.0%	N.D.	N.D.	N.D.	0.0%
46-21		N.D.	N.D.	0.0%	N.D.	N.D.	N.D.	0.0%
46-26		100.0%	0.0%	11.9%	0.0%	41.9%	20.9%	53.3%
47-5	PB	100.0%	0.0%	4.1%	N.D.	7.0%	0.0%	90.0%
47-6		100.0%	0.0%	0.0%	N.D.	34.4%	0.0%	86.2%
47-7		100.0%	4.0%	20.0%	N.D.	92.3%	0.0%	34.4%
47-26		100.0%	7.6%	10.9%	N.D.	89.3%	0.0%	0.0%

Table 14. Continued

Clone	Origin	CD40L	CD45RA	CD45RO	CD56	CD94[1]
10-10-3	PB	N.D.	0.0%	22.1%	93.3%	51.3%
10-10-5		N.D.	0.0%	58.0%	100.0%	N.D.
10-10-8		N.D.	0.0%	100.0%	POS	N.D.
10-50-2		N.D.	0.0%	88.9%	100.0%	N.D.
10-50-3		N.D.	N.D.	N.D.	N.D.	N.D.
44-16	PB	0.0%	0.0%	100.0%	84.0%	12.9%
44-27		78.0%	0.0%	100.0%	34.0%	0.0%
44-40		35.0%	0.0%	100.0%	77.0%	17.7%
44-42		0.0%	0.0%	100.0%	46.0%	0.0%
46-7	CSF	0.0%	N.D.	N.D.	N.D.	N.D.
46-14		0.0%	N.D.	N.D.	N.D.	N.D.
46-21		0.0%	N.D.	N.D.	N.D.	N.D.
46-26		53.3%	0.0%	100.0%	N.D.	10.3%
47-5	PB	90.0%	N.D.	N.D.	N.D.	3.7%
47-6		86.2%	N.D.	N.D.	N.D.	7.7%
47-7		34.4%	N.D.	N.D.	N.D.	46.1%
47-26		0.0%	N.D.	N.D.	N.D.	7.8%

Clone	Origin	CD95	CD152 (CTLA-4)	HLA-DR	HLA-ABC	PERFORIN
10-10-3	PB	100.0%	0.0%	72.3%	100.0%	0.0%
10-10-5		100.0%	N.D.	90.4%	100.0%	N.D.
10-10-8		POS	N.D.	100.0%	100.0%	N.D.
10-50-2		97.0%	N.D.	100.0%	100.0%	N.D.
10-50-3		N.D.	N.D.	N.D.	N.D.	N.D.
44-16	PB	100.0%	0.0%	100.0%	100.0%	0.0%
44-27		100.0%	0.0%	56.5%	100.0%	N.D.
44-40		100.0%	0.0%	100.0%	100.0%	N.D.
44-42		0.0%	0.0%	51.0%	100.0%	0.0%
46-7	CSF	N.D.	N.D.	N.D.	N.D.	N.D.
46-14		N.D.	N.D.	N.D.	N.D.	N.D.
46-21		N.D.	N.D.	N.D.	N.D.	N.D.
46-26		100.0%	N.D.	89.3%	100.0%	N.D.
47-5	PB	100.0%	N.D.	98.9%	N.D.	N.D.
47-6		100.0%	N.D.	95.3%	N.D.	N.D.
47-7		100.0%	N.D.	76.9%	N.D.	N.D.
47-26		100.0%	N.D.	76.2%	N.D.	N.D.

Clone		CD94[2]	CD158a	CD158b	NKB1P
47-5	PB	7.0%	0.0%	0.0%	0.0%
47-6		10.8%	0.0%	0.0%	0.0%
47-7		57.9%	0.0%	0.0%	0.0%
47-26		15.2%	0.0%	0.0%	0.0%

primarily responsible for down regulating T cell responses, the constitutive lack of this molecule suggests it may play a role in the HVS induced growth transformation process. Similarly, clone 44-42 was unique in its lack of the apoptosis marker, CD95, which again may be implicated in its immortalised nature. Increasing evidence supports close parallels between γδ T cell effector functions and NK cells and a survey of the *t*-γδ T cell clones listed in Table 14 identified avenues to address these similarities. The majority of the *t*-γδ T cell lines and clones analysed to date did not express Fc receptors (FcRIII), however clone 10-50-2 was found to constitutively express FcRIII, lending it for further investigations into Fc mediated events such as antibody dependent cell cytotoxicity. A current paradox in MS pathogenesis, is the apparent promiscuous cytotoxic nature of γδ T cells towards CNS glial cells healthy or otherwise (117). One possible avenue of control stems from NK-like killer inhibitory receptors (KIR) such as CD94 which was found extensively expressed on a number of *t*-γδ T cell clones. To date, other KIRs (CD158a and b, NKB1P) have not been found on a limited panel of screened *t*-γδ T cell clones. Despite being indirectly implicated in the mechanism of γδ T cell killing (127), perforin was never detected using the mAb Delta G9 in combination with intracytoplasmic staining of *t*-γδ T cell lines or clones.

In summary, the results of characterisation studies on human PB and CSF derived *t*-γδ T cell lines and clones has extensively broadened the knowledge base of these poorly understood and described cells. Rare γδ T cell subtypes have been identified and panels of immortalised clones displaying unique phenotypes have been isolated allowing for correlation studies between phenotype and γδ T cell function to be performed.

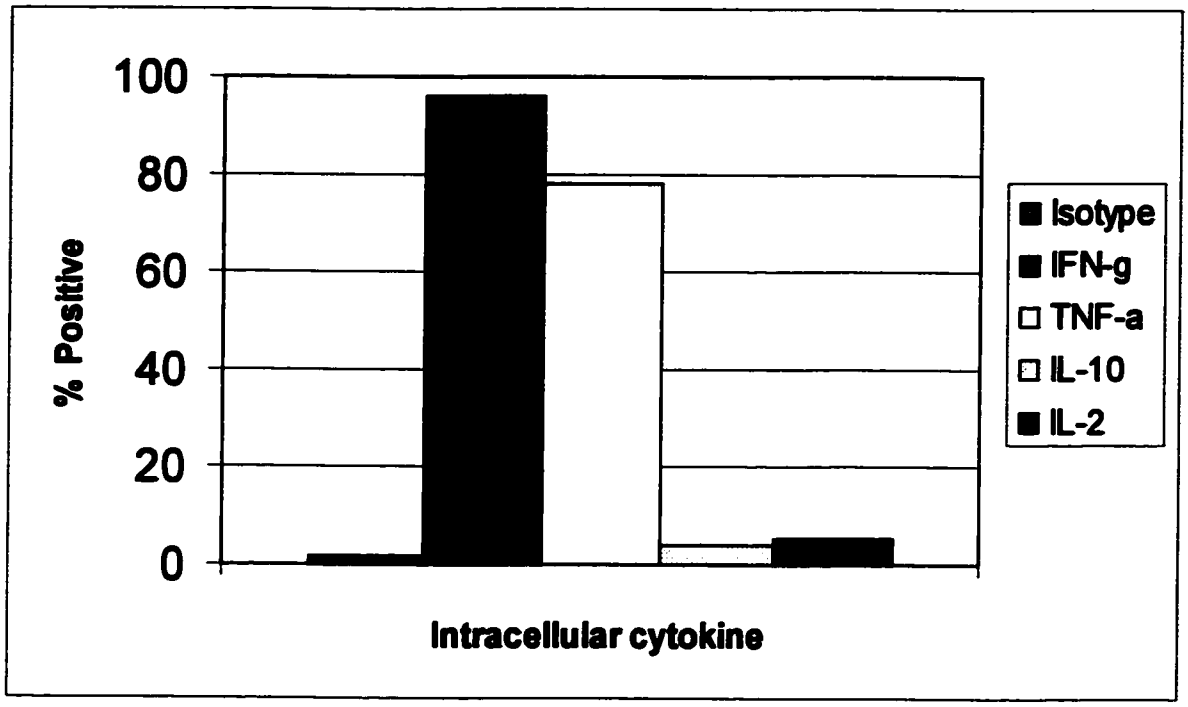
3.4.2 MS transformed γδ T cells: Cytokine profiles

The focus of γδ T cell research has centred primarily on their cytotoxic abilities, however their potential role in immunomodulation is now being recognised. In particular, the potential role of CNS γδ T cells in terms of immunomodulation and resulting MS pathogenesis is not known. To address this question, PMA/ Ca^{2+} ionophore stimulated *t*-γδ T cell lines and clones were evaluated for their IC production of relevant cytokines. The intracytoplasmic cytokine profile

depicted in Figure 23 was representative of all *t*-γδ T cell lines analysed to date. Upon stimulation, *t*-γδ T cells were strongly positive for the proinflammatory cytokines IFN-γ and TNF-α and produced little anti-inflammatory cytokine such as IL-10 or IL-4. *T*-γδ T cells interestingly did not generate significant levels of the autocrine growth factor IL-2 as assessed in a number of experiments, thus explaining their complete reliance on exogenous IL-2 addition for growth. Although the transformation process may skew cytokine production towards a proinflammatory phenotype (202), parental non-transformed γδ T cells were also predominantly proinflammatory in nature. Correlation of Vδ subtype with IC production on polyclonal *t*-γδ T cell lines was difficult as illustrated in Figure 24, owing in large part to the small numbers of non-Vδ2 subtypes present in most samples. IFN-γ and TNF-α positive cells were marginally lower in the Vδ1 vs. Vδ2 subset (IFN-γ: 22.2 vs. 33.5%; TNF-α: 33.5 vs. 45.8% respectively) but the relative trend was similar and comparable to the whole mixture. Vδ1 positive cells also displayed a small amount of IL-4 positivity (9.8%) which was not seen in either the Vδ2 or the complete mixture, however this number was based on a small number of Vδ1 positive cells (3% of total) and consequently was associated with greater uncertainty. A more accurate manner to address correlations between γδ T cell phenotype and IC production was to compare IC profiles of phenotypically defined *t*-γδ T cell clones (Table 15). Although data was collected in three separate experiments, the overwhelming trend was strong production of both IFN-γ and TNF-α regardless of VγVδ subtype. Clone 10-10-3 (Vγ4Vδ?) and 10-50-2 (Vγ?Vδ1) represented two unique VγVδ subtypes with essentially similar surface molecule expression with the exception of CD16 (Table 14). Regardless, ~90% of both clones were IFN-γ positive producing similar levels of cytokine (MCF: 6.2 vs. 7.5) after PMA/ Ca^{2+} ionophore stimulation (Table 15). Levels of IL-2 and the representative Th2 cytokine, IL-4, were low to none. Within the 44-series of clones, representing three other distinct VγVδ phenotypes, 44-16 (Vγ4Vγ9Vδ1) was markedly more TNF-α positive (99.2%) relative to 44-27 (48.3%; Vγ9Vδ?) and 44-42 (48.0%; Vγ9Vδ3). IFN-γ positivity was similar for 44-16 and 44-27 (90.3 vs. 92.4%) and moderately lower for clone 44-42 (75%). However, relative cytokine levels per cell (MCF values) indicated the concentration of IFN-γ per

Figure 23. Intracytoplasmic cytokine profile for representative proinflammatory and anti-inflammatory cytokines within a PMA/ Ca^{2+} ionophore stimulated CSF *t*-γδ T cell line. The CSF *t*-γδ T cell line was rested in IL-2 free medium for 48 h prior to stimulation with PMA/ Ca^{2+} ionophore in the presence of Brefeldin A for 6 h, followed by γδ TcR surface staining and fixation. Permeabilised cells were treated with optimised amounts of fluorochrome labelled anti-cytokine mAbs as indicated and data acquired by FACS analysis. Results are presented either as the % of γδ T cells positive for cytokine (Panel A) or as relative changes in cytokine level (Panel B). Negative controls consisted of treating cells with labelled isotype control mAbs in a similar fashion.

(A)


100
80
60
40
20
0
% Positive
Isotype
IFN-g
TNF-a
IL-10
IL-2
Intracellular cytokine

(B)

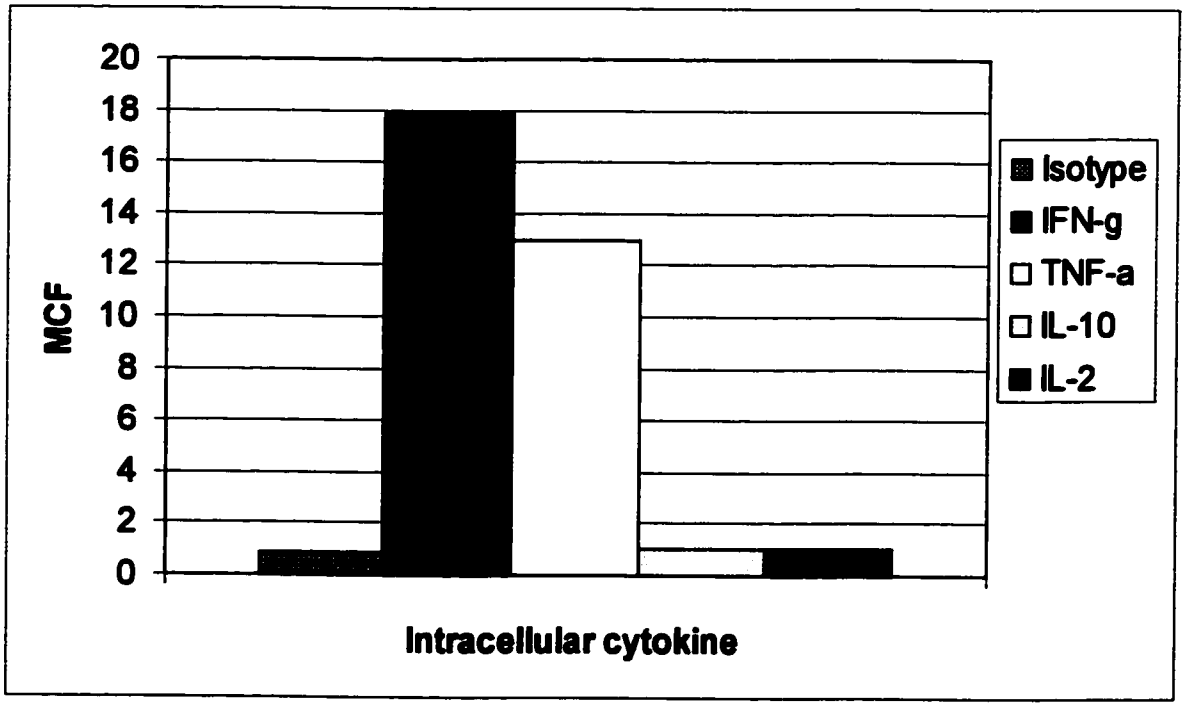

20
18
16
14
12
10
8
6
4
2
0
MCF
Isotype
IFN-g
TNF-a
IL-10
IL-2
Intracellular cytokine

Figure 24. Expression of intracytoplasmic cytokine by γδ TcR subtype populations present within a PB *t*-γδ T cell line. A PB *t*-γδ T cell line, rested for 48 h in IL-2 free medium, was stimulated with PMA/ Ca^{2+} ionophore for 6 h in the presence of Brefeldin A. Stimulated cells were surfaced stained with either pan γδ TcR, Vδ1 specific or Vδ2 specific mAbs, followed by fixation. Permeabilised cells were treated with fluorochrome labelled IFN-γ, TNF-α, or IL-4 and data acquired by FACS. The small percentage of Vδ1 positive cells (3%) present within the polyclonal line resulted in gating difficulties and correspondingly, larger uncertainties in the numbers of cytokine positive Vδ1 cells.

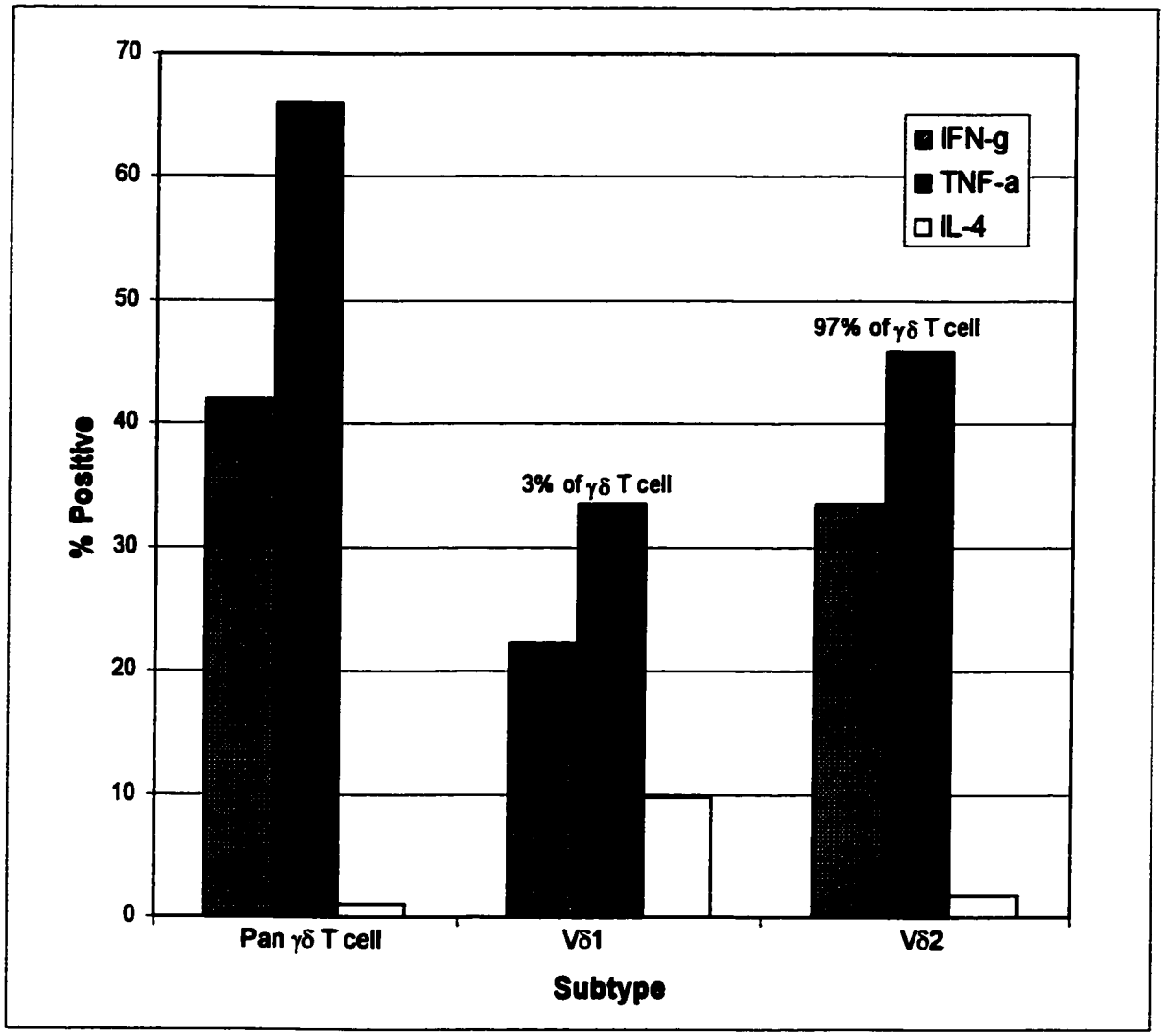

70
60
50
40
30
20
10
0
% Positive
IFN-g
TNF-a
IL-4
97% of γδ T cell
3% of γδ T cell
Pan γδ T cell
Vδ1
Vδ2
Subtype

Table 15. Intracytoplasmic cytokine analysis of phenotypically defined *t*-γδ T cell clones. Intracytoplasmic cytokine profiles of pure subtype defined *t*-γδ T cell clones were determined upon PMA/ Ca^{2+} ionophore stimulation using standard conditions and FACS analysis. Data is expressed both as the cytokine positive fraction of the *t*-γδ T cell clones as well as the relative change in cytokine level after stimulation. Vγ?: non- Vγ4 or 9; Vδ?: non- Vδ1, 2, or 3.

Clone	VγVδ subtype	IFN-γ		TNF		Other		
		%positive	MCF	%positive	MCF	cytokine	%positive	MCF
10-10-3	Vγ4Vδ?	88.5	6.2	78.5	8.9	IL-2	6.2	0.23
						IL-4	0.6	0.28
10-50-2	Vγ?Vδ1	89.5	7.5	N.D.	N.D.	IL-4	0.8	0.17
44-16	Vγ4Vγ9Vδ1	90.3	10.5	99.2	57.4	N.D.		
44-27	Vγ9Vδ?	92.4	9.7	48.3	4.6	N.D.		
44-42	Vγ9Vδ3	75.0	5.1	48.0	5.2	N.D.		
46-26	Vγ9Vδ1	97.5	10.1	91.8	17.6	IL-10	23.4	0.99
47-6	Vγ9Vδ?	95.5	16.8	85.7	16.0	IL-10	7.9	0.61
47-26	Vγ9Vδ2	92.7	7.2	92.0	16.9	IL-10	1.3	0.37

44-16 or 44-27 clone was twice the level found in 44-42 (10.5 and 9.7 vs. 5.1), despite comparable numbers of cells positive for IFN-γ. Similarly, clone 44-16 produced markedly more TNF-α per cell relative to 44-27 and 44-42 (MCF: 57.4 vs. 4.6 and 5.2). A number of *t*-γδ T cell clones demonstrated a natural tendency to produce IFN-γ despite the adverse condition of IL-2 starvation. Figure 25 depicts the production of IFN-γ and TNF-α after 48 h of culture without IL-2 prior to and post PMA/ Ca^{2+} ionophore stimulation. The numbers of spontaneous TNF-α producing cells were low for both clones 44-42 and 44-27 (1.3 and 2.1% respectively) while only clone 44-42 showed low numbers of basal IFN-γ producing cells (5.9%). Comparatively, there were significant numbers of IFN-γ producing 44-27 cells (58.1%) prior to any stimulation.

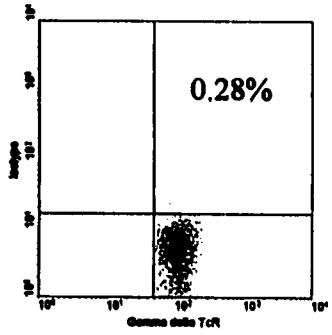
A third series of *t*-γδ T cell clones encompassing three distinct VγVδ subtypes including the PB dominant Vγ9Vδ2 subtype were compared for IC profiles upon PMA/ Ca^{2+} ionophore stimulation (Table 15). All three clones, regardless of VγVδ phenotype behaved in a similar manner exhibiting virtually identical IC profiles. The lone exception was found with clone 46-26 (Vγ9Vδ1) which was slightly IL-10 positive (23.4 vs. 7.9 and 1.3% for 46-26 vs. 47-6 and 47-26 respectively) but in terms of cytokine produced per cell was only marginally higher (0.99 vs. 0.61 and 0.37 MCF). This series of clones is dealt with in more detail in Section 3.5.2.

In summary, overwhelmingly all transformed γδ T cell lines or clones displayed IC profiles that were strongly proinflammatory (IFN-γ and TNF-α) rather than anti-inflammatory (IL-4 or IL-10) and in general, produced little to no IL-2. There were no apparent correlations between VγVδ phenotype and the type of IC cytokine produced, from data obtained in three separate series of *t*-γδ T clones analysed. Differences were seen however, in terms of the level of cytokine produced per cell with some clones producing markedly more cytokine compared to their siblings. Whether these differences are correlative to VγVδ phenotype can only be addressed upon further comparisons.

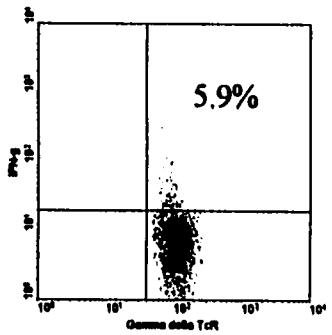
Figure 25. Flow cytometric profiles illustrating the tendency for high basal production of IFN-γ by some *t*-γδ T cells . Two sibling *t*-γδ T cell clones were rested in IL-2 free medium for 48 h. Portions of the rested clones were either stimulated with PMA/ Ca^{2+} ionophore or medium alone, both in the presence of Brefeldin A for 6 h, followed by γδ TcR surface staining and fixation. Permeabilised cells were stained with either fluorochrome labelled IFN-γ or TNF-α, or matched non-specific isotype controls, and data was acquired by FACS analysis. The cytokine positive fractions of the *t*-γδ T cell clones are indicated in the upper right-hand quadrants.

44-42:

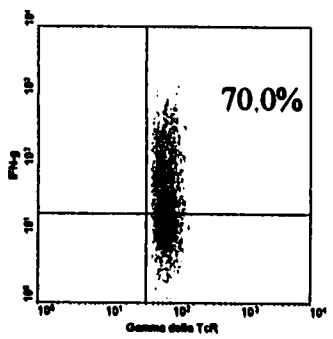
Isotype control



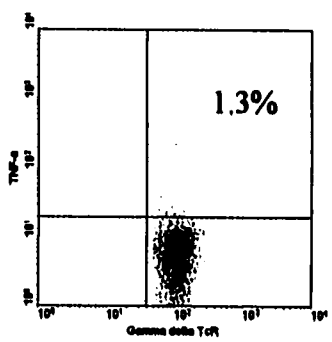
Spontaneous IFN-γ



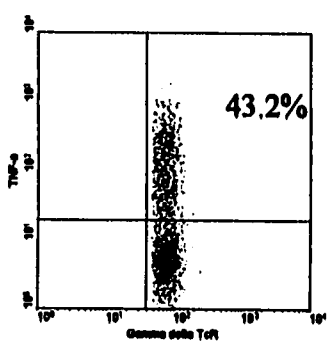
IFN-γ



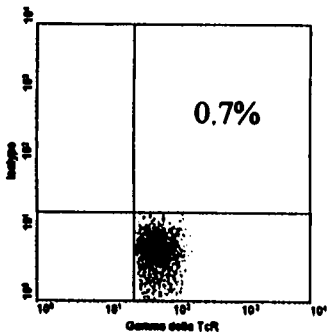
Spontaneous TNF-α

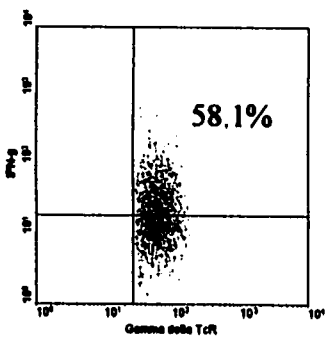


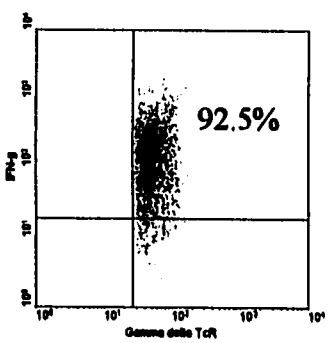
TNF-α

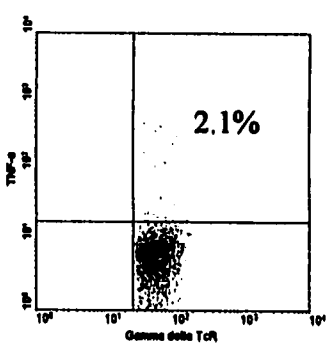


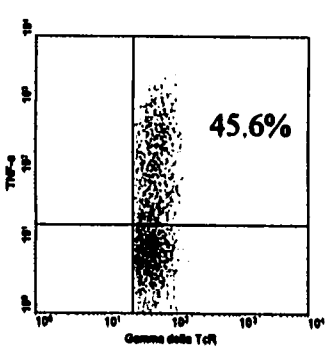
44-27:











3.4.3 MS transformed γδ T cells: Cytotoxicity

Interpretation of results from short term γδ T cell cytotoxicity studies towards relevant targets (Section 3.1.4) were hindered in part by the high variability encountered. Specifically, it was difficult to determine whether the induced cytotoxicity was due to certain γδ T cell subtypes or cytotoxic subpopulations within the heterogeneous sample or whether all γδ T cells within the short term line behaved as one. The panel of phenotypically defined *t*-γδ T cell clones was screened for cytotoxicity towards a number of different cell targets in an effort to address this question. As a first measure, the cytotoxicity of three *t*-γδ T cell clones towards Daudi B cells (old lot- see below) was directly compared to a polyclonal short term γδ T cell line (Figure 26). Clone 10-10-3 data was acquired in a separate experiment using similar assay conditions but the reproducible nature of the JAM assay enabled this type of comparison. γδ T cell subtypes based on VγVδ expression (Table 14) clearly showed no marked correlation to Daudi B cell cytotoxicity by the different clones and cytotoxicity was comparable to that generated by the polyclonal, predominantly Vγ9Vδ2 expressing short term γδ T cell line (% kill at E:T= 6.3: 11.7, 14.6, 17.3 for clones 10-5, 50-2, line HOD respectively; % kill at E:T= 8.8: 13 for 10-3). Cytotoxic potentials of the *t*-γδ T cell clones showed no correlation to the variable expression of CD8 on the cell surface (Table 14). Cytotoxic profiles were similar for 10-10-5 and 10-50-2 which expressed abundant CD8 (~60-70% of cells) and 10-10-3 which expressed relatively little CD8 (19%) suggesting that CD8 restriction was not a factor or CD8 levels were sufficiently expressed for effector function. Evidence for the former situation was seen in MHC class I blocking studies using either normally poorly killed Colo-205 cells or highly sensitive U937 target cells. In both circumstances, target susceptibility was unaffected by prior blocking with saturating amounts of an anti-HLA-ABC mAb when compared to targets treated with control IgG1 (Colo-205 at E:T= 10: IgG 41.2, Class I 40.4% kill; U937 at E:T= 2.5: IgG 51.3, Class I 54.2% kill; Figure 27). Similarly, the ability of the alternate MHC class I ligand, CD94 and abundantly expressed on clone 10-10-3 (51%), to modulate cytotoxicity, was not evident from these same blocking studies. Cytotoxicity

Figure 26. Evaluation of possible γδ TcR subtype to cytotoxicity correlations using a series of distinct PB *t*-γδ T cell clones and a short term PB γδ T cell line. *t*-γδ T cell clones with defined γδ TcR expression as indicated, together with a short term γδ T cell line consisting primarily of Vγ9Vδ2 cells were evaluated for their cytotoxic responses towards Daudi B cell lymphoma targets in a standard 4 h JAM assay. The level of kill represents the mean ± SD of quadruplicate wells. Clone 10-10-3 was evaluated in a separate experiment using similar conditions. Vδ?: non-Vδ1,Vδ, or Vδ3; Vγ?: non-Vγ4 or Vγ9.

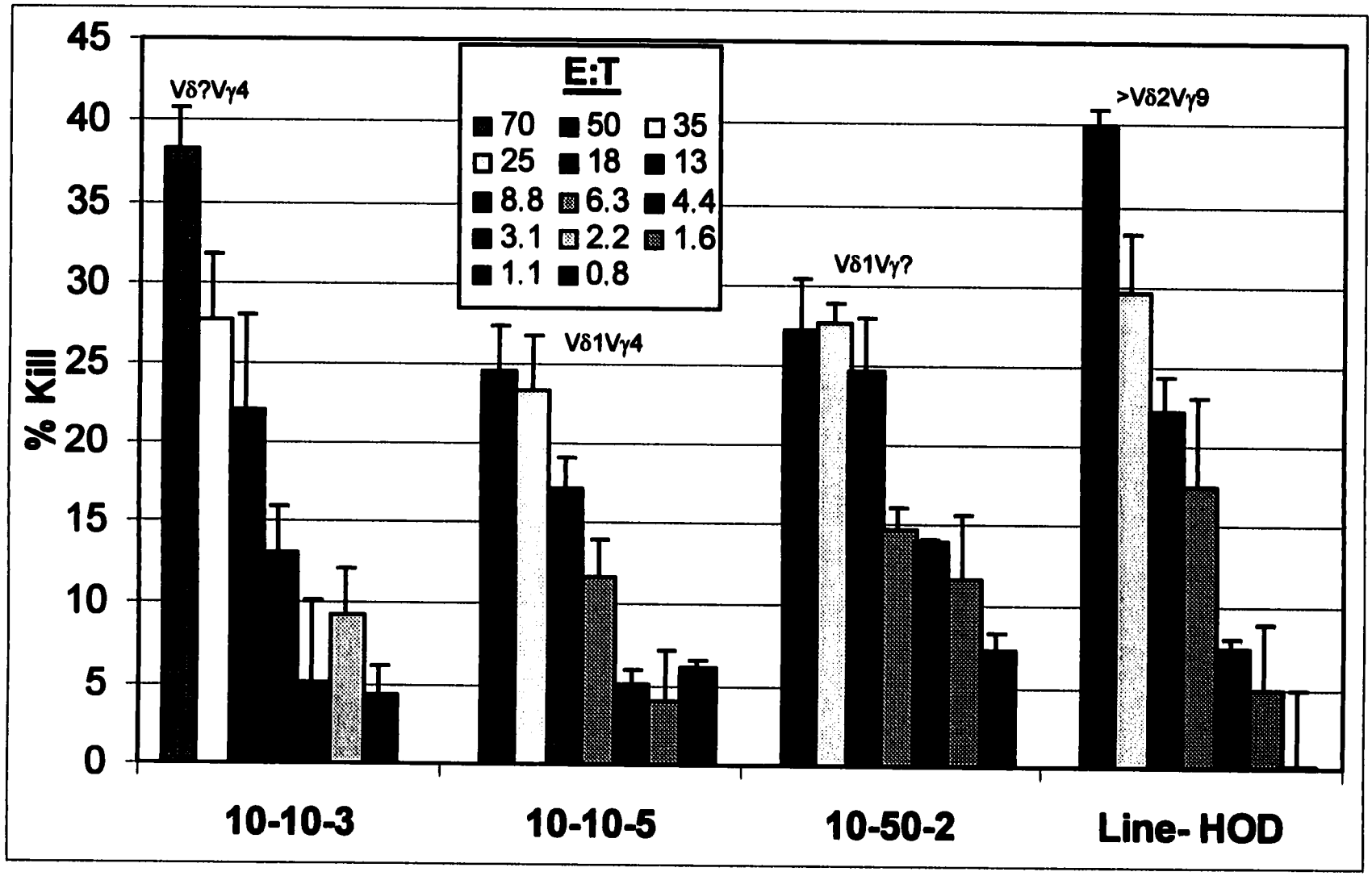
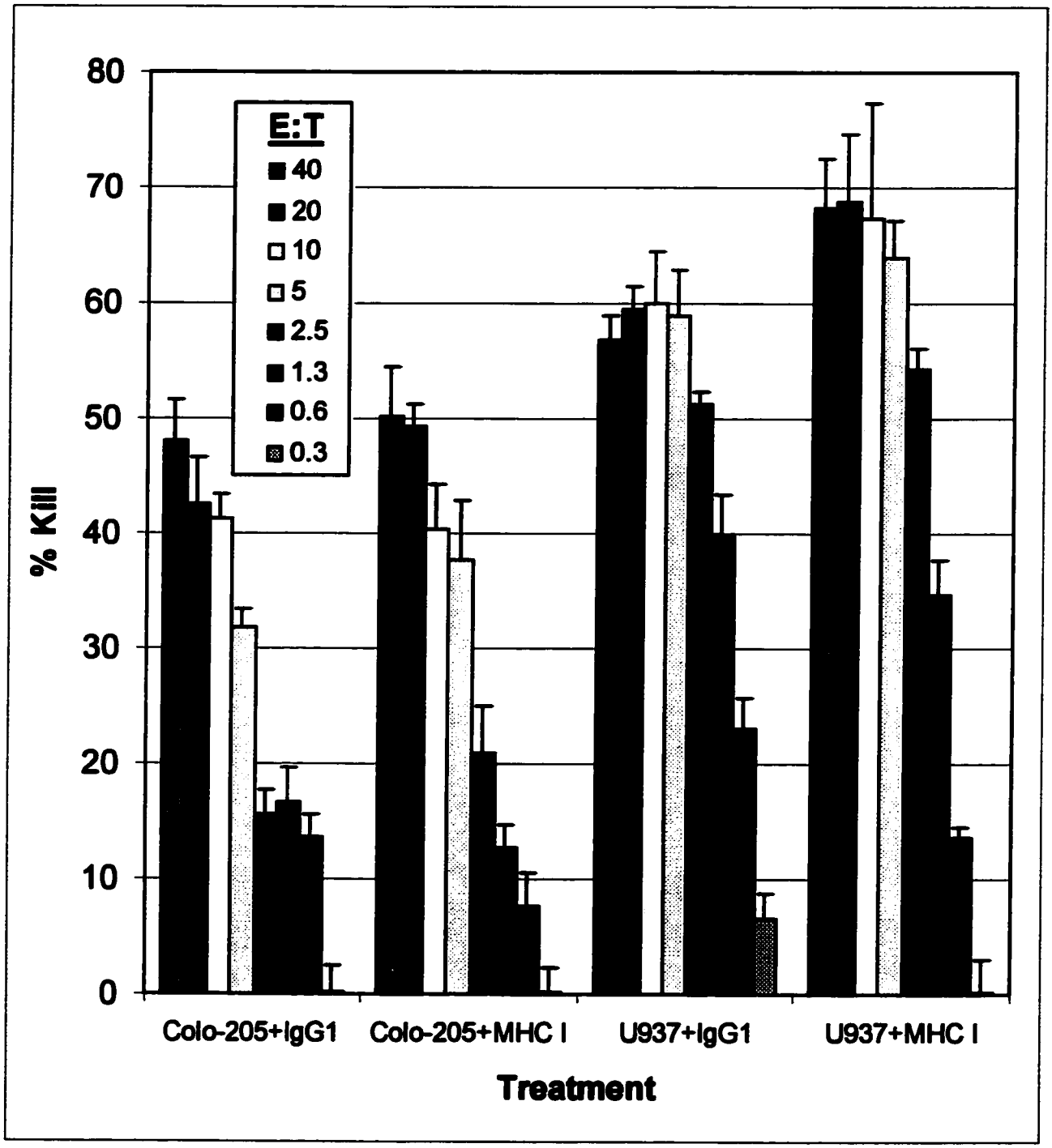

45
40
35
30
25
20
15
10
5
0
% Kill
E:T
70 50 35
25 18 13
8.8 6.3 4.4
3.1 2.2 1.6
1.1 0.8
Vδ?Vγ4
Vδ1Vγ4
Vδ1Vγ?
>Vδ2Vγ9
10-10-3
10-10-5
10-50-2
Line- HOD

Figure 27. The effect of MHC Class I blocking on the cytotoxic response towards two tumour cell targets by a PB *t*-γδ T cell clone. [*methyl*-^{3}H] thymidine labelled Colo-205 and U937 target cells were incubated with saturating amounts of either anti-HLA-ABC mAb (1 μg/ 200K cells) or mouse IgG1 as negative controls, for 1 h prior to their use as targets in a 4 h JAM assay using a PB *t*-γδ T cell clone (10-10-3) as effectors. The results are representative of the mean kill ± SD of quadruplicate wells.

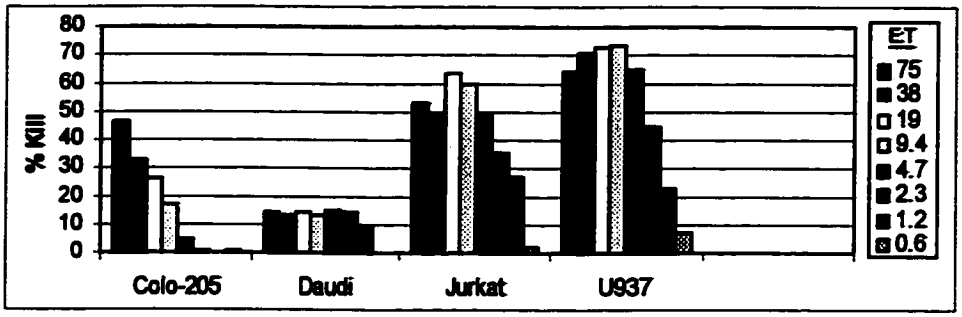

80
70
60
50
40
30
20
10
0
% Kill
E:T
40
20
10
5
2.5
1.3
0.6
0.3
Colo-205+IgG1
Colo-205+MHC I
U937+IgG1
U937+MHC I
Treatment

comparisons were further expanded by profiling the cytotoxicities of a second series of *t*-γδ T cell clones towards a tumour cell panel (Figure 28). Overall, the *t*-γδ T cell clones were cytotoxic to all targets, however Daudi B cell killing was surprisingly poor. Although this consistent low killing of Daudi cells cannot be explained at this time, the change in sensitivity occurred only after new Daudi stocks (from ATCC) were introduced for routine work. U937 monocytes and Jurkat T cells were the most sensitive γδ T cell targets followed by the KG-1 myeloblasts. The colorectal carcinoma, Colo-205, displayed the lowest sensitivity to *t*-γδ T cell killing apart from the aberrant Daudi B cells. Over the range of E:T used, some clones displayed high levels of killing towards their targets that did not titrate down significantly (i.e. clone 44-27 against Jurkat). Subsequent assays over a broader range of E:T, reproduced consistently the pattern of kill seen in Figure 28 but also titrated down as expected when E:Ts were lowered to smaller values. For comparative purposes, the % Kill for each clone at an E:T of 3.2 (2.3 for the clone 10-10-3) was plotted against cell type (Figure 29). Although direct comparisons between 10-10-3 and the 44-series of clones cannot be made based on the differing E:T ratios used, the pattern of kill followed that seen with the 44-series of clones. Uniformly across the various cell targets, 44-27 (Vδ?Vγ9) and 44-16 (Vδ1Vγ4Vγ9) displayed greater killing potentials relative to the remaining three clones (Colo-205: 43, 29 vs. 11, 11 and 1%; Jurkat: 63, 62, vs. 39, 21 and 35%; U937: 74, 74, vs. 57, 41 and 44%; KG-1: 52, 34, vs. 15 and 19% for 44-27, 44-16, 44-42, 44-40, and10-10-3 respectively). Clone 44-40 (Vδ3Vγ4) uniformly displayed the poorest killing potential and interestingly was the only clone with a CD4 phenotype. Similar studies were performed on a series of CSF and PB derived *t*-γδ T cell clones, the results of which can be found in Section 3.5.3.

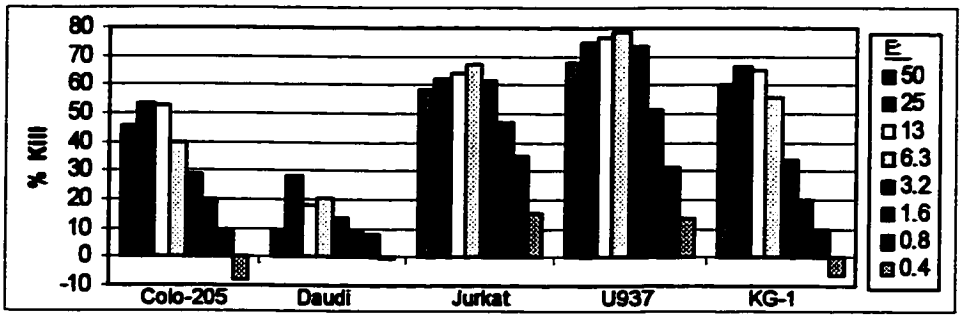
Studies with *t*-αβ T cells suggested previously non-cytolytic αβ T cells acquired a non-specific cytolytic ability after transformation due to hyperreactivity mediated between target cell CD58 interaction with *t*-αβ T cell CD2 molecules (186). To address this issue with *t*-γδ T cells, a series of blocking experiments were performed specifically disrupting the CD2:CD58 interaction between effector and target cells using blocking CD2 and CD58 mAbs at concentrations shown to be effective with *t*-αβ T cells, and assessing their effect on cytotoxicity in JAM assays (Figure 30).

Figure 28. Further evaluation of possible γδ TcR subtype to cytotoxicity correlations using a series of distinct PB *t*-γδ T cell clones towards a tumour cell target panel. *t*-γδ T cell clones with defined γδ TcR expression as indicated were evaluated for their cytotoxic responses towards either Colo-205, Daudi, Jurkat, U937, or KG-1 cell targets in a standard 4 h JAM assay. The level of kill represents the mean of quadruplicate wells. Clone 10-10-3 was evaluated in a separate experiment using similar conditions. Vδ?: non-Vδ1,Vδ, or Vδ3; Vγ?: non-Vγ4 or Vγ9.

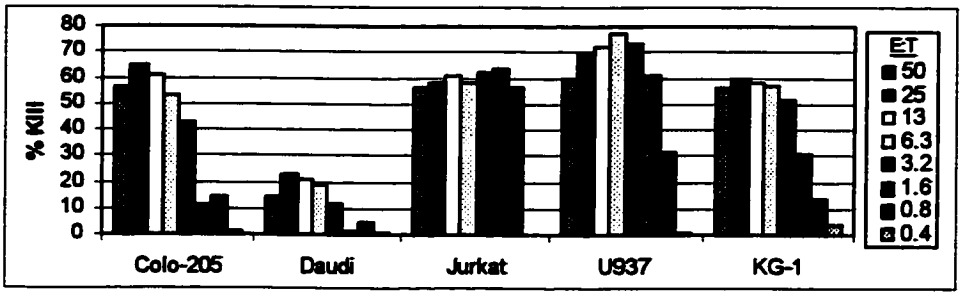
A) Vδ?Vγ4
clone 10-10-3



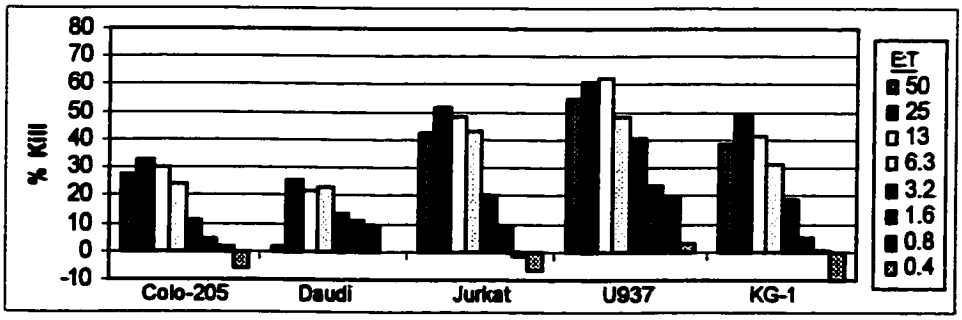
B) Vδ1Vγ4Vγ9
clone 44-16



C) Vδ?Vγ9
clone 44-27



D) Vδ1Vγ4
clone 44-40



E) Vδ3Vγ9
clone 44-42

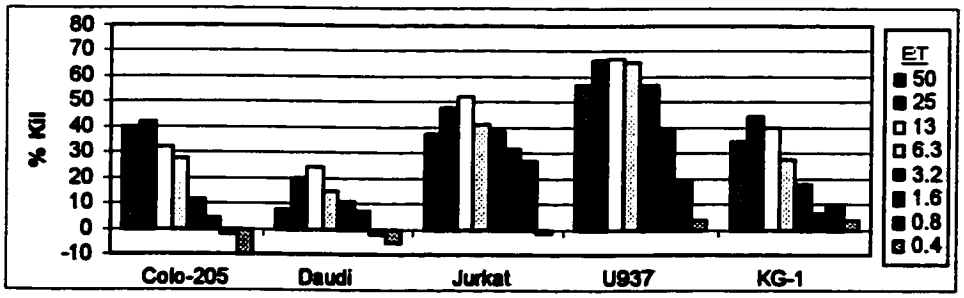


Figure 29. Relative cytotoxic responses at a single E:T ratio for the series of phenotypically distinct *t*-γδ T cell clones and tumour cell targets depicted in Figure 29. The % kill for the five *t*-γδ T cell clones was determined at an E:T of 3.2 with the exception of clone 10-10-3 which was determined at an E:T of 2.3 due to data acquisition in a separate experiment. The data as shown represent the mean % kill ± SD of 5 replicate wells.

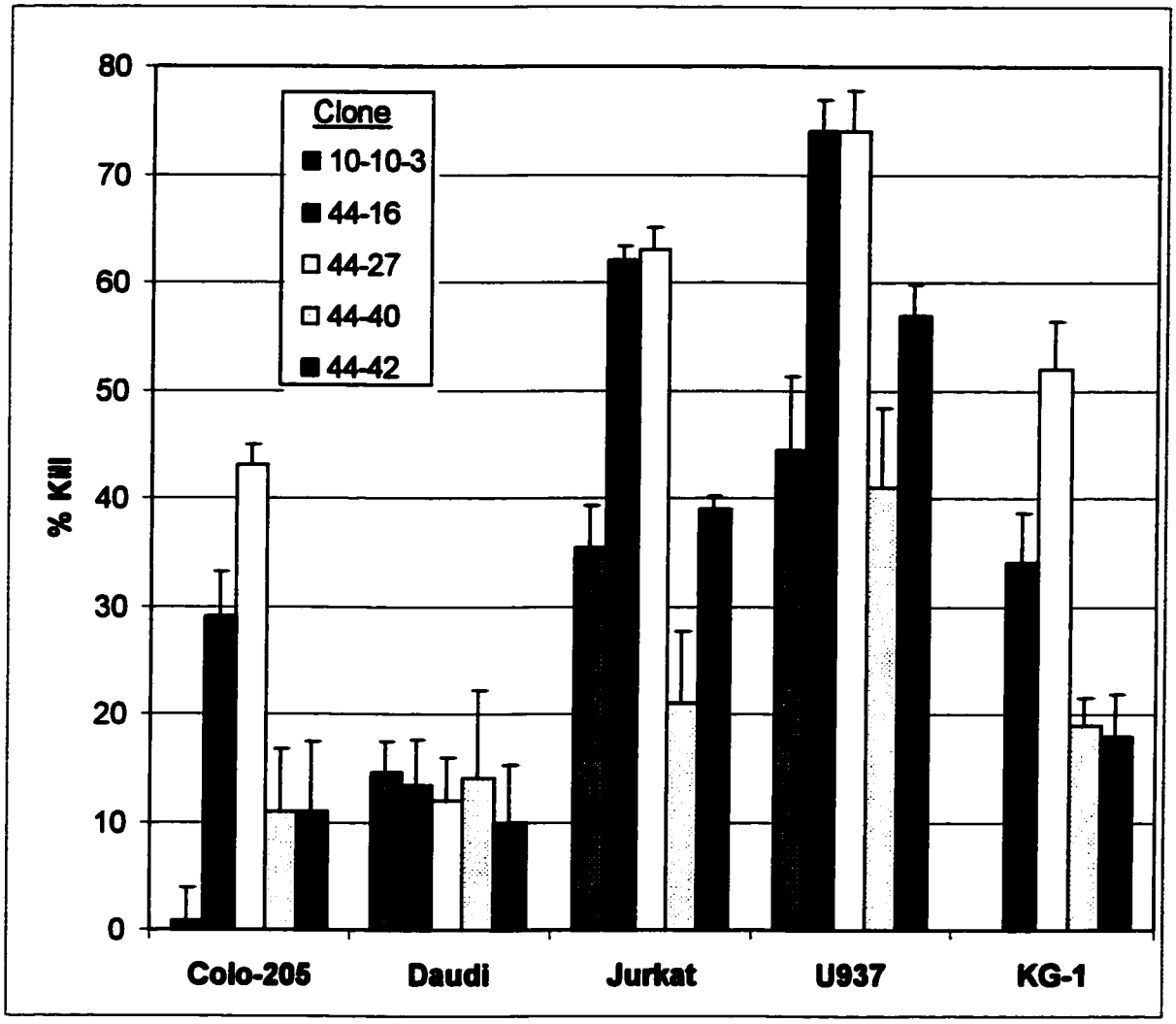
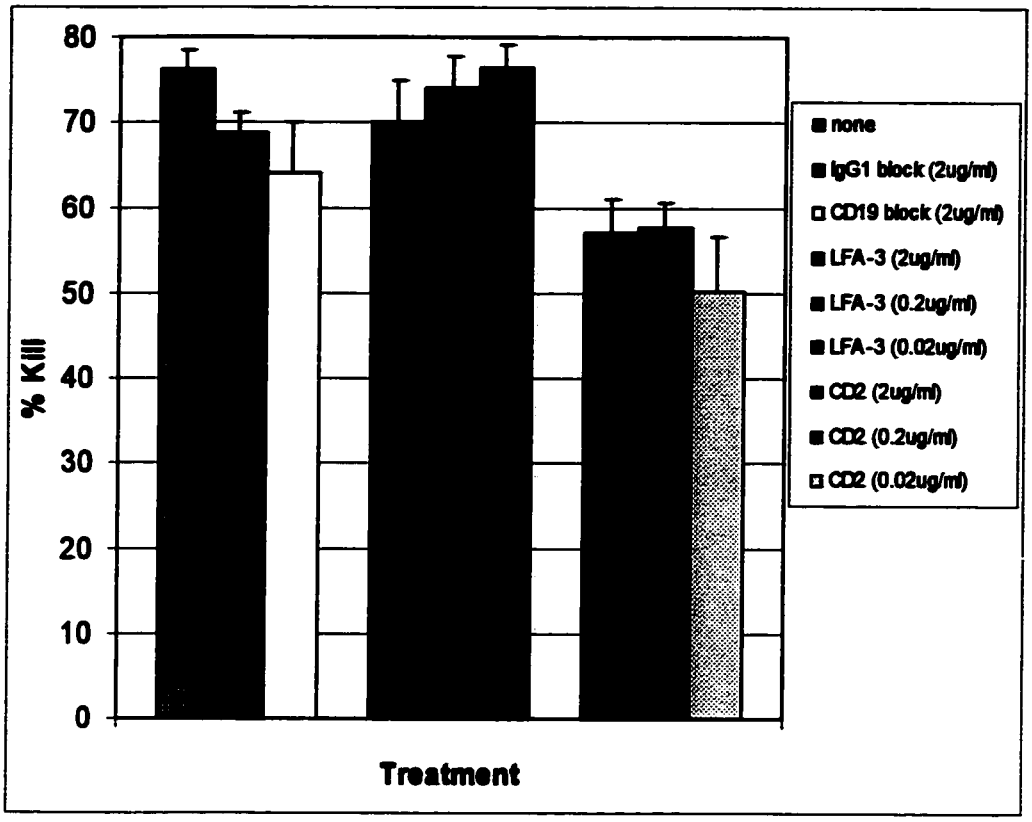
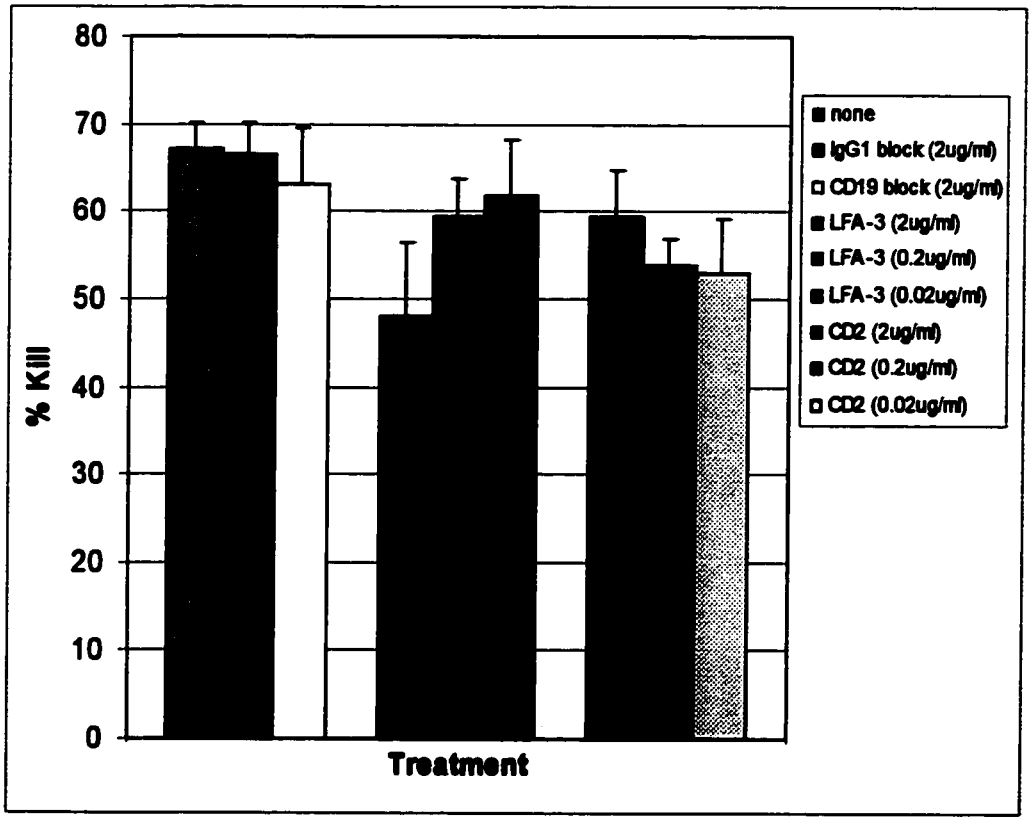

80
70
60
50
40
30
20
10
0
% Kill
Clone
10-10-3
44-16
44-27
44-40
44-42
Colo-205
Daudi
Jurkat
U937
KG-1

Figure 30. The effect of the CD2/ CD58 activation pathway on the cytotoxic response of *t*-γδ T cells as assessed through blocking studies. U937 (Panel A) and Jurkat (Panel B) tumour targets were blocked with graded amounts of anti- CD58 mAb prior to determining their susceptibility to lysis by a CSF *t*-γδ T cell line at an E:T ratio of 10:1 in a standard JAM assay. Similarly, the CD2/ CD58 pathway was also disrupted by pre-blocking effector cells with an anti-CD2 mAb. Negative controls consisted of either effector or target cells treated with medium alone, non-specific IgG1, or irrelevant CD19 mAbs. Results as shown represent the mean % kill ± SD of 5 replicate wells.

(A)


80
70
60
50
40
30
20
10
0
% Kill
Treatment
none
IgG1 block (2ug/ml)
CD19 block (2ug/ml)
LFA-3 (2ug/ml)
LFA-3 (0.2ug/ml)
LFA-3 (0.02ug/ml)
CD2 (2ug/ml)
CD2 (0.2ug/ml)
CD2 (0.02ug/ml)

(B)


80
70
60
50
40
30
20
10
0
% Kill
Treatment
none
IgG1 block (2ug/ml)
CD19 block (2ug/ml)
LFA-3 (2ug/ml)
LFA-3 (0.2ug/ml)
LFA-3 (0.02ug/ml)
CD2 (2ug/ml)
CD2 (0.2ug/ml)
CD2 (0.02ug/ml)

Targets treated with blocking CD58 were killed as efficiently as targets treated with medium alone, irrelevant IgG, or CD19 controls (U937: 70% CD58 (2μg) vs. 68% IgG (2μg); Jurkat: 49% CD58 (2μg) vs. 66% IgG (2μg)). Similarly, effector cells treated with blocking CD2 mAb killed U937 and Jurkat targets as efficiently as effector cells treated with irrelevant IgG (U937: 57% CD2 (2μg) vs. 68% IgG (2μg); Jurkat: 59% CD2 (2μg) vs. 66% IgG (2μg)). These results suggest that unlike *t*-αβ T cells, non-specific CD2:CD58 mediated target cell cytotoxicity is not a major mechanism for *t*-γδ T cell killing of target cells. In support of this finding, was the relative expression of surface CD2 and CD58 on the tumour target cell panel as determined by flow cytometry (Table 16).

Table 16. CD2 and CD58 expression levels (MCF) on target cell panel

	Colo-205	Daudi B	Jurkat T	KG-1	U937
CD2 (MCF)	0	0	39	0	0
CD58 (MCF)	1.8	3.1	4.3	2.9	2.2

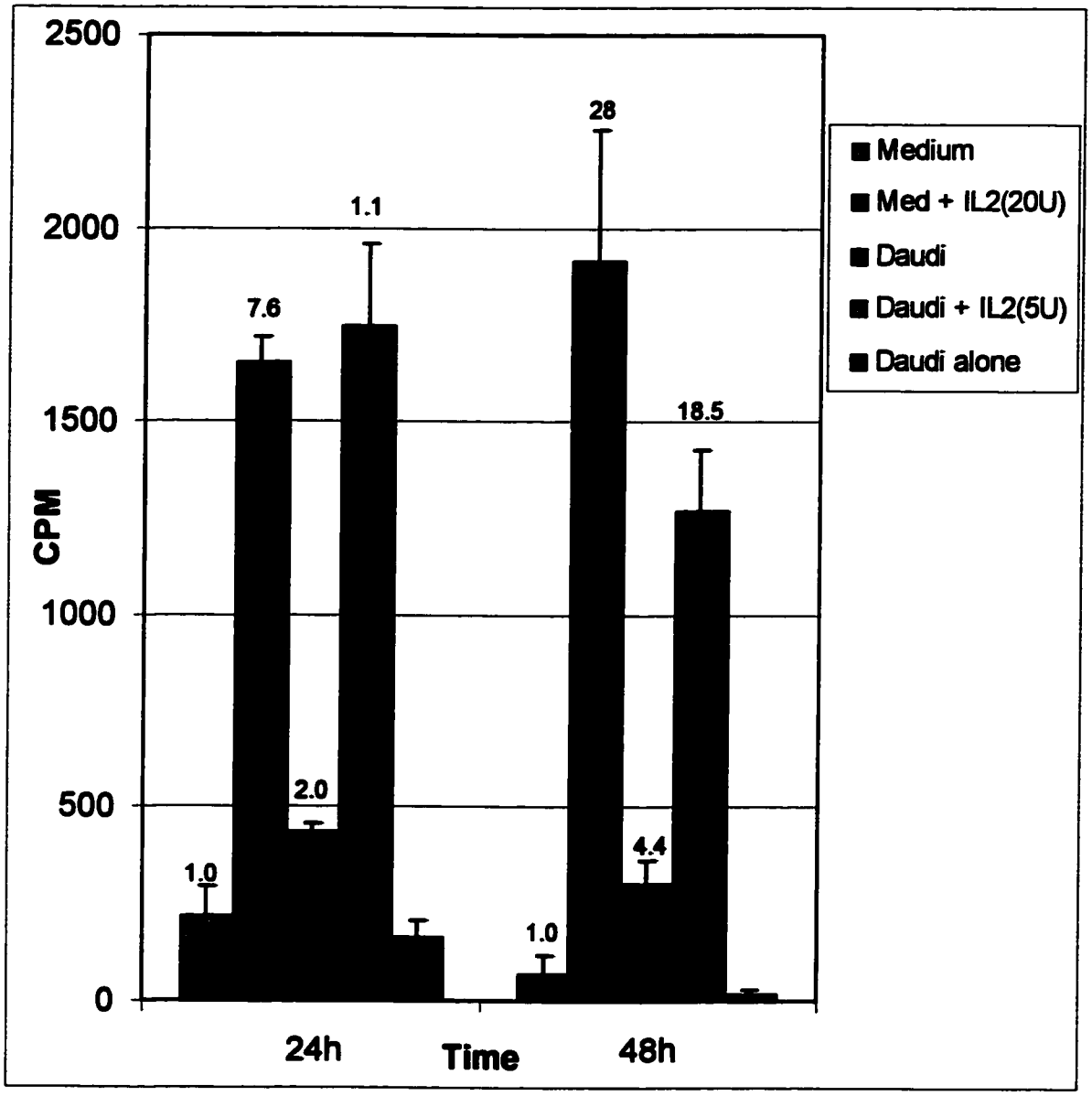
Contrary to the pattern of kill expected if mediated solely based on CD2:CD58 interactions, Daudi B cells displaying the second highest level of CD58 surface molecules, were found to be virtually insensitive to *t*-γδ T cell clone killing while the lowest CD58 expressing target, U937, exhibited the highest degree of sensitivity to *t*-γδ T cell clone cytotoxicity.

In summary, these results indicated that γδ TcR subtype populations uniformly possessed the ability to kill cellular targets and did not display any overt cytotoxicity differences between VγVδ subtypes. The cytotoxic potentials of two clones (44-27: Vδ?Vγ9 and 44-16: Vδ1Vγ4Vγ9) towards a target cell panel consistently killed 2-4-fold greater than other VγVδ subtypes. Preliminary studies suggested that CD8 restriction as well as CD94 modulation were not factors for efficient killing of target cells.

3.4.4 MS transformed cells: Antigen recognition events

Results have now been presented which demonstrated that HVS transformed MS γδ T cells can be used to study effector mechanisms which may be relevant in the MS disease setting. What antigen(s) or type of antigen(s) may trigger these γδ T cell effector functions are largely unknown, especially antigens found within the CNS. It was therefore of interest to examine whether *t*-γδ T cells could be applied to studying antigen recognition events. To accomplish this, it was first necessary to determine an appropriate manner to measure antigen recognition which was further compounded by the lack of known *t*-γδ T cell reactive antigens. Antigen recognition measured by *t*-γδ T cell proliferation and ^{3}[H]- thymidine uptake was found to be problematic due primarily to the poor proliferative capacity of the *t*-γδ T cells without exogenous IL-2. Illustrating this point, clone 10-10-3 rested for 24 h without IL-2, was stimulated with the cytotoxicity target, Daudi B cells (new lot), with and without IL-2 (Figure 31). After 24 h, transformed cells stimulated with the irradiated Daudi B cells had a stimulation index (SI) of 2.0 vs. medium alone which compared to a SI of 7.6 for the positive control (medium + IL-2 (20 U/ml)). Cells incubated in the presence of a small amount of IL-2 (5 U/ml) and irradiated Daudi B cells incorporated thymidine to the same extent as the positive IL-2 (20 U/ml) control (1744 CPM vs. 1651 CPM for Daudi cells + IL-2 (5 U/ml) and medium + 20 U/ml IL-2 respectively). After 48 h of stimulation, SIs for both positive control and irradiated Daudi B cell treatment increased substantially (SI: 28 +'ve control; SI: 4.4 Daudi B cell) due primarily to a lower thymidine incorporation for 10-10-3 treated with medium alone. 10-10-3 stimulated for 48 h with irradiated Daudi B cells and IL-2 (5 U/ml) incorporated thymidine at a level lower than the positive control (1264 vs. 1912 CPM respectively) but substantially higher than medium alone. It was difficult to determine what contribution Daudi B cells had to the amount of thymidine incorporated vs. the contribution from the medium + IL-2 (5 U/ml) since this control was omitted. Indeed, based on the aberrant cytotoxicity behaviour of new lot Daudi B cells (Section 3.4.3), whether Daudi cells served as an appropriate *t*-γδ T cell antigen source remains questionable. Because the driving force behind proliferation assays is the production of IL-2 upon antigen recognition, it was not surprising that the poor IL-2 producing *t*-γδ T cells always resulted in low thymidine incorporation levels when IL-2 was absent. Assays

Figure 31. Daudi B cell lymphoma (new lot) recognition by a PB *t*-γδ T cell clone determined through proliferation and thymidine uptake. The PB *t*-γδ T cell clone 10-10-3 was rested in IL-2 free medium for 24 h prior to being subjected to either medium alone as a measure of background, medium + IL-2 as a positive control, a 0.5 cell equivalent of irradiated Daudi B cells, irradiated Daudi B cells + IL-2, and irradiated Daudi B cells alone. Cells were cultured for either 24 or 48 h with the addition of [*methyl*- ^{3}H] thymidine during the last 18 h of culture. Incorporated thymidine (CPM) as shown represents the mean ± SD of triplicate wells. Numbers above the curve are stimulation index values based on either medium alone or in the case of Daudi B cells + IL-2, based on medium + IL-2.


2500
2000
1500
1000
500
0
CPM
7.6
1.1
28
18.5
2.0
4.4
1.0
1.0
24h
Time
48h
Medium
Med + IL2(20U)
Daudi
Daudi + IL2(5U)
Daudi alone

performed with a small presence of IL-2 (1-5 U/ml) on the other hand, resulted in high background counts due directly to the small IL-2 presence, hindering potential antigen recognition events.

Measurement of intracytoplasmic cytokine was found to be a more sensitive and suitable method to evaluate antigen responses by *t*-γδ T cells. This method was used to evaluate the responsiveness of a series of phenotypically defined PB *t*-γδ T cell clones to the cellular antigens Daudi B and U937 cells as well as to a potential etiologic antigen for MS, *Chlamydia pneumoniae* (215). *T*-γδ T cell clones were rested in IL-2 free medium for 48 h to minimise background spontaneous IC, prior to exposure to graded amounts of stimulator cells for 6 h. To minimise stimulator cell cytokine production which may activate the *t*-γδ T cells indirectly, stimulator cells were first treated with brefeldin A (2 h). Detection of IC IFN-γ and/ or TNF-α was used to measure *t*-γδ T cell clone responsiveness to antigen and an example of the flow cytometric results given in Figure 32 using IFN-γ and Figure 33 using TNF-α as readout cytokines. TNF-α IC production was superior to IFN-γ IC production for all Ag studies performed due mainly to the lower spontaneous production and therefore higher signal to noise found with TNF-α measurement. This was illustrated by comparing PMA/ Ca^{2+} ionophore induced cytokine stimulation indices (cSI) based on IFN-γ positivity (cSI= 7.1) relative to TNF-α positivity (cSI= 15.3). Clone 44-42 preferentially recognised U937 vs. Daudi B cells which was most evident with a 1:1 effector:stimulator (E:S) ratio (IFN-γ: 44.7 vs. 24.3% positive, cSI 4.7 vs. 2.6; TNF-α: 29.6 vs. 12% positive, cSI 10.2 vs. 4.1 for U937 vs. Daudi cells respectively) and was a titratable phenomenon (e.g. U937-TNF-α: cSI= 4.7, 2.1, 1.2 for 1:1, 10:1, 100:1 E:S ratio). Antigen recognition comparisons with two additional sibling PB *t*-γδ T cell clones (44-27 and 42) to cellular antigens are represented graphically in Figures 34 and 35 with summaries found in Tables 17 and 18. Determination of changes in overall cytokine levels (MCF values) upon stimulation were more sensitive than changes in the number of cytokine positive cells. For example, the cSI for clone 44-16 stimulated with PMA/ Ca^{2+} ionophore based on % positive cells was 7.5 compared to a cSI of 19.4 based on MCF differences (Table 18). Of the three clones, only one of the clones

Figure 32. Tumour cell antigen recognition by a PB *t*-γδ T cell clone using intracytoplasmic IFN-γ production and flow cytometric detection as a readout method. PB *t*-γδ T cell clone (44-42) was rested in IL-2 free medium for 48 h prior to the assay. Graded amounts of either Daudi B cells (top panels) or U937 monocytes (bottom panels) (1, 0.1, 0.01 cell eq.) that had themselves been maintained for 2 h in the presence of Brefeldin A, were combined with clone 44-42 together with Brefeldin A. Positive controls consisted of treating clone 44-42 with PMA/ Ca^{2+} ionophore in the standard manner, while background controls consisted of treating clone 44-42 with medium alone. After a 6 h incubation, cells were surfaced stained with γδ TcR mAb and fixed. Cytokine detection was performed in the standard manner on permeabilised cells with fluorochrome labelled IFN-γ and data was accumulated through FACS analysis. The proportion of clone 44-42 producing intracytoplasmic IFN-γ in response to the stimuli can be found in the upper right quadrants and is based on cells falling above a threshold set on γδ TcR positive cells alone. Antigen response expressed as a cytokine stimulation index (cSI) is based on the ratio of the stimulated % positive cells to the spontaneous % positive cells that received medium alone.

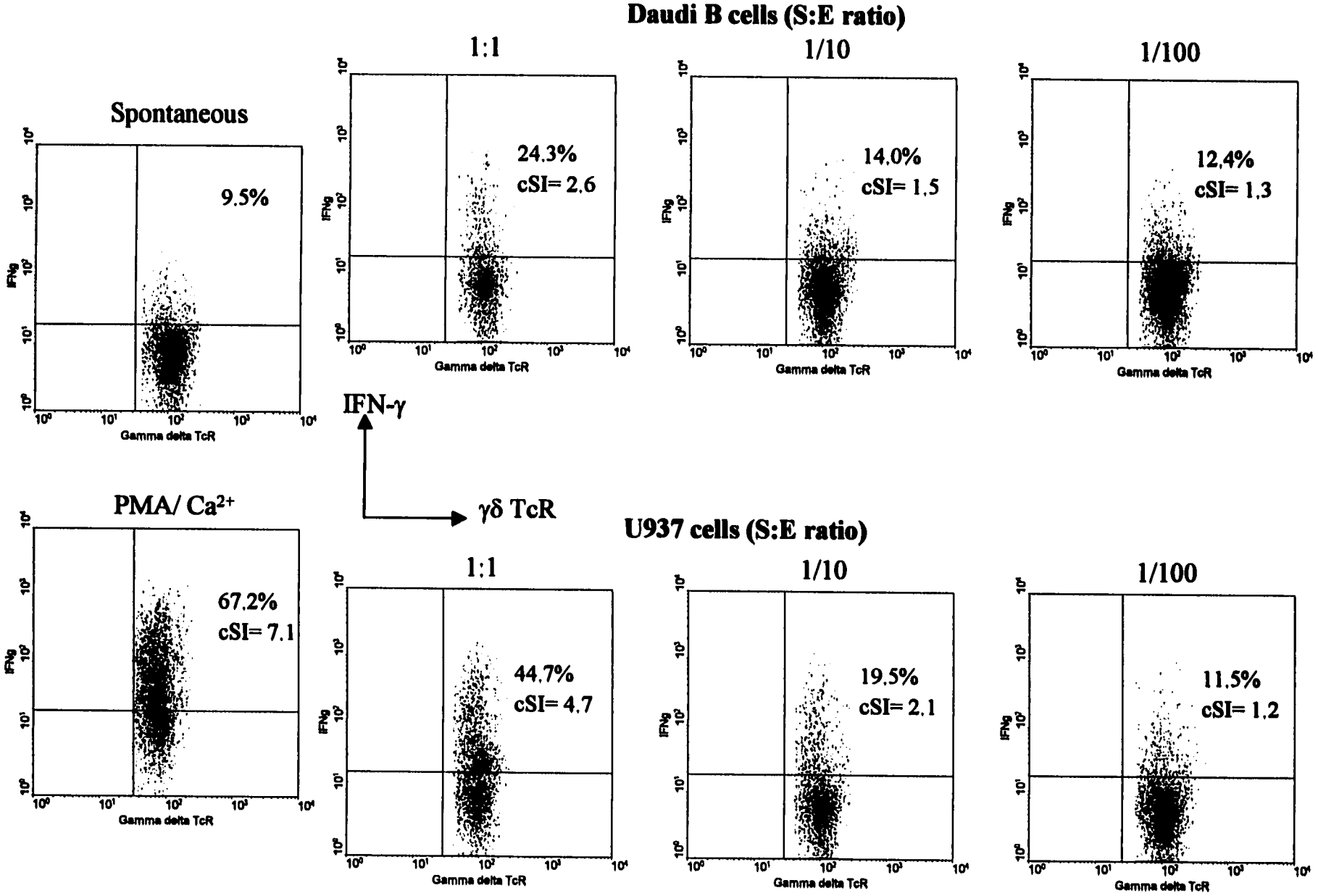

Daudi B cells (S:E ratio)
1:1
1/10
1/100
Spontaneous
9.5%
24.3%
cSI= 2.6
14.0%
cSI= 1.5
12.4%
cSI= 1.3
IFN-γ
γδ TcR
PMA/ Ca2+
67.2%
cSI= 7.1
U937 cells (S:E ratio)
1:1
1/10
1/100
44.7%
cSI= 4.7
19.5%
cSI= 2.1
11.5%
cSI= 1.2
IFNg
Gamma delta TcR

Figure 33. Tumour cell antigen recognition by a PB *t*-γδ T cell clone using intracytoplasmic TNF-α production and flow cytometric detection as a readout method. PB *t*-γδ T cell clone (44-42) was rested in IL-2 free medium for 48 h prior to the assay. Graded amounts of either Daudi B cells (top panels) or U937 monocytes (bottom panels) (1, 0.1, 0.01 cell eq.) that had themselves been maintained for 2 h in the presence of Brefeldin A, were combined with clone 44-42 together with Brefeldin A. Positive controls consisted of treating clone 44-42 with PMA/ Ca^{2+} ionophore in the standard manner, while background controls consisted of treating clone 44-42 with medium alone. After a 6 h incubation, cells were surfaced stained with γδ TcR mAb and fixed. Cytokine detection was performed in the standard manner on permeabilised cells with fluorochrome labelled TNF-α and data was accumulated through FACS analysis. The proportion of clone 44-42 producing intracytoplasmic TNF-α in response to the stimuli can be found in the upper right-hand quadrants and is based on cells falling above a threshold set on γδ TcR positive cells alone. Antigen response expressed as a cytokine stimulation index (cSI) is based on the ratio of the stimulated % positive cells to the spontaneous % positive cells that received medium alone.

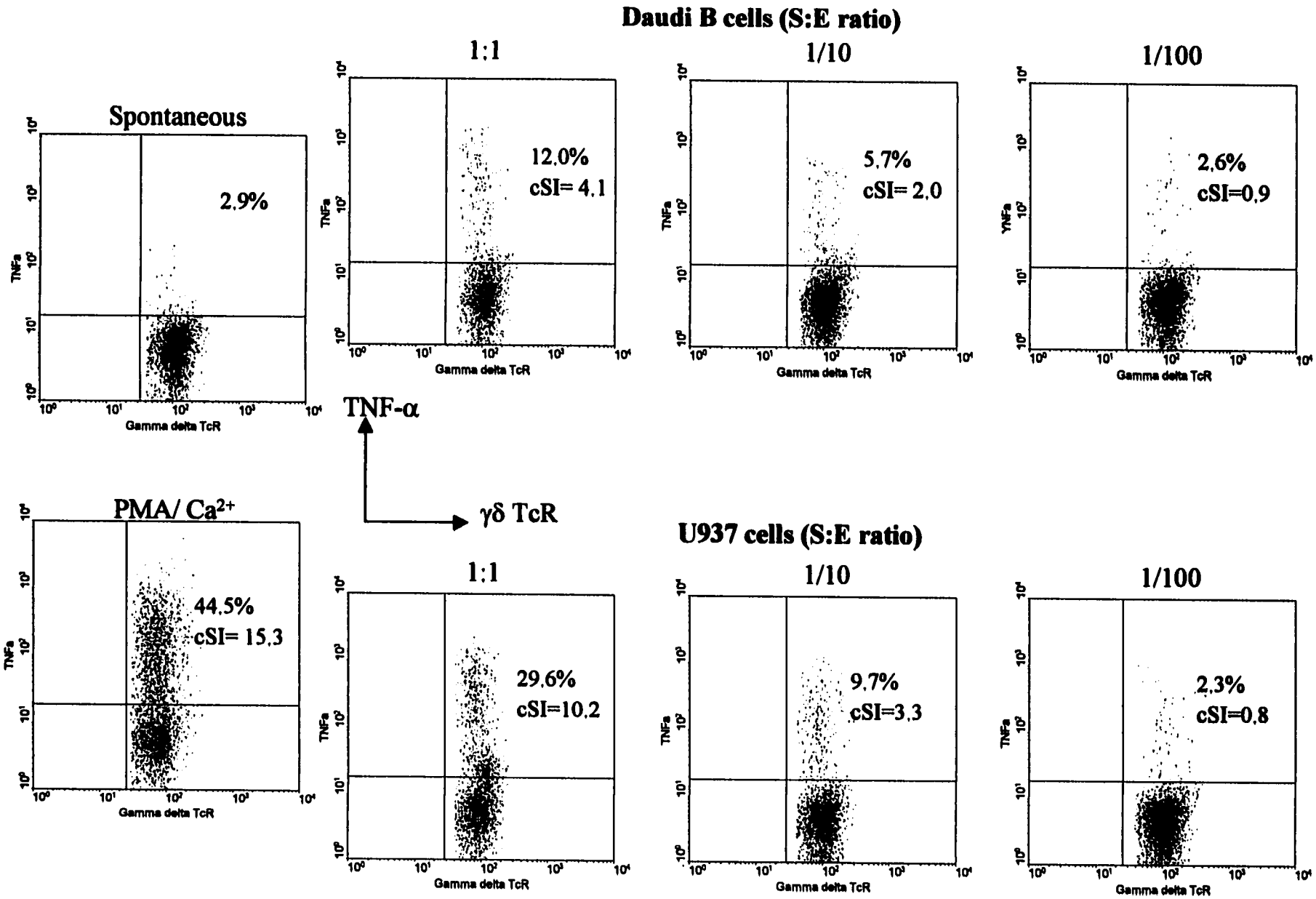
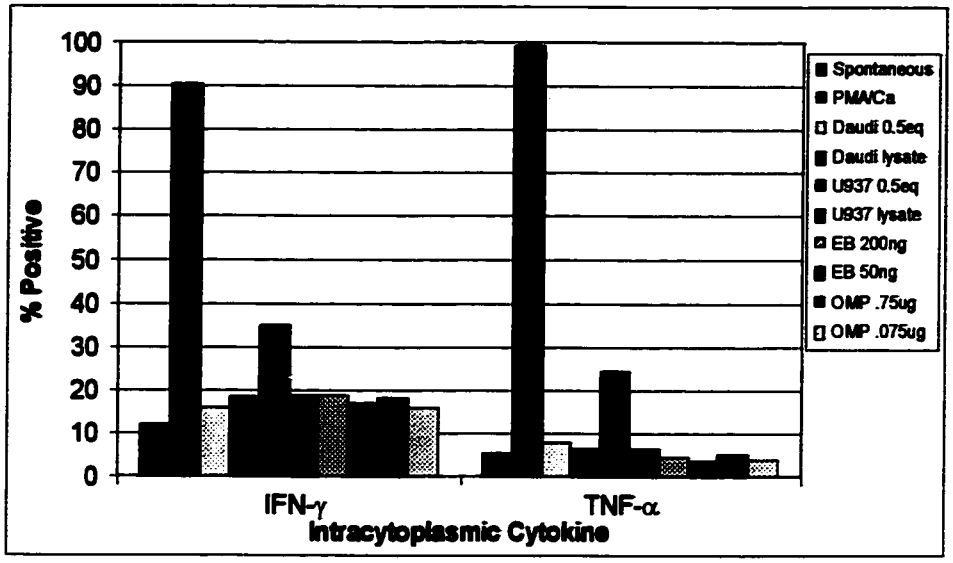
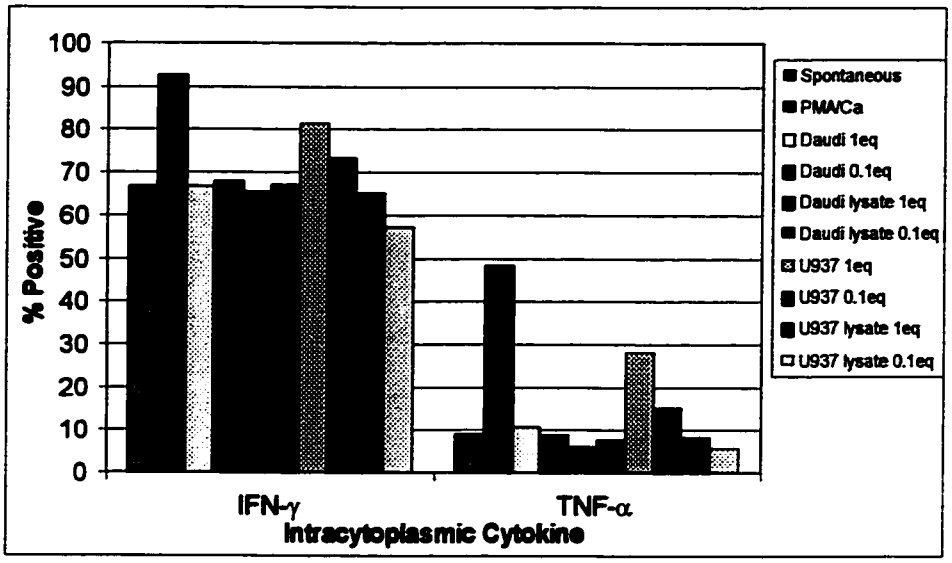

Daudi B cells (S:E ratio)
1:1
1/10
1/100
Spontaneous
2.9%
12.0%
cSI= 4.1
5.7%
cSI= 2.0
2.6%
cSI=0.9
TNF-α
γδ TcR
PMA/ Ca2+
44.5%
cSI= 15.3
U937 cells (S:E ratio)
1:1
1/10
1/100
29.6%
cSI=10.2
9.7%
cSI=3.3
2.3%
cSI=0.8
TNFa
Gamma delta TcR

Figure 34. Cellular antigen recognition comparison between three PB *t*-γδ T cell clones using IFN-γ or TNF-α intracytoplasmic cytokine positivity as a measure of response. Antigen recognition experiments were conducted as described in the legend for Figure 33 for clones 44-16, 44-27, and 44-42. In addition to the graded amounts of either Daudi or U937 tumour antigens as indicated, all *t*-γδ T cell clones were screened against tumour cell lysates prepared by repetitive freeze-thaw cycles of an equivalent number of tumour cells. Clone 44-16 was further screened against putative MS antigens, *Chlamydia pneumoniae* elementary body (EB) and outer membrane protein (OMP) at the concentrations indicated. The % of *t*-γδ T cell clones that were positive for either IFN-γ or TNF-α were calculated based on cells found above a threshold set on cells positive for γδ TcR alone.

Clone 44-16



Clone 44-27



Clone 44-42

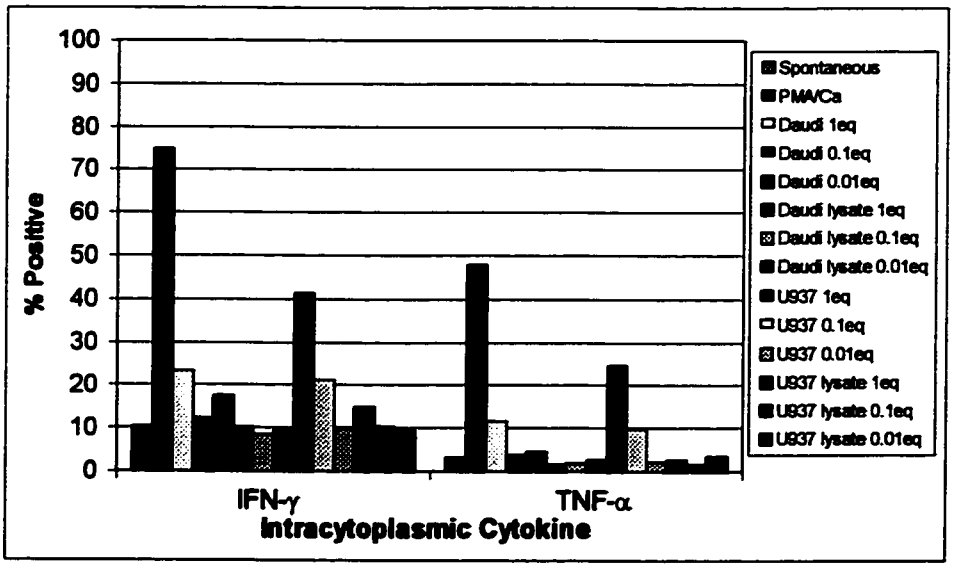
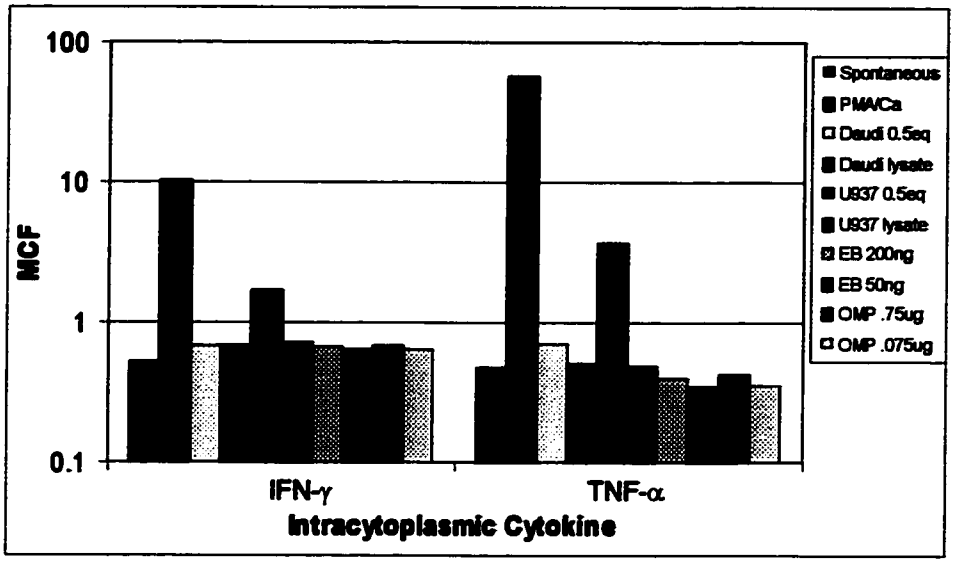
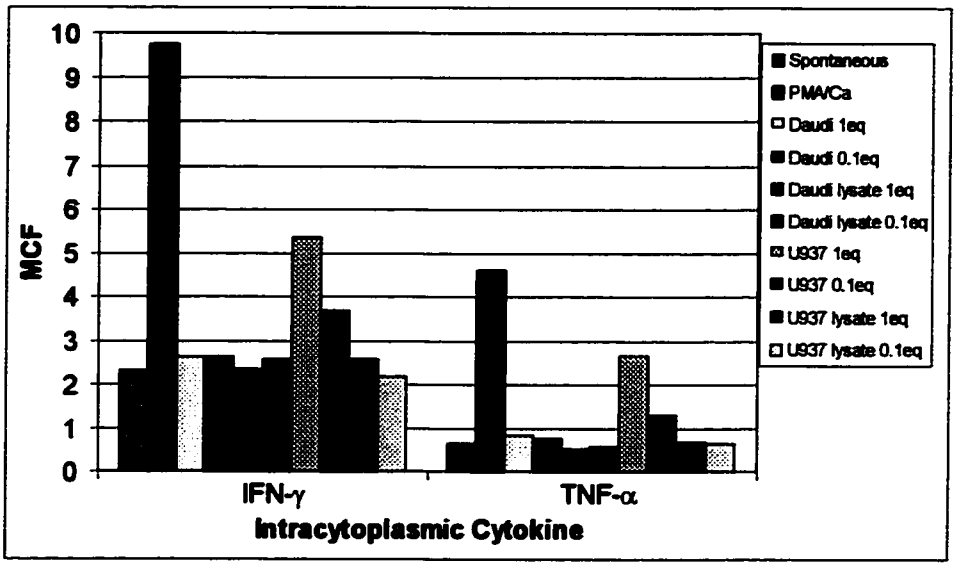


Figure 35. Cellular antigen recognition comparison between three PB *t*-γδ T cell clones using the relative change in IFN-γ or TNF-α intracytoplasmic cytokine levels as a measure of response. Antigen recognition experiments were conducted as described in the legend for Figure 33 for clones 44-16, 44-27, and 44-42. In addition to the graded amounts of either Daudi or U937 tumour antigens as indicated, all *t*-γδ T cell clones were screened against tumour cell lysates prepared by repetitive freeze-thaw cycles of an equivalent number of tumour cells. Clone 44-16 was further screened against putative MS antigens, *Chlamydia pneumoniae* elementary body (EB) and outer membrane protein (OMP) at the concentrations indicated. Changes in the overall level of intracytoplasmic cytokine (MCF) produced as a result of antigenic stimuli were calculated as described in *Materials and methods*.

Clone 44-16



Clone 44-27



Clone 44-42

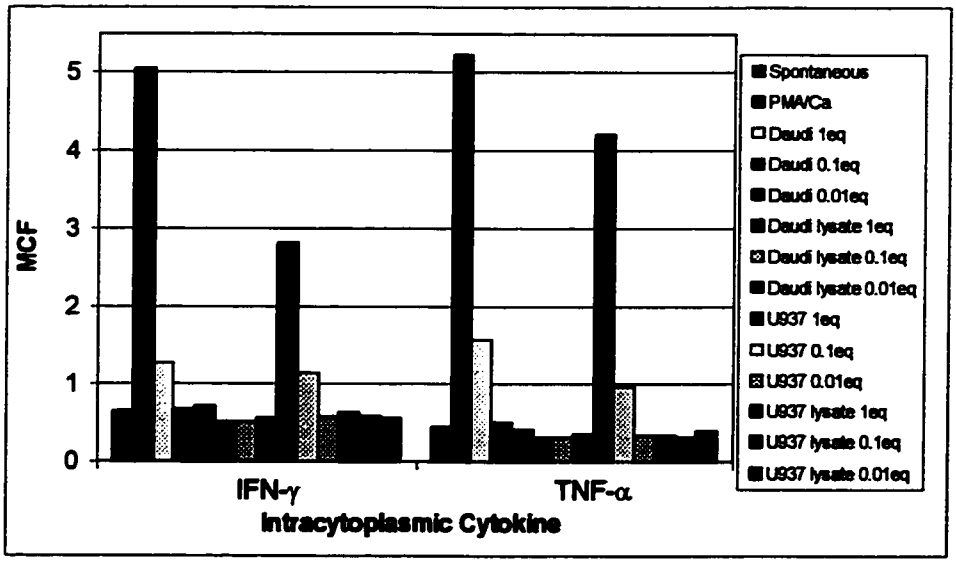


Table 17. Summary of cellular antigen recognition studies for three PB *t*-γδ T cell clones using intracytoplasmic cytokine production as a measure of response. %: percentage of *t*-γδ T cell clones positive for cytokine; MCF: relative level of cytokine produced per cell; Spon: *t*-γδ T cell clones treated with medium alone; PMA/Ca: PMA/ Ca^{2+} ionophore stimulation as positive control; D-eq: cell equivalents of Daudi B lymphoma antigen; DL-eq: cell equivalents of Daudi B lymphoma lysates; U-eq: cell equivalents of U937 cells; UL: cell equivalents of U937 lysates; EB: *Chlamydia pneumoniae* elementary body; OMP: *Chlamydia pneumoniae* outer membrane protein.

Clone	Cytokine	Unit	Spon.	PMA/Ca	D-0.5eq	DL-0.5eq	U-0.5eq	UL -0.5eq	EB (200ng)	EB (50ng)	OMP (75μg)	OMP (0.75μg)
44-16	IFN-γ	%	12	90.3	15.7	18.2	35	18.6	18.5	17	18.1	15.7
Vδ1Vγ4Vγ9		MCF	0.53	10.3	0.67	0.67	1.69	0.72	0.66	0.63	0.67	0.64
	TNF-α	%	5.3	99.2	7.8	6.5	24.3	6.3	4.4	3.7	5.1	4
		MCF	0.47	57.4	0.71	0.51	3.63	0.48	0.40	0.35	0.43	0.36

Clone	Cytokine	Unit	Spon	PMA/Ca	D-1eq	D-0.1eq	DL-1eq	DL–0.1eq	U-1eq	U-0.1eq	UL-1eq	UL-0.1eq
44-27	IFN-γ	%	66.8	92.4	66.7	67.8	65.4	67	81.3	73	65	57.1
Vδ7Vγ9		MCF	2.31	9.74	2.63	2.62	2.35	2.57	5.35	3.67	2.57	2.19
	TNF-α	%	9.1	48.3	10.7	8.7	5.8	7.6	28.1	15.1	8.1	5.6
		MCF	0.64	4.6	0.82	0.74	0.51	0.58	2.66	1.31	0.66	0.66

Clone	Cytokine	Unit	Spon	PMA/Ca	D-1	D-0.1	D-0.01	DL-1	DL-0.1	DL-0.01	U-1	U-0.1	U-0.01	UL–1	UL–0.1	UL–0.01
44-42	IFN-γ	%	10.2	75.0	23.3	12.2	17.5	10.1	8.5	9.6	41.2	21.2	9.7	14.8	10.3	9.5
Vδ3Vγ9		MCF	0.64	5.05	1.27	0.67	0.71	0.50	0.51	0.55	2.81	1.14	0.57	0.63	0.58	0.56
	TNF-α	%	3.1	48.0	11.7	3.8	4.5	1.7	1.7	2.5	24.6	9.9	2.0	2.6	1.5	3.5
		MCF	0.43	5.22	1.57	0.50	.41	0.30	0.31	0.35	4.21	0.96	0.33	0.34	0.30	0.40

Table 18. Summary of cytokine stimulation indices for the recognition of cellular antigen by three PB *t*-γδ T cell clones. Cytokine stimulation indices were calculated based on the ratio of either the % positive cells or cytokine level (MCF) following stimulation to either the spontaneous % positive or cytokine level. %: percentage of *t*-γδ T cell clones positive for cytokine; MCF: relative level of cytokine produced per cell; Spon: *t*-γδ T cell clones treated with medium alone; PMA/Ca: PMA/ Ca^{2+} ionophore stimulation as positive control; D-eq: cell equivalents of Daudi B lymphoma antigen; DL-eq: cell equivalents of Daudi B lymphoma lysates; U-eq: cell equivalents of U937 cells; UL: cell equivalents of U937 lysates; EB: *Chlamydia pneumoniae* elementary body; OMP: *Chlamydia pneumoniae* outer membrane protein.

Clone	Cytokine	Unit	PMA/Ca	D-0.5eq	DL-0.5eq	U-0.5eq	UL -0.5eq	EB (200ng)	EB (50ng)	OMP (75μg)	OMP (0.75μg)
44-16	IFN-γ	cSI-%	7.5	1.3	1.5	2.9	1.5	1.5	1.4	1.5	1.3
Vδ1Vγ4Vγ9		cSI-MCF	19.4	1.3	1.3	3.2	1.4	1.2	1.2	1.3	1.2
	TNF-α	cSI-%	18.7	1.5	1.2	4.6	1.2	0.8	0.7	1.0	0.8
		cSI-MCF	122	1.5	1.1	7.7	1.0	0.9	0.7	0.9	0.8

Clone	Cytokine	Unit	PMA/Ca	D-1eq	D-0.1eq	DL-1eq	DL–0.1eq	U-1eq	U-0.1eq	UL-1eq	UL-0.1eq
44-27	IFN-γ	cSI-%	1.4	1.0	1.0	1.0	1.0	1.2	1.0	1.1	0.9
Vδ7Vγ9		cSI-MCF	4.2	1.1	1.1	1.0	1.1	2.3	1.6	1.1	1.0
	TNF-α	cSI-%	5.3	1.2	1.0	0.6	0.8	3.1	1.7	0.9	0.6
		cSI-MCF	7.2	1.3	1.2	0.8	0.9	4.2	2.0	1.0	1.0

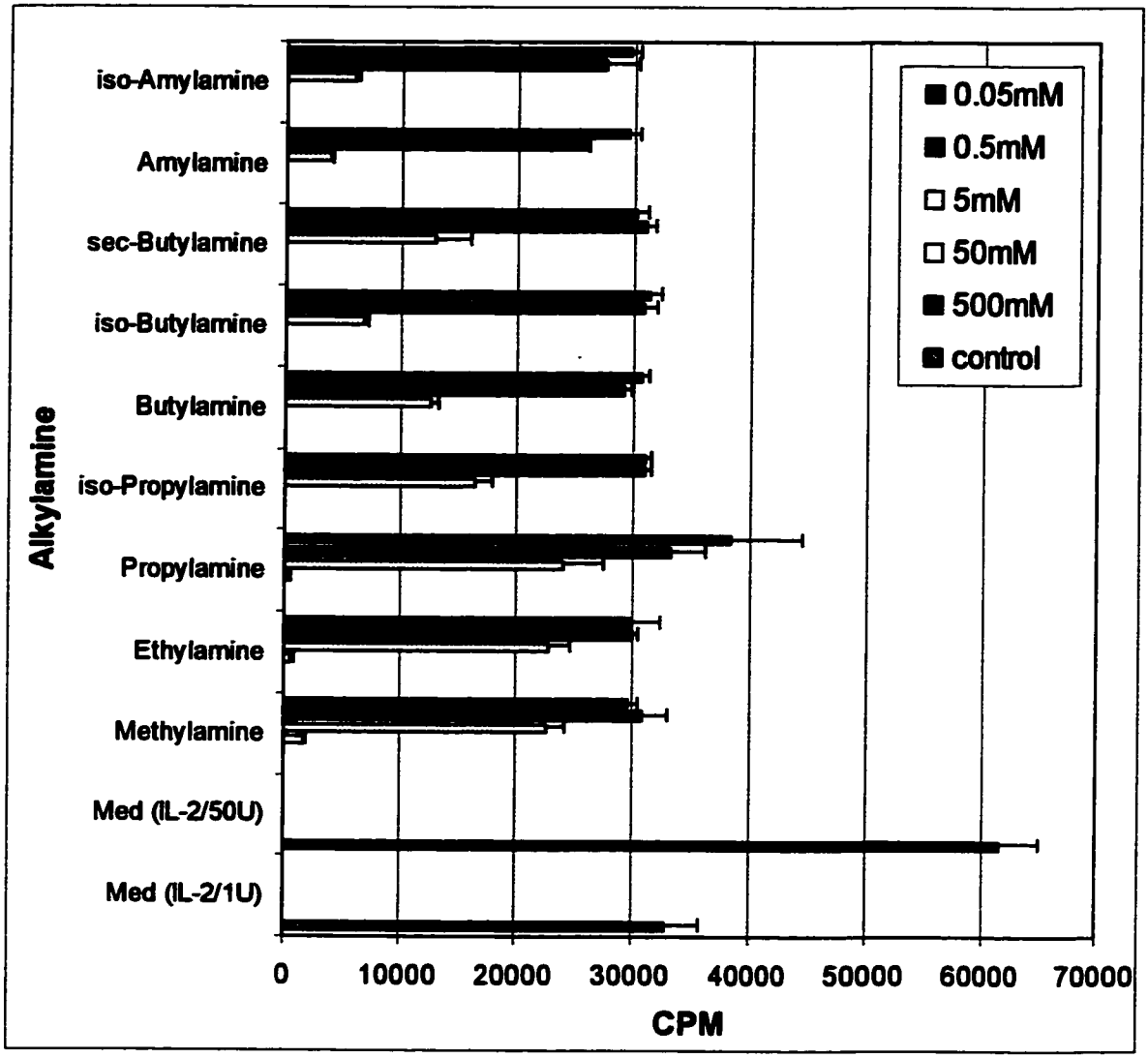
Clone	Cytokine	Unit	PMA/Ca	D-1	D-0.1	D-0.01	DL-1	DL-0.1	DL-0.01	U-1	U-0.1	U-0.01	UL–1	UL–0.1	UL–0.01
44-42	IFN-γ	cSI-%	7.4	2.3	1.2	1.7	1.0	0.8	0.9	4.0	2.1	1.0	1.5	1.0	0.9
Vδ3Vγ9		cSI-MCF	7.9	2.0	1.0	1.1	0.8	0.8	0.9	4.4	1.8	0.9	1.0	0.9	0.9
	TNF-α	cSI-%	15.5	3.8	1.2	1.5	0.5	0.5	0.8	7.9	3.2	0.6	0.8	0.5	1.1
		cSI-MCF	12.1	3.7	1.2	1.0	0.7	0.7	0.8	9.8	2.2	0.8	0.8	0.7	0.9

(44-42) recognised Daudi B cells (cSI 3.7 for TNF-α MCF at a 1:1 E:S ratio vs. 1.5 and 1.3 for clones 44-16 and 27 respectively). Clones 44-42 and 44-16 both recognised U937 cells to an approximate equal extent (cSI 9.8 for TNF-α MCF at a 1:1 E:S ratio and cSI 7.7 for TNF-α MCF at a 1:0.5 E:S ratio for 44-42 and 16 respectively) followed by a lower response with clone 44-27 (cSI 4.2 for TNF-α MCF at 1:1 E:S ratio). Only cellular antigen was recognised since an equivalent amount of cell lysate prepared by repetitive freeze-thaw cycles, failed to generate either a IFN-γ or TNF-α response (i.e. 44-16 cSI 7.7 for cellular U937 vs. 1.0 for an equivalent amount of U937 lysate). Clone 44-16 did not recognise either *Chlamydia pneumoniae* EB or OMP at the two concentrations tested (cSI of ~1 for all combinations). This result was consistent with similar studies performed with other *t*-γδ T cell clones against *C. pneumoniae* antigens using proliferation assays.

The Vγ4Vγ9Vδ1 clone, 44-16, was further tested for responsiveness towards a number of antigens belonging to a recently reported, γδ T cell reactive antigen class known as alkylamines (110) using proliferation assays. While the significance of this antigen class is not presently known, their ubiquitous presence in microbes and presumably in human tissues make them candidate CNS autoantigens. After resting, clone 44-16 was stimulated with graded amounts of alkylamine antigen in the presence of 1 U/ml IL-2 for 48 h followed by an 18 h pulse with ^{3}[H]-thymidine (Figure 36). In no case did clone 44-16 respond to alkylamine antigen with a level greater than medium + IL-2 alone. Indeed at higher alkylamine concentrations, (50-500 mM), clone 44-16 displayed CPM counts markedly lower than medium + IL-2 alone, indicating cell stasis or toxicity.

In summary, a sensitive method to evaluate antigen responsiveness by intracytoplasmic cytokine production was established. Antigen recognition studies to cellular and *C. pneumoniae* antigens for a series of phenotypically defined *t*-γδ T cell clones have identified selective and differential recognition of Daudi vs. U937 cells and complete non-recognition of the *C. pneumoniae* antigens. In addition, the Vγ4Vγ9Vδ1 clone, 44-16, did not respond to any of a series of alkylamine antigens in proliferation assays. Results from additional antigen recognition studies with other non-cellular candidate antigens can be found in the following section.

Figure 36. The response of a PB *t*-γδ T cell clone to a series of non-peptidic alkylamine antigens as assessed by proliferation and thymidine incorporation. Clone 44-16 (Vγ4Vγ9Vδ1) was rested for 36 h in IL-2 free medium prior to culture with graded amounts of alkylamine antigens as indicated in the presence of IL-2 (1 U/ml) for 36 h followed by a further 18 h pulse with [*methyl*- ^{3}H] thymidine. Medium containing IL-2 (1 U/ml) served as background control while medium containing IL-2 (50 U/ml) served as positive control. Data as shown represents the mean thymidine incorporation values (CPM) ± SD for triplicate cultures as measured by scintillation counting.


iso-Amylamine
Amylamine
sec-Butylamine
iso-Butylamine
Butylamine
iso-Propylamine
Propylamine
Ethylamine
Methylamine
Med (IL-2/50U)
Med (IL-2/1U)
Alkylamine
0.05mM
0.5mM
5mM
50mM
500mM
control
0
10000
20000
30000
40000
50000
60000
70000
CPM

3.5 Peripheral blood vs. CSF derived γδ T cells

A major focus at the outset of this work was to apply the HVS technology on a patient to patient basis in order to comprehensively study potential differences between CSF and PB derived γδ T cells. Due to time constraints, comprehensive PB/ CSF comparisons were limited to immortalised PB and CSF γδ T cells from a single MS patient and as such, represented only a narrow view of γδ T cells derived from these two compartments.

3.5.1 PB and CSF phenotype comparisons

Phenotypic comparisons between the PB and CSF *t*-γδ T cells were previously tabulated (Table 13 patient sample F and I respectively). There was a dramatic difference between the two compartments based on VγVδ subtype expression with the PB line composed of Vδ1 (13.7%), Vδ2 (82.8%), Vδ3 (3.2%), Vγ4 (3.8%), and Vγ9 (92.7%) positive cells in ratios that were typical of normal PB distributions. The CSF transformed line however, was exclusively composed of Vδ1Vγ9 positive cells. This limited distribution was not the result of the HVS transformation technique since phenotypic analysis of the short term CSF γδ T cell line revealed a homogeneous $V\delta1^{+}$ and heterogeneous Vγ population (70% Vγ9, 30% Vγother). Whether short term CSF expansion selected for this limited VγVδ population or whether this population was representative of the sole γδ T cell population present within the patient's CSF, is not known. Other notable differences between the transformed PB and CSF lines were the relatively low expression of CD25 on PB (7.0%) vs. CSF (42.9%) cells, a 3-fold higher expression of CD40L on PB (61.7%) vs. CSF (22.7%) cells and 55% lower expression of the KIR, CD94 on PB (22.0%) vs. CSF (49.1%) transformed cells.

T-γδ T cell clones were also generated from both the *t*-PB and *t*-CSF lines using the methods outlined above (Section 3.2.5). Thirty one clones were generated from PB of which 4 were selected for further study and 27 clones generated from CSF of which 1 was selected for further study (Table 14). Out of the 31 PB clones, 9 were subtyped for VγVδ usage and 7/9 were

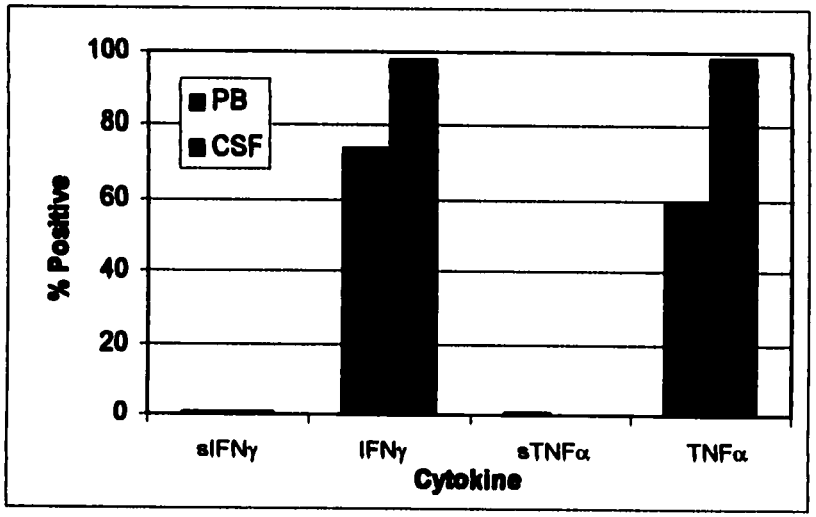
of the Vδ2Vγ9 subtype, while the remaining two were either Vδ?Vγ9 or Vδ?Vγ?. Of the 27 CSF clones, 11 were subtyped for VγVδ usage and all were found to be Vδ1Vγ9. To assess for other CSF clone differences, CD8 and CD40L expression was determined and the levels of expression for the four clones tested were identical. Clone 46-26 was chosen as the representative CSF clone sample and other than the Vγ9Vδ1 phenotype, the only distinguishing feature of this clone was a moderate level of CD28 expression (21%) compared to none for its matched PB clone counterparts. Among the 4 PB *t*-γδ T cell clones selected for study, levels of CD8 were marginally different (0-20%), CD25 and CD40L expression ranged from low to high (CD25: 7-92%; CD40L: 0-90%), and one clone expressed relatively high levels of CD94 (50% vs. 3-7%). Testing for other KIR molecules (CD158a, CD158b, NKB1P) failed to detect their presence on the surface of the PB *t*-γδ T cell clones.

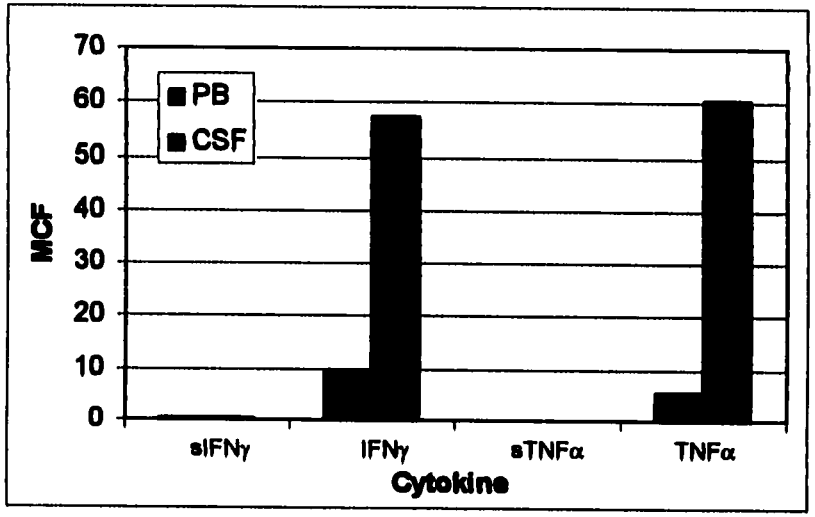
3.5.2 Intracytoplasmic cytokine comparisons

Representative proinflammatory (IFN-γ,TNF-α, IL2) and anti-inflammatory (IL-10) IC profiles were determined post PMA/ Ca^{2+} ionophore stimulation of PB and CSF *t*-γδ T cells derived from two patients (Figure 37). In both cases, the majority of the PB and CSF *t*-γδ T cells were positive for IFN-γ and TNF-α, with Patient CG favouring greater CSF vs. PB cytokine positivity (IFN-γ: 98 vs. 74%; TNF-α: 98 vs. 59% for CSF vs. PB respectively). Virtually all PB and CSF *t*-γδ T cells were negative for IL-10 and IL-2 production (Patient FI). Analysis of the relative level of cytokine produced (MCF), revealed some surprising differences. CSF derived γδ T cells from Patient CG produced between 6-11 fold greater concentrations of IFN-γ and TNF-α per cell than PB derived cells (IFN-γ: 58 vs. 9 MCF units; 61 vs. 5.6 MCF units for CSF vs. PB). This was not the case for patient FI where comparable PB and CSF IFN-γ levels (15 and 18 MCF units respectively) and moderately skewed PB vs. CSF TNF-α levels (26.9 vs. 13 MCF units respectively) were found. Patient CG also differed from patient FI by the very low % of PB and CSF positive cells spontaneously producing IFN-γ and TNF-α (0.2-0.6%). Although patient FI

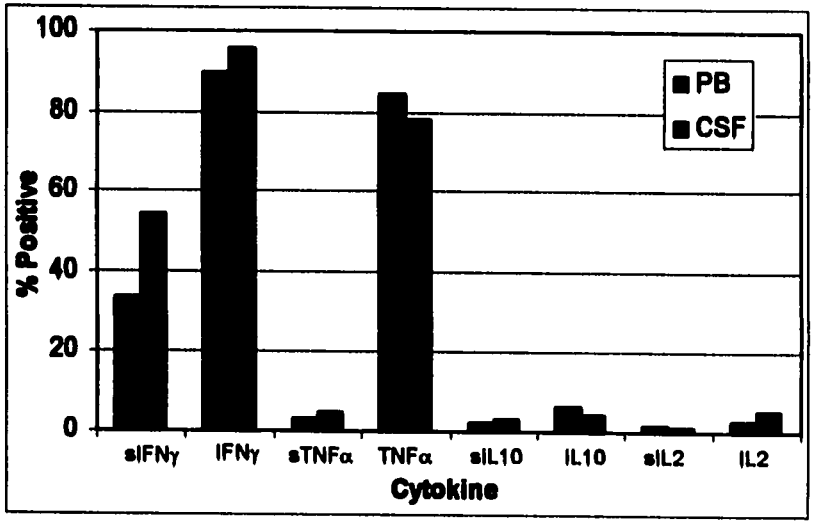
Figure 37. Comparison of intracytoplasmic cytokine profiles between PB and CSF *t*-γδ T cells of two MS patients. PB and CSF *t*-γδ T cells from Patient CG (upper panels) and Patient FI (lower panels) were stimulated with PMA/ Ca^{2+} ionophore for 6h in the presence of Brefeldin A, after being rested in IL-2 free medium during the previous 48 h. Permeabilised γδ TcR surfaced stained cells were treated with fluorochrome labelled cytokine mAbs and enumerated through FACS analysis. Left hand panels depict the % of γδ T cells staining positive for the indicated cytokines based on gates set on γδ TcR positive cells alone. Right hand panels depict the relative cytokine level per cell based on calculated MCF values. sIFN-γ and sTNF-α: spontaneous IFN-γ and TNF-α resulting from cells treated with medium alone.

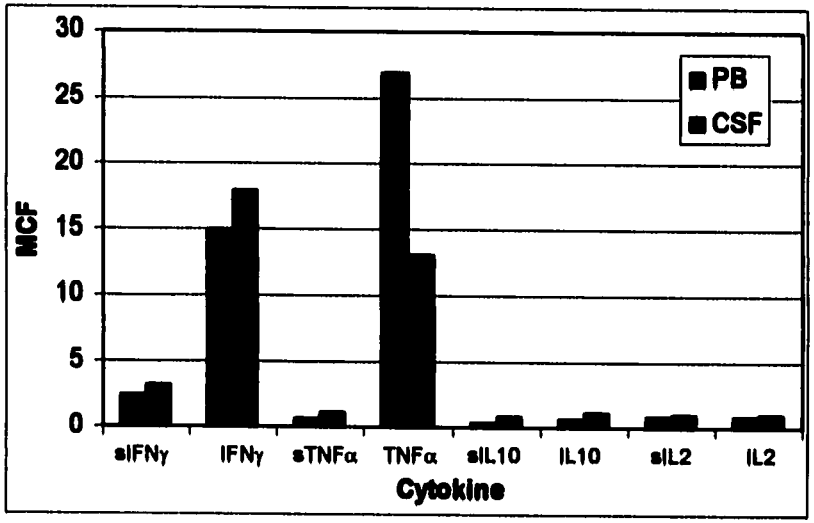
Patient CG





Patient FI





also had low basal numbers of TNF-α positive PB and CSF cells (0.6 and 1%), spontaneous PB and CSF IFN-γ was elevated (33 and 54%) despite a 48 h prior rest period.

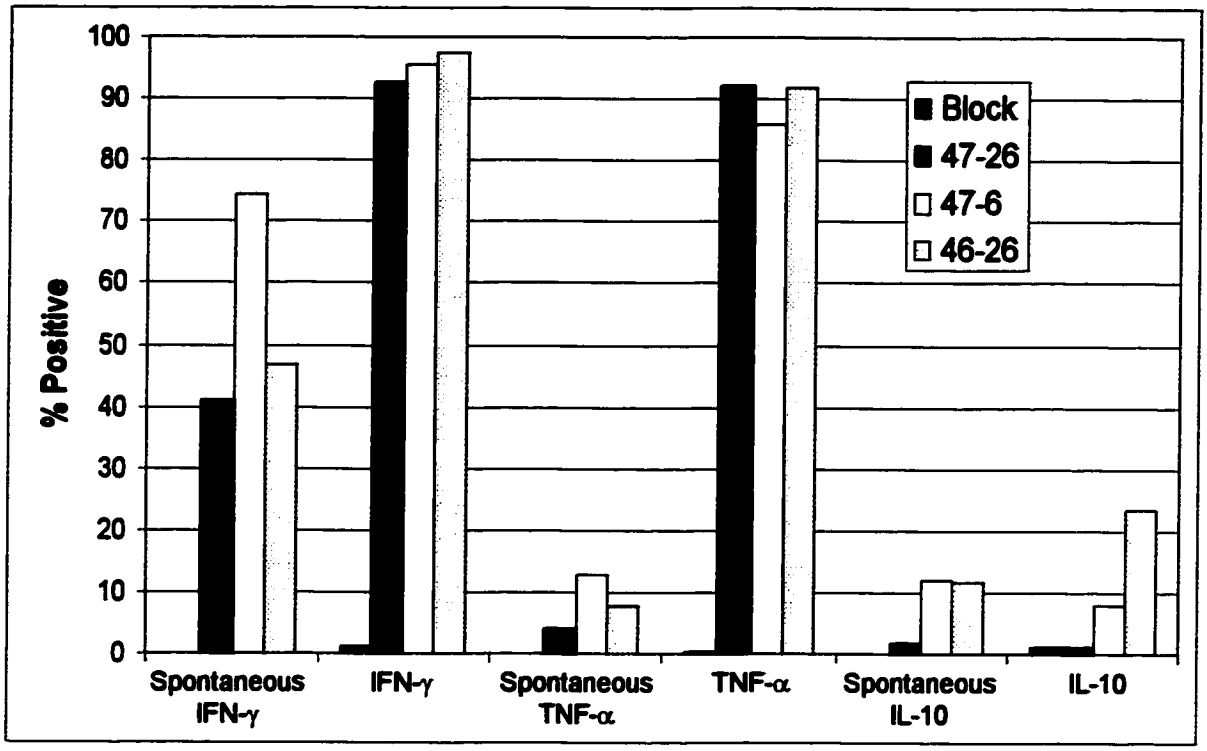
IC profiles were also evaluated for representative *t*-γδ T cell clones derived from patient FI (PB: clones 47-6 and 47-26; CSF: clone 46-26) (Figure 38). As in the parental lines, both the PB and CSF clones spontaneously produced IFN-γ (40-75%) but not TNF-α (3-12%), with clone 47-6 (Vγ9Vδ?) producing markedly more spontaneous IFN-γ relative to 47-26 (Vγ9Vδ2) and 46-26 (Vδ1Vγ9) (MCF: 4.1 vs. 1.6 and 1.8 for clones 47-6 vs. 47-26 and 46-26 respectively). Both the PB and CSF clones post PMA/ Ca^{2+} ionophore stimulation responded similarly to their parental lines with IFN-γ and TNF-α but little to no IL-10 production. There was a small PMA/ Ca^{2+} ionophore induced IL-10 contribution within the CSF clone 46-26 relative to basal levels (23.4 vs. 11%) but this increase was not as marked when relative cytokine levels were compared (MCF: 0.99 vs. 0.77). Consistent with its greater basal IFN-γ producing capacity, clone 47-6 upon stimulation produced ~2-fold higher amounts of cytokine per cell relative to the others (MCF: 17 vs. 7 and 10 for 47-6 vs. 47-26 and 46-26 respectively) despite all clones being equally IFN-γ positive (93, 96, and 98% IFN-γ positive for 47-6, -26, and 46-26 respectively). TNF-α production, in terms of cell numbers (86-92%) and relative cytokine levels (16-17.6 MCF), was equivalent for all three clones post PMA/ Ca^{2+} ionophore stimulation.

3.5.3 Cytotoxicity comparisons

Comparative cytotoxicity studies directed towards various cell targets were performed with the matched PB and CSF *t*-γδ T cell samples from Patient FI using JAM assays (Figure 39). Similar to previous results, there was a graded ability of both CSF and PB *t*-γδ T cells to kill the various cell targets. At an E:T ratio of 0.2, PB and CSF *t*-γδ T cells demonstrated their greatest killing potential towards Jurkat (PB: 49.2%, CSF: 48.3%) and U937 (PB: 36.4%, CSF: 39.5%), followed by the intermediary killing of KG-1 (PB: 7.1%, CSF:13.8%) and low killing of Colo-205 cells (PB: 4.4%, CSF: none) (Table 19).

Figure 38. Comparison of the intracytoplasmic cytokine profiles for two representative PB and one CSF *t*-γδ T cell clone derived from Patient FI. PB *t*-γδ T cell clones (47-6 and 47-26) and the CSF *t*-γδ T cell clone (46-26) from Patient FI were stimulated with PMA/ Ca^{2+} ionophore for 6h in the presence of Brefeldin A, after being rested in IL-2 free medium during the previous 48 h. Permeabilised γδ TcR surfaced stained cells were treated with fluorochrome labelled cytokine mAbs and enumerated through FACS analysis. Panel A depicts the % of γδ T cells staining positive for the indicated cytokines based on gates set on γδ TcR positive cells alone. Panel B depicts the relative cytokine level per cell based on calculated MCF values. Spontaneous IFN-γ, TNF-α, and IL-10 represent cytokine values from *t*-γδ T cell clones treated with medium alone.

(A)


100
90
80
70
60
50
40
30
20
10
0
% Positive
Block
47-26
47-6
46-26
Spontaneous IFN-γ
IFN-γ
Spontaneous TNF-α
TNF-α
Spontaneous IL-10
IL-10

(B)

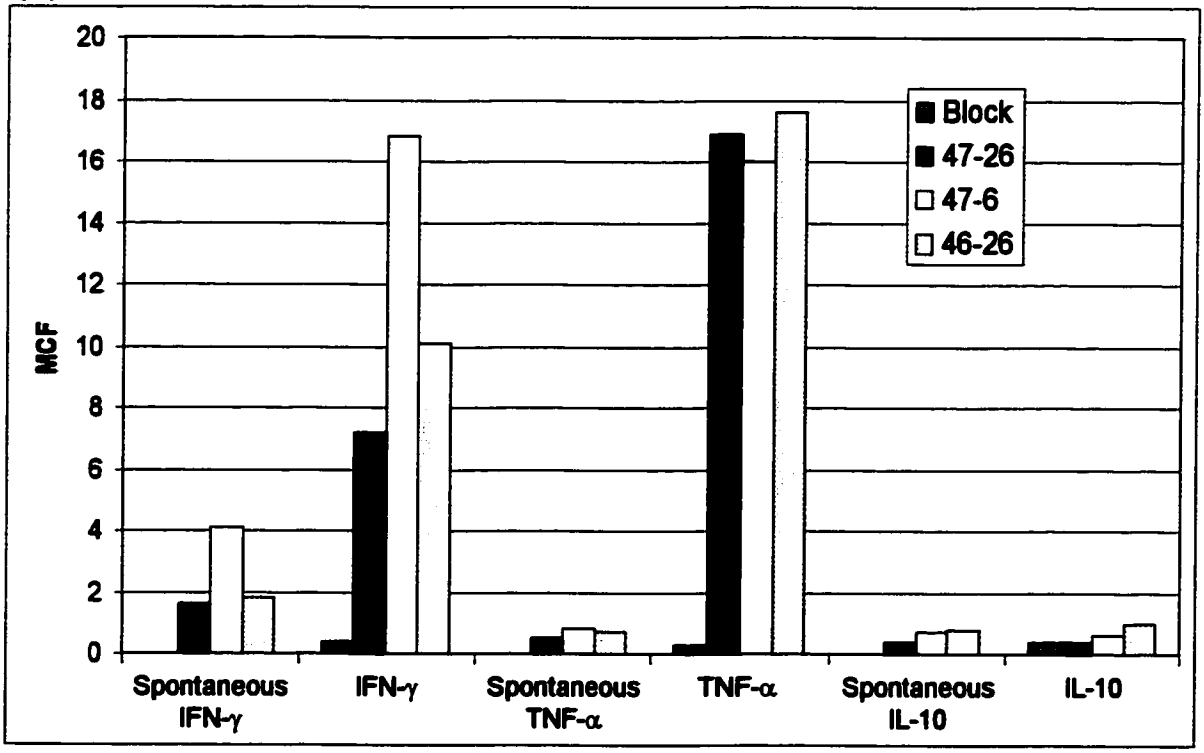
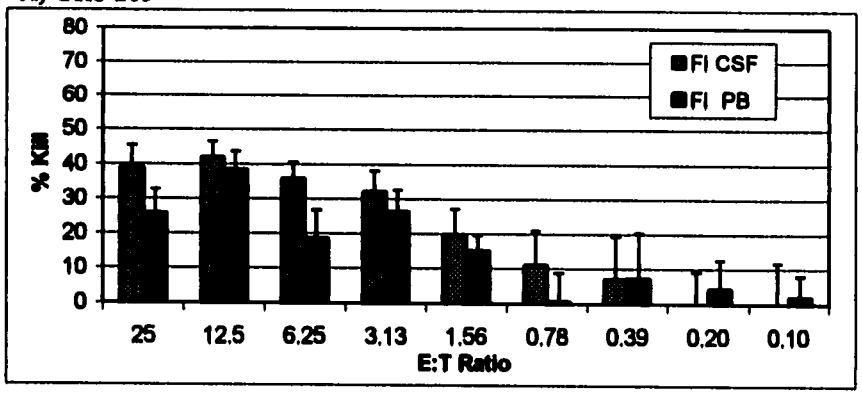
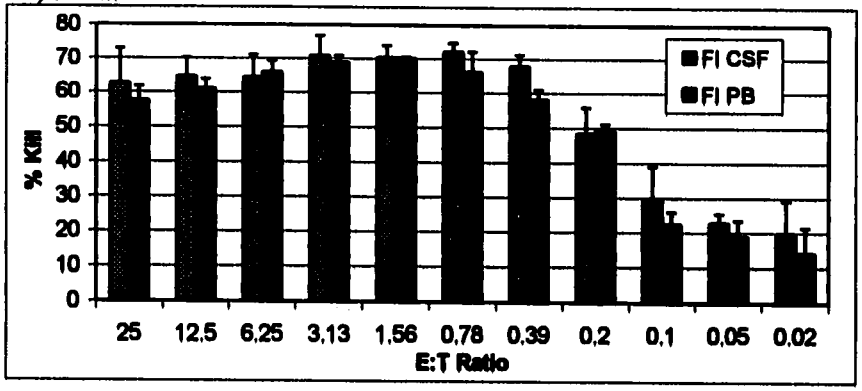

20
18
16
14
12
10
8
6
4
2
0
MCF
Block
47-26
47-6
46-26
Spontaneous IFN-γ
IFN-γ
Spontaneous TNF-α
TNF-α
Spontaneous IL-10
IL-10

Figure 39. Tumour cell cytotoxicity comparisons between the PB and CSF *t*-γδ T cells obtained from Patient FI. Cytotoxicity responses of the PB and CSF *t*-γδ T cell lines derived form Patient FI, were evaluated against tumour target cells (panels A-E) in standard 4 h JAM assays. Panel E: Daudi targets were "new lot" cells that behaved aberrantly (see text). Panel F: For comparison purposes, the cytotoxicity responses of PB vs CSF *t*-γδ T cells derived from a second patient sample (EV), towards "old lot" Daudi B cell targets were included. The results shown are representative of the mean % kill ± SD for quadruplicate cultures.

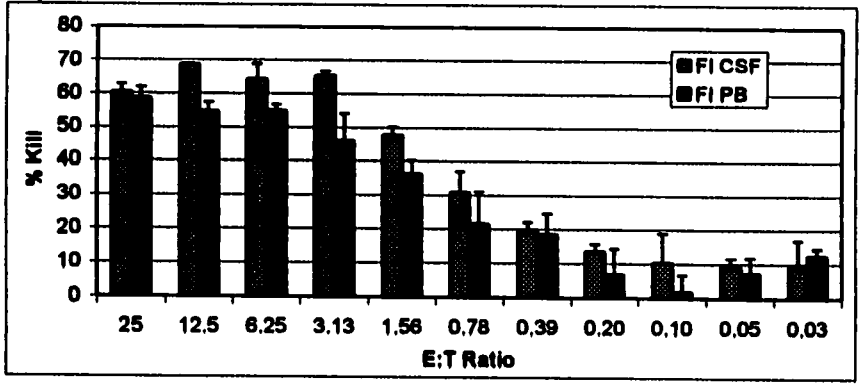
A) Colo-205



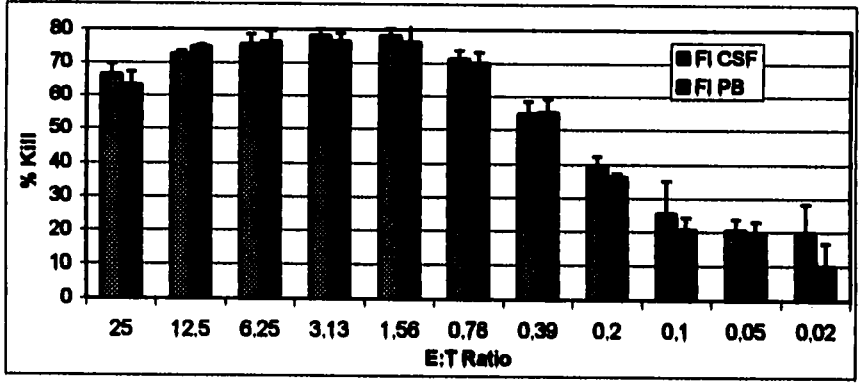
B) Jurkat



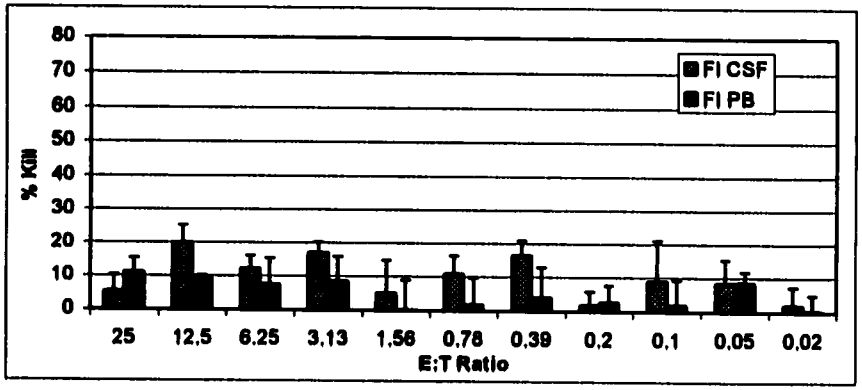
C) KG-1



D) U937



E) Daudi



F) Daudi

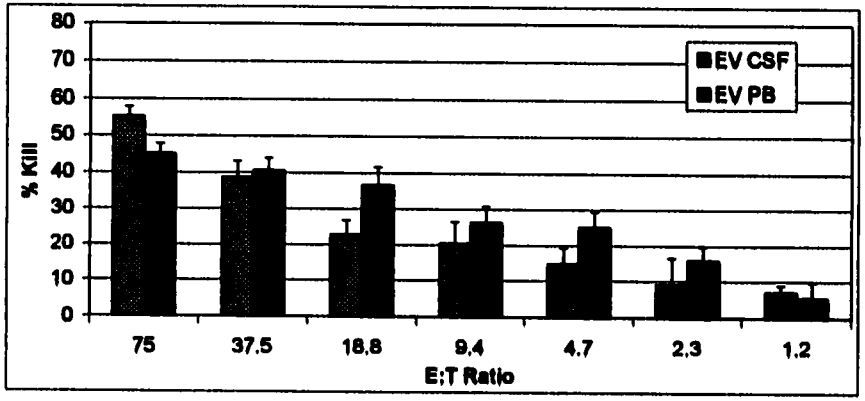


Table 19. Comparison of PB vs. CSF *t*-γδ T cell cytotoxicity (Patient FI) against a series of tumour cell targets

Cell Target	PB (% Kill)[1]	CSF (% Kill)
Colo-205	4.4 ± 8	-0.91 ± 9
Jurkat	49.2 ± 2	48.3 ± 7
KG-1	7.1 ± 7	13.8 ± 2
U937	36.4 ± 1	39.5 ± 3
Daudi[2]	2.4 ± 5	1.6 ± 4

[1] % Kill calculated at an E:T of 0.2
[2] Cytotoxicity did not follow a dose response

Despite major differences in the CSF and PB VγVδ makeup (Table 13), both CSF and PB γδ T cells killed their targets in a highly comparable manner without preference to the compartment of origin. PB and CSF induced cytotoxicities towards Daudi B cells (new lot) were erratic and not dose responsive due primarily to poor signal to noise. For comparison purposes, paired PB and CSF *t*-γδ T cell cytotoxicity towards Daudi B cells (original lot) obtained from a previous patient (EV) was included in Figure 39. Consistent with Patient FI results, there was no marked difference between PB and CSF in their ability to kill Daudi B cells.

Three PB (47-6, 7, and 26; Figure 40) and 1 CSF *t*-γδ T cell clone (46-26; Figure 41) derived from Patient FI were selected for cytotoxicity studies towards the tumour target cell panel. All PB *t*-γδ T cell clones as well as the CSF clone behaved in an analogous manner to their parent transformed lines displaying the same killing patterns towards the cell targets. Within the PB *t*-γδ T cell clone series, the two Vγ9Vδ2 clones (47-7 and 26) patterned the recognition and cytotoxicity of the Vγ9Vδ? clone (47-6) with the possible exception of decreased Vγ9Vδ? Jurkat cytotoxicity (24.9 vs. 56.5 and 38.2% for 47-6 vs. 47-7 and 26 at an E:T of 0.2). Although PB

Figure 40. Cytotoxicity responses of three PB *t*-γδ T cell clones derived from Patient FI against a tumour target cell panel. Clones 47-6, 7, and 26 were evaluated for their cytotoxicity potentials towards U937, Jurkat, Colo-205, and new lot Daudi cells (A-D) in a standard 4 h JAM assay. The results shown represent the mean % kill ± SD for quadruplicate wells.

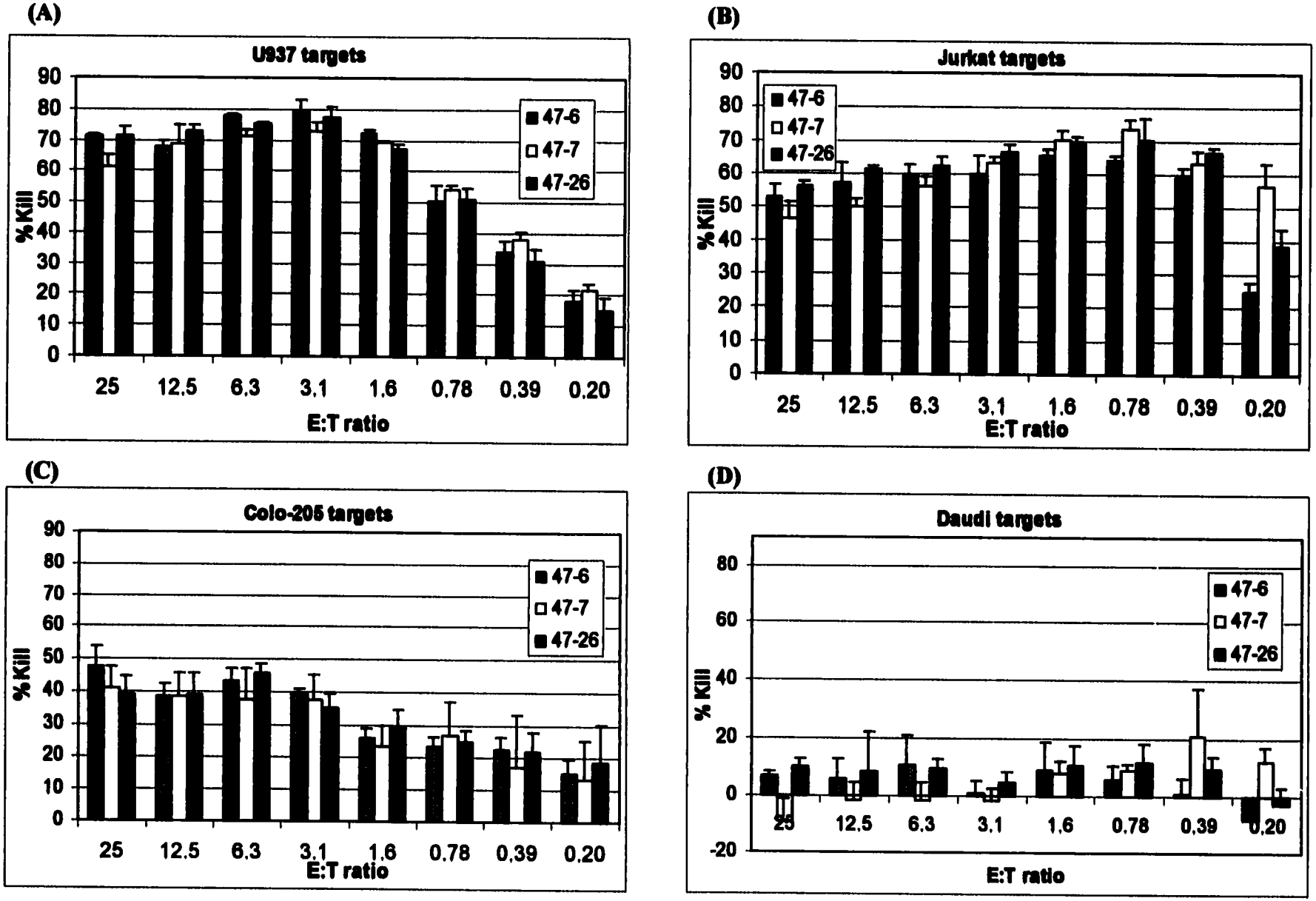
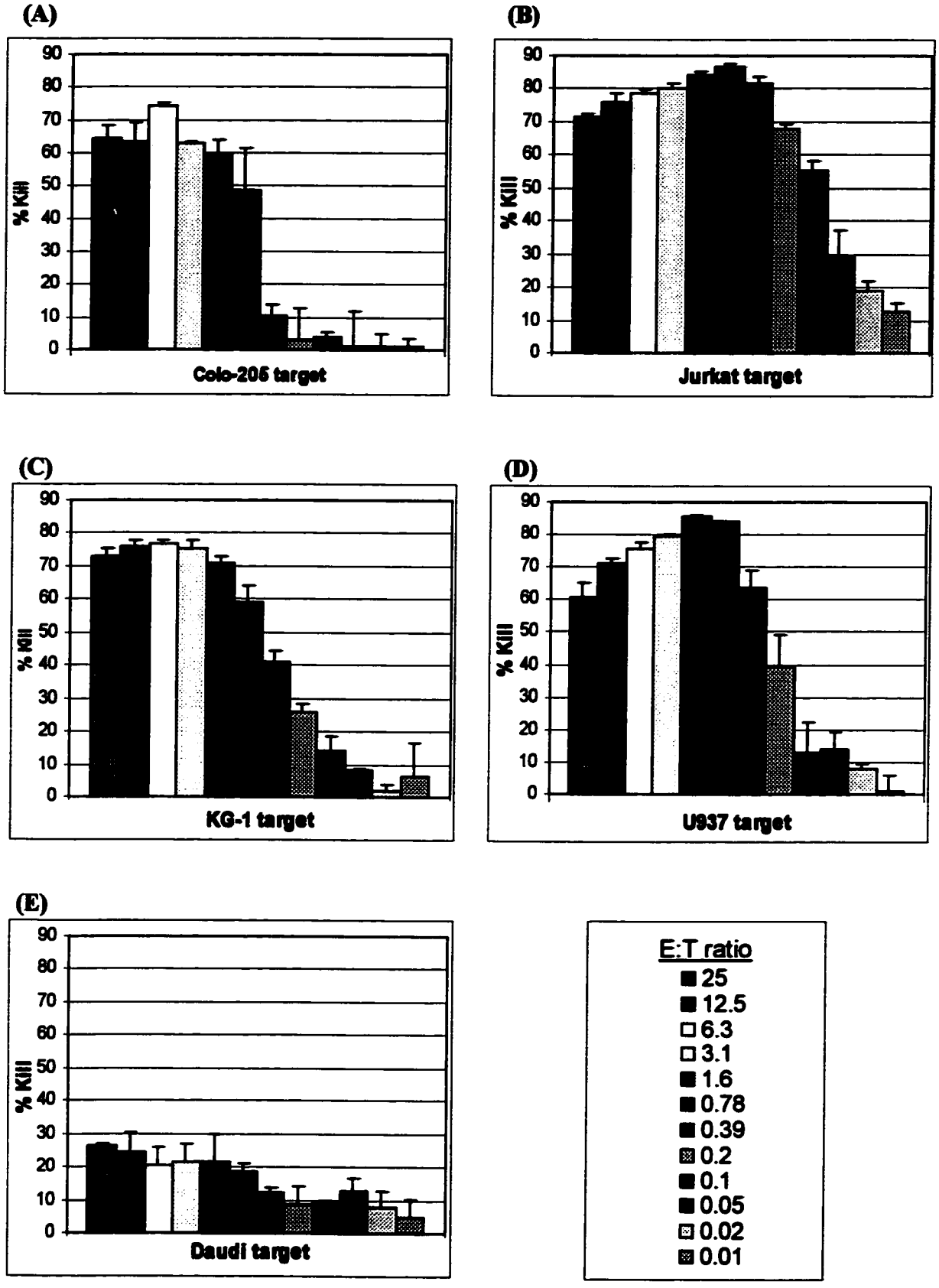

(A)
U937 targets
% Kill
E:T ratio
25
12.5
6.3
3.1
1.6
0.78
0.39
0.20
47-6
47-7
47-26
(B)
Jurkat targets
% Kill
E:T ratio
47-6
47-7
47-26
(C)
Colo-205 targets
% Kill
E:T ratio
47-6
47-7
47-26
(D)
Daudi targets
% Kill
E:T ratio
47-6
47-7
47-26

Figure 41. Cytotoxicity profiles of a CSF *t*-γδ T cell clone derived from Patient FI towards a tumour target cell panel. Clone 47-26 was evaluated for its cytotoxicity response towards Colo-205, Jurkat, KG-1, U937, and new lot Daudi cells (A-E) in a 3.5 h JAM assay. The results shown represent the mean % kill ± SD for triplicate wells. Effector to target ratios used are listed in the bottom right panel.


(A)
% Kill
Colo-205 target
(B)
% Kill
Jurkat target
(C)
% Kill
KG-1 target
(D)
% Kill
U937 target
(E)
% Kill
Daudi target
E:T ratio
25
12.5
6.3
3.1
1.6
0.78
0.39
0.2
0.1
0.05
0.02
0.01

clones induced Colo-205 death, the level of kill as the E:T was lowered decreased gradually compared to steeper rates encountered with other target cells. New lot Daudi B cells were not killed to any significant extent by all PB *t*-γδ T cell clones. Although direct comparisons to its PB counterparts could not be made due to experimental differences, the cytotoxic profile of the CSF transformed clone behaved similarly. Moreover, Colo-205 cell targets were efficiently killed by the CSF clone (60-70% kill) and titrated as expected when E:Ts were decreased.

3.5.4 Antigen recognition comparisons

Paired PB and CSF *t*-γδ T cell lines were tested for their reactivities towards five different classes of antigens using IC cytokine production as a readout. The first class examined were the cellular antigens which served as targets in cell cytotoxicity studies. In general, TNF-α relative to IFN-γ production was a more sensitive readout for antigen stimuli owing to the high basal number of IFN-γ producing cells observed in both PB and CSF *t*-γδ T cell lines (Table 20 and Figure 42 Panel A vs. Panel B). Similarly, the ratio of induced cytokine level per cell to spontaneous cytokine (MCF) was a more sensitive manner to calculate cytokine stimulation indices (cSI) in comparison to changes in cell positivity (e.g. cSI%= 24.9 vs. cSImcf= 44.1 for PMA stimulation of PB cells and TNF-α detection; Table 20). Cellular antigens were treated with brefeldin A just prior to their use as stimulators in order to minimise γδ T cell reactivity to possible soluble products released by the stimulator cells. In addition, cellular antigens were used at both a 1:1 and a 1:10 ratio of stimulator to effector cells to examine dose responsiveness. Looking at TNF-α concentration changes (Figure 42 panel B: PB and CSF MCF), the transformed PB γδ T cell line responded to KG-1 > Jurkat ≅ U937 and not Colo-205 cells (cSImcf: 4.6> 3.6≅ 3.4, 0.9 respectively) while the CSF line recognised U937 > Jurkat > Daudi > Colo-205 (cSImcf: 6.9> 4.0> 2.7> 2.1 respectively). Cytokine stimulation indices decreased as the number of stimulator cells decreased in a dose dependent manner (i.e. cSImcf: 6.9 vs. 2.3 for U937 stimulation of CSF γδ T cells at a 1:1 and 1:10 S:E ratio respectively).

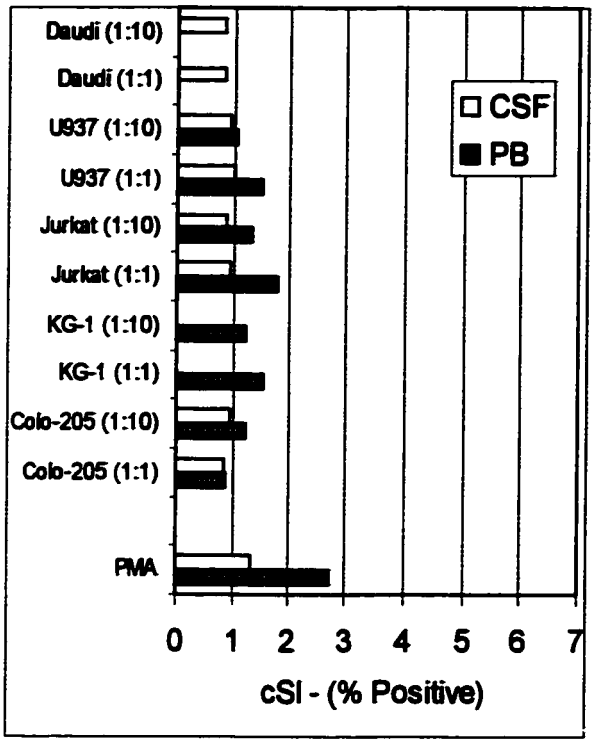
Table 20. Cellular antigen recognition studies comparing PB vs. CSF *t*-γδ T cells derived from Patient FI. PB and CSF *t*-γδ T cells were rested for 48 h prior to a 6 h stimulation with graded amounts of tumour cell targets, themselves treated with Brefeldin A for 2 h prior to the assay. Background controls consisted of cells treated with medium alone (spontaneous) whereas PMA/ Ca^{2+} ionophore stimulation served as a positive control. Following γδ TcR surface staining and fixation, permeabilised cells were subjected to either fluorochrome labelled IFN-γ or TNF-α mAbs. Cytokine specificity was demonstrated by blocking either IFN-γ or TNF-α signals upon PMA/ Ca^{2+} ionophore stimulation with the use of unlabelled IFN-γ or TNF-α blocking treatment (PMA/ block). % Pos: % of cytokine positive γδ T cells based on cell numbers found above a threshold set on γδ TcR positive cells alone; MCF: Overall relative level of cytokine per γδ T cell; cSI%: cytokine stimulation index based on % positive cells (ratio of stimulated % positive/ spontaneous % positive); cMCF: cytokine stimulation index based on MCF values (ratio of stimulated MCF/ spontaneous MCF); tumour cell 1:1 or 1:10: indicates stimulation with either 1 or 0.1 cell eq. of tumour cells to γδ T cells.

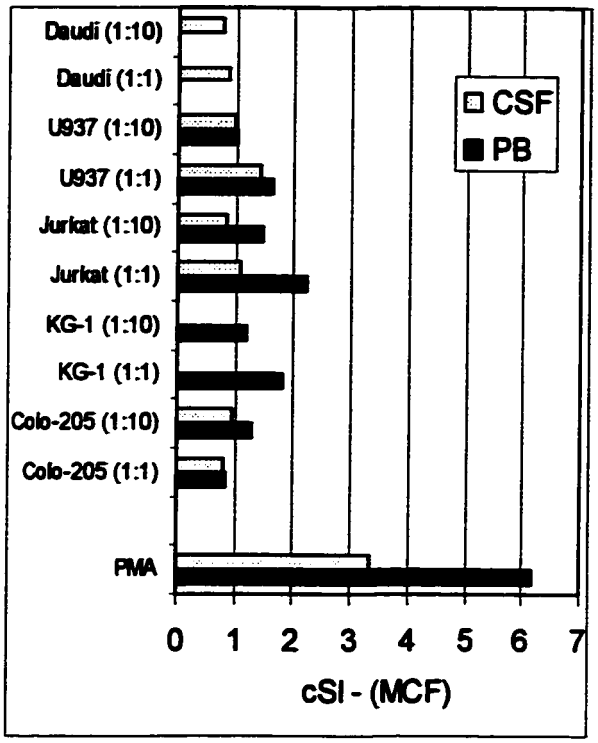
Pt. FI PB	IFNγ				TNFα			
	% Pos	MCF	cSI%	cSImcf	% Pos	MCF	cSI%	cSImcf
Spontaneous	33.4	2.40			3.4	0.61		
PMA/ block	8.8	0.75			1.3	0.65		
PMA	90.2	14.80	2.70	6.17	84.7	26.90	24.91	44.10
Colo-205 1:1	28.9	2.01	0.87	0.84	6.0	0.56	1.76	0.92
Colo-205 1:10	41.2	3.06	1.23	1.28	5.3	0.91	1.56	1.49
KG-1 1:1	51.7	4.38	1.55	1.83	11.0	2.82	3.24	4.62
KG-1 1:10	40.7	2.86	1.22	1.19	5.7	1.20	1.68	1.97
Jurkat 1:1	59.5	5.33	1.78	2.22	15.0	2.20	4.41	3.61
Jurkat 1:10	43.7	3.47	1.31	1.45	6.1	1.00	1.79	1.64
U937 1:1	50.1	3.94	1.50	1.64	12.0	2.10	3.53	3.44
U937 1:10	35.4	2.46	1.06	1.03	6.0	1.01	1.76	1.66
Daudi 1:1	ND							
Daudi 1:10	ND							

Pt. FI CSF	IFNγ				TNFα			
	% Pos	MCF	cSI%	cSImcf	% Pos	MCF	cSI%	cSImcf
Spontaneous	72.9	3.70			5.8	0.76		
PMA/ block	4.2	0.27			2.6	0.21		
PMA	96.1	12.40	1.32	3.35	74.2	15.60	12.80	20.50
Colo-205 1:1	61.9	2.90	0.85	0.80	12.1	1.56	2.09	2.05
Colo-205 1:10	67.6	3.44	0.93	0.93	7.1	0.90	1.22	1.19
KG-1 1:1	ND							
KG-1 1:10	ND							
Jurkat 1:1	68.8	4.02	0.94	1.09	18.3	3.00	3.16	3.96
Jurkat 1:10	63.1	3.00	0.87	0.82	8.3	1.33	1.43	1.75
U937 1:1	73.0	5.34	1.00	1.44	28.0	5.10	4.80	6.86
U937 1:10	69.4	3.61	0.95	0.98	10.9	1.78	1.90	2.33
Daudi 1:1	62.0	3.19	0.85	0.86	10.2	2.09	1.80	2.74
Daudi 1:10	61.6	2.86	0.84	0.77	6.5	1.06	1.12	1.39

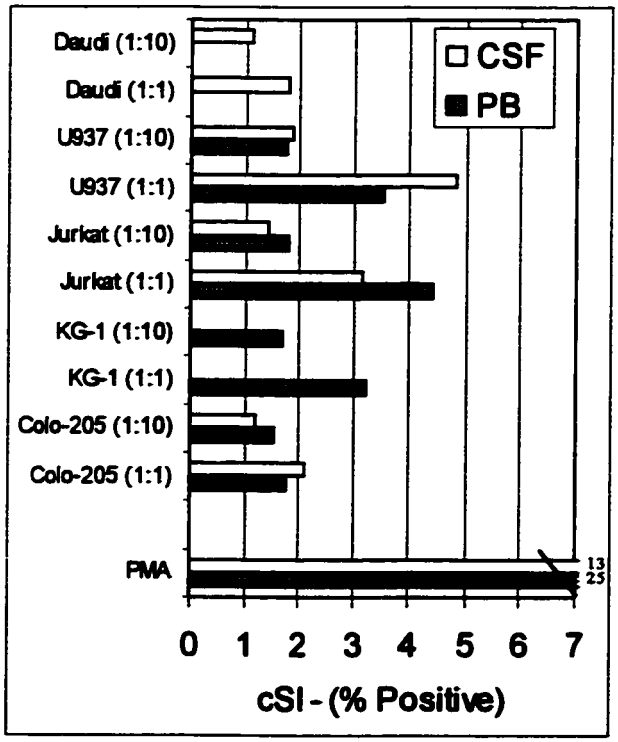
Figure 42. Comparison of the cytokine stimulation indices generated in response to tumour cell antigens by the PB vs CSF *t*-γδ T cells obtained from Patient FI. Graphical summary of the data found in Table 18. Panel A: Cytokine stimulation indices based on the number of responding cells producing IFN-γ (left) or on the relative level of IFN-γ (right). Panel B: Cytokine stimulation indices based on the number of responding cells producing TNF-α (left) or on the relative level of TNF-α (right). PB *t*-γδ T cells were not subjected to Daudi B cell stimulation and similarly, CSF cells were not subjected to KG-1 stimulation.

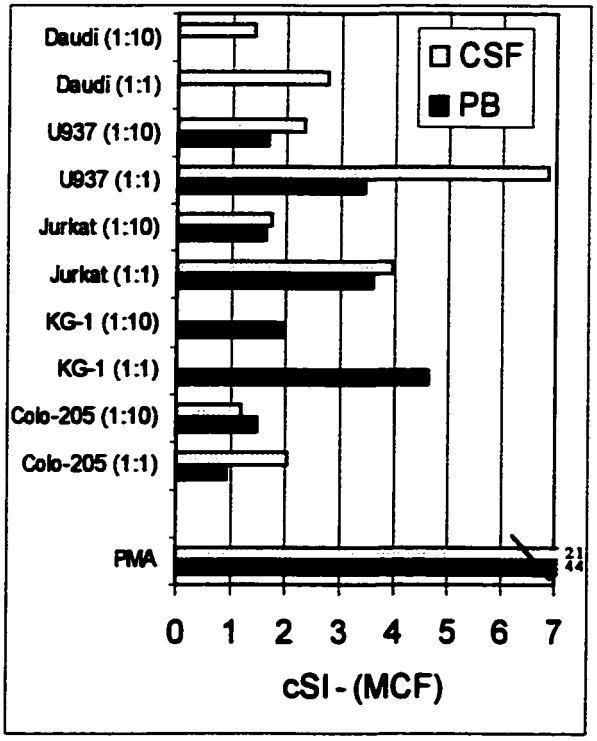
(A) IFN-γ


Daudi (1:10)
Daudi (1:1)
U937 (1:10)
U937 (1:1)
Jurkat (1:10)
Jurkat (1:1)
KG-1 (1:10)
KG-1 (1:1)
Colo-205 (1:10)
Colo-205 (1:1)
PMA
CSF
PB
0 1 2 3 4 5 6 7
cSI - (% Positive)


Daudi (1:10)
Daudi (1:1)
U937 (1:10)
U937 (1:1)
Jurkat (1:10)
Jurkat (1:1)
KG-1 (1:10)
KG-1 (1:1)
Colo-205 (1:10)
Colo-205 (1:1)
PMA
CSF
PB
0 1 2 3 4 5 6 7
cSI - (MCF)

(B) TNF-α


Daudi (1:10)
Daudi (1:1)
U937 (1:10)
U937 (1:1)
Jurkat (1:10)
Jurkat (1:1)
KG-1 (1:10)
KG-1 (1:1)
Colo-205 (1:10)
Colo-205 (1:1)
PMA
13
25
CSF
PB
0 1 2 3 4 5 6 7
cSI - (% Positive)


Daudi (1:10)
Daudi (1:1)
U937 (1:10)
U937 (1:1)
Jurkat (1:10)
Jurkat (1:1)
KG-1 (1:10)
KG-1 (1:1)
Colo-205 (1:10)
Colo-205 (1:1)
PMA
21
44
CSF
PB
0 1 2 3 4 5 6 7
cSI - (MCF)

The remaining four classes of antigens examined were composed of candidate protein MS autoantigens (human αB crystallin (AB) and bovine myelin basic protein (MBP)), the newly described γδ T cell ligands, small alkyl phosphates (dimethylallyl pyrophosphate (DA) and isopentenyl pyrophosphate (IP)) and alkylamines (isobutylamine (IB)), and a CD1 restricted mycolic acid (trehalose 6,6' dimycolate (TD)) . The results of the PB and CSF recognition assays are summarised in Table 21 and the subsequent cSIs based on TNF-α responses, graphically represented in Figure 43. The CSF *t*-γδ T cell lines uniformly did not respond to any of the antigens tested, whether by IFN-γ or TNF-α production (Table 21 and Figure 43- Panels A and B). PB γδ T cells on the other hand, responded to both small phosphate antigens and the candidate alkylamine antigen but did not respond to any measurable extent with either the protein antigens (AB and MBP) or the CD1 restricted antigen (TD). The highest cSI was obtained with IP (cSImcf= 8.3 at 100 uM) followed by IB (cSImcf= 7.2 at 5 mM) then DA (cSImcf= 2.4 at 100 uM) when looking at TNF-α responses (Figure 43). Graded amounts of all three antigens resulted in a dose response for TNF-α production (i.e. cSImcf= 8.3 vs. 1.9 vs. 1.0 for 100, 10, and 1 uM of IP respectively). IB at the highest concentration tested (50mM) resulted in γδ T cell toxicity as determined by low cell yields post stimulation and flow cytometric FS/ SS profiles. Cytokine stimulation indices based on the increased number of TNF-α producing cells (% positive), produced identical recognition patterns however with smaller magnitude cSIs (Table 21).

Two representative PB *t*-γδ T cell clones (47-6, Vγ9Vδ? and 47-26, Vγ9Vδ2) were further analysed for their reactivity towards the various antigen classes described above. Only clone 47-26 (Vγ9Vδ2) demonstrated a positive antigen response as assessed by TNF-α output, and recognition was restricted to the same antigens as for the parental PB *t*-γδ T cell line above (Table 22 and Figure 44-A and B). The strongest response to antigen was again directed to IP (cSImcf= 12.3; cSI%= 5.7 at 100 μM) and followed by DA (cSImcf= 4.9; cSI%= 3.8 at 100 μM). Clone 47-26 also responded to the candidate alkylamine antigen, IB, although to a lesser extent (cSImcf= 2.8; cSI%= 3.1 at 5mM). All antigens screened against clone 47-6 resulted in TNF-α

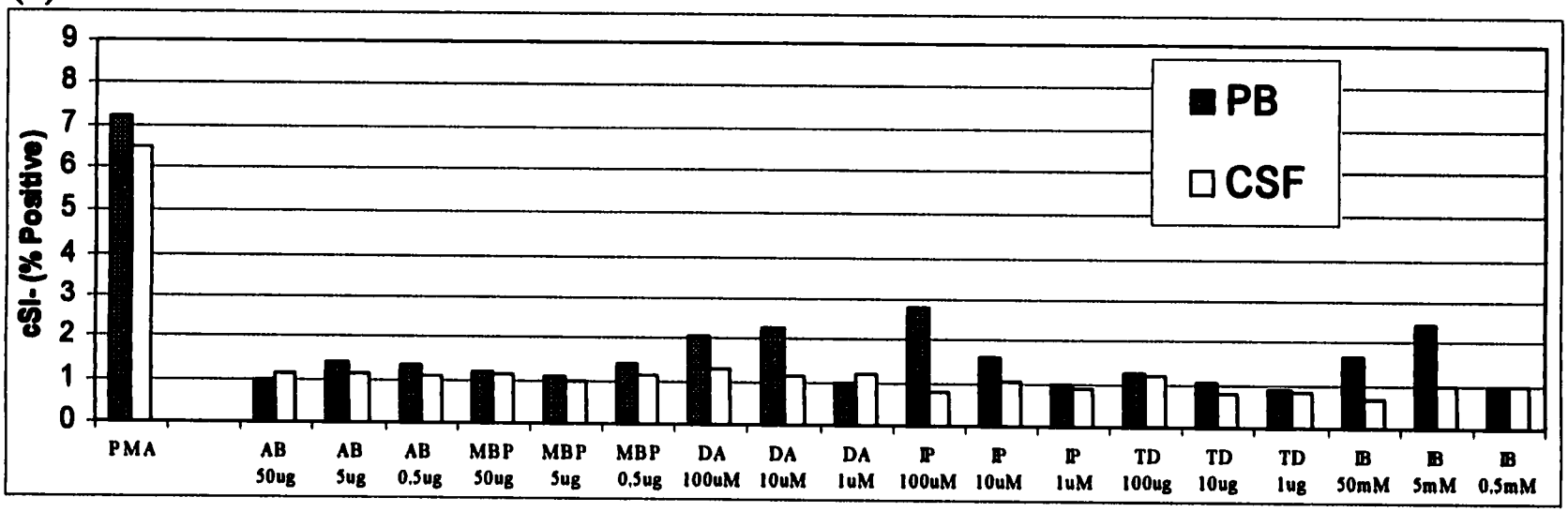
Table 21. Small molecule antigen recognition studies comparing PB vs. CSF *t*-γδ T cells derived from Patient FI. PB and CSF *t*-γδ T cells were rested for 48 h prior to a 6 h stimulation with graded amounts of the putative MS antigens αB crystallin and myelin basic protein (MBP), candidate phospholigand antigens dimethylallyl (DA) and isopentenyl (IP) pyrophosphates, candidate alkylamine antigen isobutylamine (IB), and the candidate mycolic acid trehalose dimycolate (TD). Spontaneous controls consisted of cells treated with medium alone whereas PMA/ Ca^{2+} ionophore stimulation served as a positive control. Permeabilised γδ TcR stained cells were subjected to either fluorochrome labelled IFN-γ or TNF-α mAbs. % Pos: % of cytokine positive γδ T cells based on cell numbers found above a threshold set on γδ TcR positive cells alone; MCF: Overall relative level of cytokine per γδ T cell; cSI%: cytokine stimulation index based on % positive cells (ratio of stimulated % positive/ spontaneous % positive); cMCF: cytokine stimulation index based on MCF values (ratio of stimulated MCF/ spontaneous MCF).

Pt. FI PB	IFNγ				TNFα			
	% Pos	MCF	cSI%	cSImcf	% Pos	MCF	cSI%	cSImcf
Spontaneous	58.0	1.56			12.5	0.61		
PMA	96.2	23.40	1.66	15.00	89.8	101.70	7.18	166.72
αβ crystallin 50ug	69.2	1.85	1.19	1.19	12.5	0.64	1.00	1.05
αβ crystallin 5ug	54.8	1.52	0.94	0.97	17.6	0.74	1.41	1.21
αβ crystallin 0.5ug	58.8	1.60	1.01	1.03	17.0	0.73	1.36	1.20
MBP 50ug	57.7	1.56	0.99	1.00	15.0	0.71	1.20	1.16
MBP 5ug	44.7	1.34	0.77	0.86	13.8	0.65	1.10	1.07
MBP 0.5ug	43.1	1.30	0.74	0.83	17.7	0.73	1.42	1.19
DA 100uM	55.2	1.66	0.95	1.06	26.0	1.46	2.08	2.39
DA 10uM	55.4	1.54	0.96	0.99	28.4	0.95	2.27	1.56
DA 1uM	45.0	1.37	0.78	0.88	12.2	6.08	0.98	1.00
IP 100uM	56.8	2.13	0.98	1.37	34.7	5.08	2.78	8.33
IP 10uM	54.2	1.62	0.93	1.04	19.9	1.17	1.59	1.92
IP 1uM	39.1	1.22	0.67	0.78	12.2	0.62	0.98	1.01
TD 100ug	54.1	1.54	0.93	0.99	15.3	0.68	1.22	1.11
TD 10ug	51.6	1.47	0.89	0.94	12.8	0.64	1.02	1.06
TD 1ug	50.3	1.43	0.87	0.92	11.1	0.58	0.89	0.96
Iso-butylamine 50mM	52.2	1.69	0.90	1.08	20.4	0.89	1.63	1.46
Iso-butylamine 5mM	52.6	2.25	0.91	1.44	30.7	4.38	2.46	7.18
Iso-butylamineB -0.5mM	42.6	1.31	0.73	0.84	11.9	0.63	0.95	1.04

Pt. FI CSF	IFNγ				TNFα			
	% Pos	MCF	cSI%	cSImcf	% Pos	MCF	cSI%	cSImcf
Spontaneous	60.1	2.62			12.7	1.56		
PMA	96.6	14.60	1.61	5.57	82.1	11.30	6.46	7.24
αβ crystallin 50ug	59.5	2.34	0.99	0.89	14.6	1.34	1.15	0.86
αβ crystallin 5ug	62.8	2.46	1.04	0.94	14.7	1.42	1.16	0.91
αβ crystallin 0.5ug	60.9	2.32	1.01	0.89	14.0	1.24	1.10	0.79
MBP 50ug	42.3	1.53	0.70	0.58	14.4	1.02	1.13	0.65
MBP 5ug	56.1	2.17	0.93	0.83	12.8	1.12	1.01	0.72
MBP 0.5ug	55.4	2.20	0.92	0.84	14.6	1.32	1.15	0.85
DA 100uM	59.7	2.48	0.99	0.95	16.7	1.57	1.31	1.01
DA 10uM	61.7	2.36	1.03	0.90	14.5	1.28	1.14	0.82
DA 1uM	59.2	2.35	0.99	0.90	14.8	1.39	1.17	0.89
Iso-butylamine 50mM	70.9	2.56	1.18	0.98	8.6	0.50	0.68	0.32
Iso-butylamine 5mM	48.3	1.80	0.80	0.69	12.6	1.00	0.99	0.64
Iso-butylamine -0.5mM	46.5	1.75	0.77	0.67	12.3	1.16	0.97	0.74

Figure 43. Comparison of the cytokine stimulation indices generated in response to small molecule antigens by the PB vs CSF *t*-γδ T cells obtained from Patient FI. Graphical summary of the cytokine stimulation indices (cSI) based on TNF-α production in response to the series of small antigens (Table 19 data). Panel A: cSIs based on the ratio of stimulated TNF-α % positive cells to spontaneous TNF-α % positive cells. Panel B: cSIs based on the ratio of the relative TNF-α level post stimulation to the TNF-α level occurring spontaneously. AB: αB crystallin; MBP: myelin basic protein; DA: dimethylallyl pyrophosphate; IP: isopentenyl pyrophosphate; TD: trehalose dimycolate; IB: isobutylamine.

(A)


cSI- (% Positive)
9
8
7
6
5
4
3
2
1
0
PB
CSF
PMA
AB 50ug
AB 5ug
AB 0.5ug
MBP 50ug
MBP 5ug
MBP 0.5ug
DA 100uM
DA 10uM
DA 1uM
IP 100uM
IP 10uM
IP 1uM
TD 100ug
TD 10ug
TD 1ug
IB 50mM
IB 5mM
IB 0.5mM

(B)

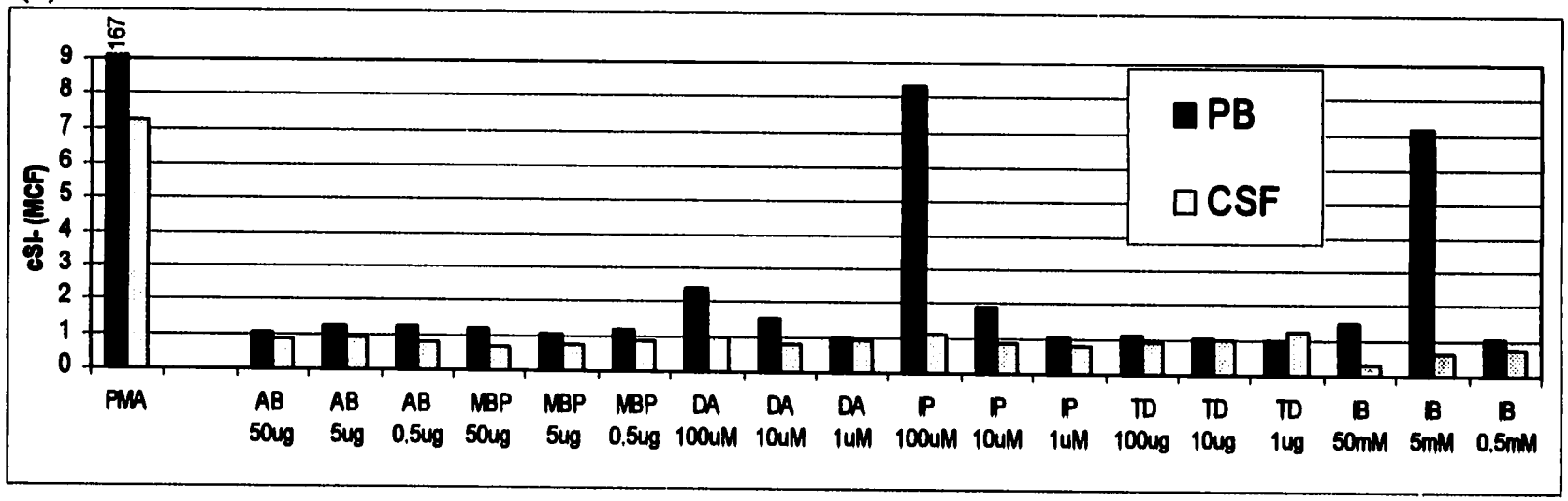
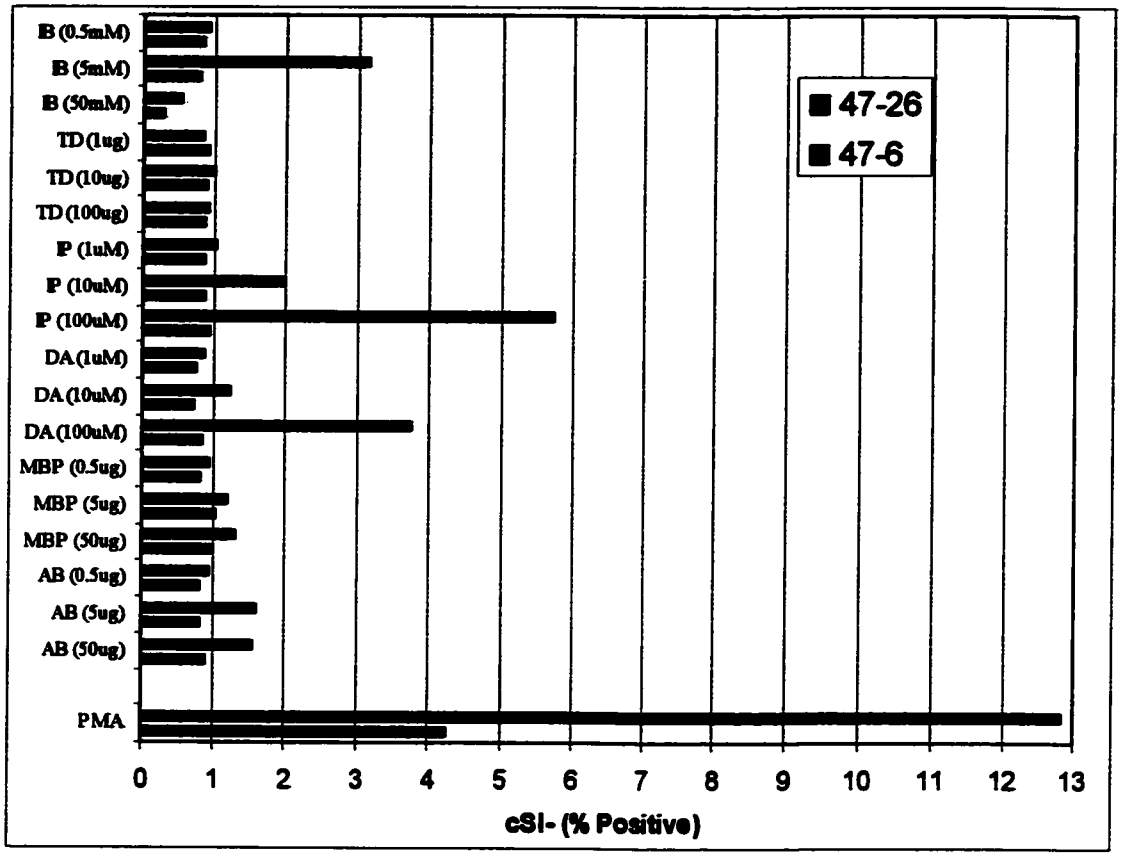

cSI- (MCF)
167
9
8
7
6
5
4
3
2
1
0
PB
CSF
PMA
AB 50ug
AB 5ug
AB 0.5ug
MBP 50ug
MBP 5ug
MBP 0.5ug
DA 100uM
DA 10uM
DA 1uM
IP 100uM
IP 10uM
IP 1uM
TD 100ug
TD 10ug
TD 1ug
IB 50mM
IB 5mM
IB 0.5mM

Table 22. Small molecule antigen recognition studies comparing representative PB *t*-γδ T cell clones derived from Patient FI. Clone 47-6 (Vγ9non-Vδ1, 2, or 3) and clone 47-26 (Vγ9Vδ2) were rested for 48 h prior to a 18 h stimulation with graded amounts of the putative MS antigens αB crystallin (AB) and myelin basic protein (MBP), candidate phospholigand antigens dimethylallyl (DA) and isopentenyl (IP) pyrophosphates, candidate alkylamine antigen isobutylamine (IB), and the candidate mycolic acid trehalose dimycolate (TD). Spontaneous controls consisted of cells treated with medium alone whereas PMA/ Ca^{2+} ionophore stimulation served as a positive control. Permeabilised γδ TcR stained cells were subjected to phycoerythrin labelled TNF-α mAb. % Pos: % of TNF-α positive γδ T cells based on cell numbers found above a threshold set on γδ TcR positive cells alone; MCF: Overall relative level of TNF-α per γδ T cell; cSI%: cytokine stimulation index based on TNF-α % positive cells (ratio of stimulated % positive/ spontaneous % positive); cMCF: cytokine stimulation index based on TNF-α MCF values (ratio of stimulated MCF/ spontaneous MCF).

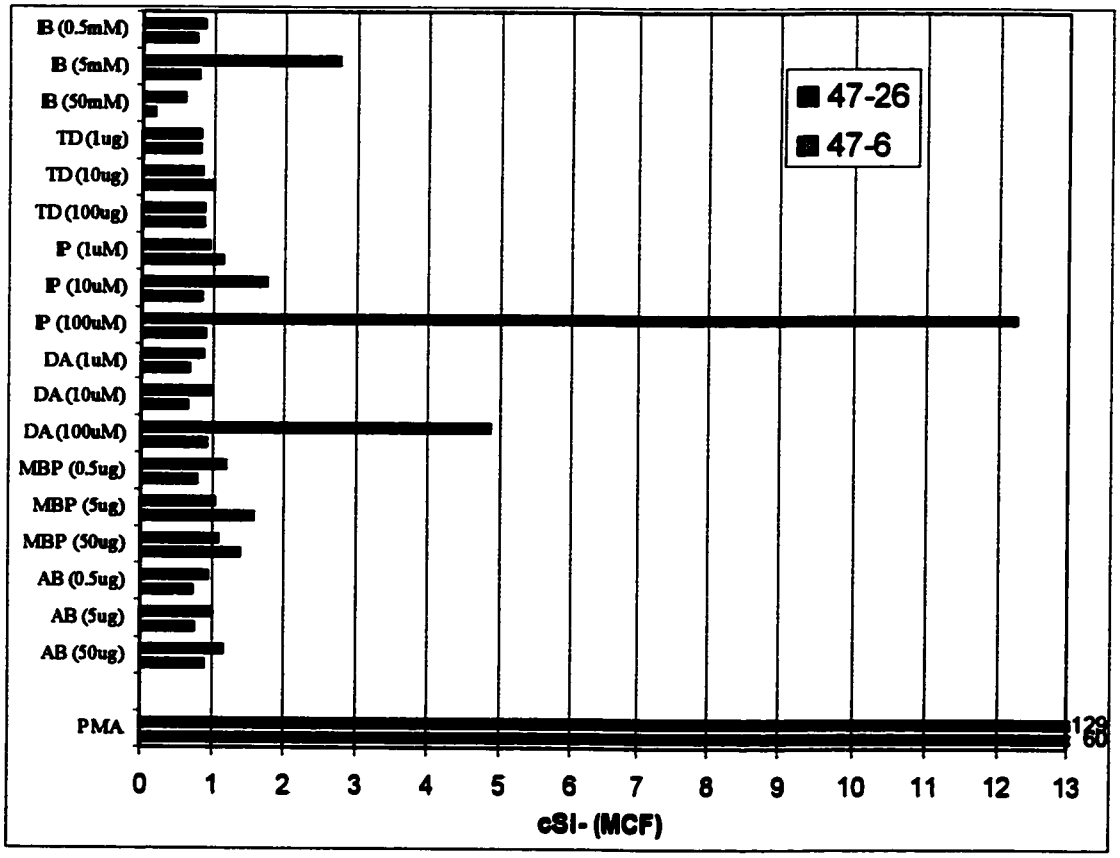
Antigen	Clone 47- 6 (Vγ9Vδ?) TNFα				Clone 47-26 (Vγ9Vδ2) TNFα			
	% Pos	MCF	cSI%	cSImcf	% Pos	MCF	cSI%	cSImcf
Spontaneous	23.1	2.70			7.6	0.80		
PMA	97.8	163	4.23	60.4	97.6	104	12.8	129
αβ crystallin 50ug	20.4	2.40	0.88	0.89	11.9	0.94	1.57	1.18
αβ crystallin 5ug	18.8	2.00	0.81	0.74	12.2	0.81	1.61	1.01
αβ crystallin 0.5ug	18.5	1.99	0.80	0.74	7.2	0.75	0.95	0.94
MBP 50ug	22.2	3.74	0.96	1.39	9.9	0.86	1.30	1.08
MBP 5ug	24.0	4.28	1.04	1.59	9.0	0.82	1.18	1.03
MBP 0.5ug	18.7	2.10	0.81	0.78	7.1	0.95	0.93	1.19
DA 100uM	19.5	2.50	0.84	0.93	28.5	3.90	3.75	4.88
DA 10uM	16.9	1.76	0.73	0.65	9.2	0.77	1.21	0.96
DA 1uM	17.1	1.81	0.74	0.67	6.5	0.70	0.86	0.88
IP 100uM	21.7	2.41	0.94	0.89	43.4	9.80	5.71	12.3
IP 10uM	19.9	2.26	0.86	0.84	14.9	1.41	1.96	1.76
IP 1uM	20.0	3.05	0.87	1.13	7.8	0.75	1.03	0.94
TD 100ug	20.0	2.32	0.87	0.86	6.9	0.70	0.91	0.88
TD 10ug	20.7	2.67	0.90	0.99	7.6	0.66	1.00	0.83
TD 1ug	21.4	2.20	0.93	0.81	6.4	0.64	0.84	0.80
IB 50mM	6.1	0.45	0.26	0.17	4.0	0.47	0.53	0.59
IB 5mM	17.9	2.10	0.77	0.78	23.8	2.20	3.13	2.75
IB -0.5mM	19.0	2.04	0.82	0.76	7.0	0.70	0.92	0.88

Figure 44. Comparison of the cytokine stimulation indices generated in response to small molecule antigens by representative PB *t*-γδ T cell clones obtained from Patient FI. Graphical summary of the cytokine stimulation indices (cSI) for clone 47-6 (Vγ9non-Vδ1, Vδ2, or Vδ3) and 47-26 (Vγ9Vδ2), based on TNF-α production in response to the series of small antigens (Table 20 data). Panel A: cSIs based on the ratio of stimulated TNF-α % positive cells to spontaneous TNF-α % positive cells. Panel B: cSIs based on the ratio of the relative TNF-α level post stimulation to the TNF-α level occurring spontaneously. AB: αB crystallin; MBP: myelin basic protein; DA: dimethylallyl pyrophosphate; IP: isopentenyl pyrophosphate; TD: trehalose dimycolate; IB: isobutylamine.

(A)


B (0.5mM)
B (5mM)
B (50mM)
TD (1ug)
TD (10ug)
TD (100ug)
P (1uM)
P (10uM)
P (100uM)
DA (1uM)
DA (10uM)
DA (100uM)
MBP (0.5ug)
MBP (5ug)
MBP (50ug)
AB (0.5ug)
AB (5ug)
AB (50ug)
PMA
47-26
47-6
0 1 2 3 4 5 6 7 8 9 10 11 12 13
cSI- (% Positive)

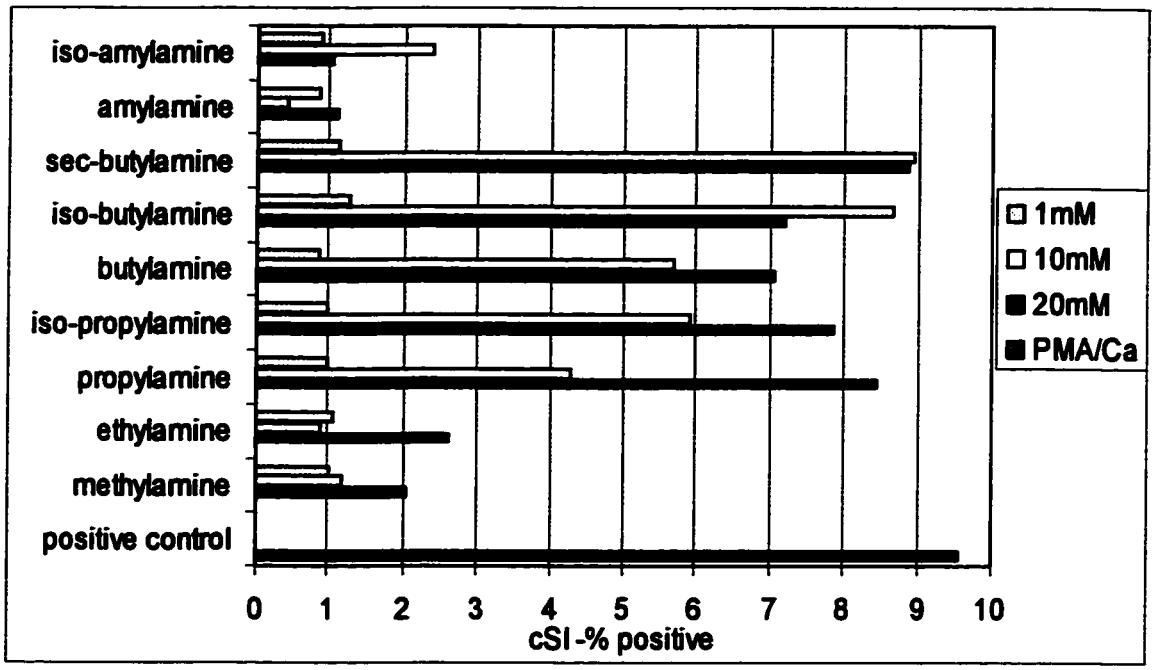
(B)


B (0.5mM)
B (5mM)
B (50mM)
TD (1ug)
TD (10ug)
TD (100ug)
P (1uM)
P (10uM)
P (100uM)
DA (1uM)
DA (10uM)
DA (100uM)
MBP (0.5ug)
MBP (5ug)
MBP (50ug)
AB (0.5ug)
AB (5ug)
AB (50ug)
PMA
47-26
47-6
129
60
0 1 2 3 4 5 6 7 8 9 10 11 12 13
cSI- (MCF)

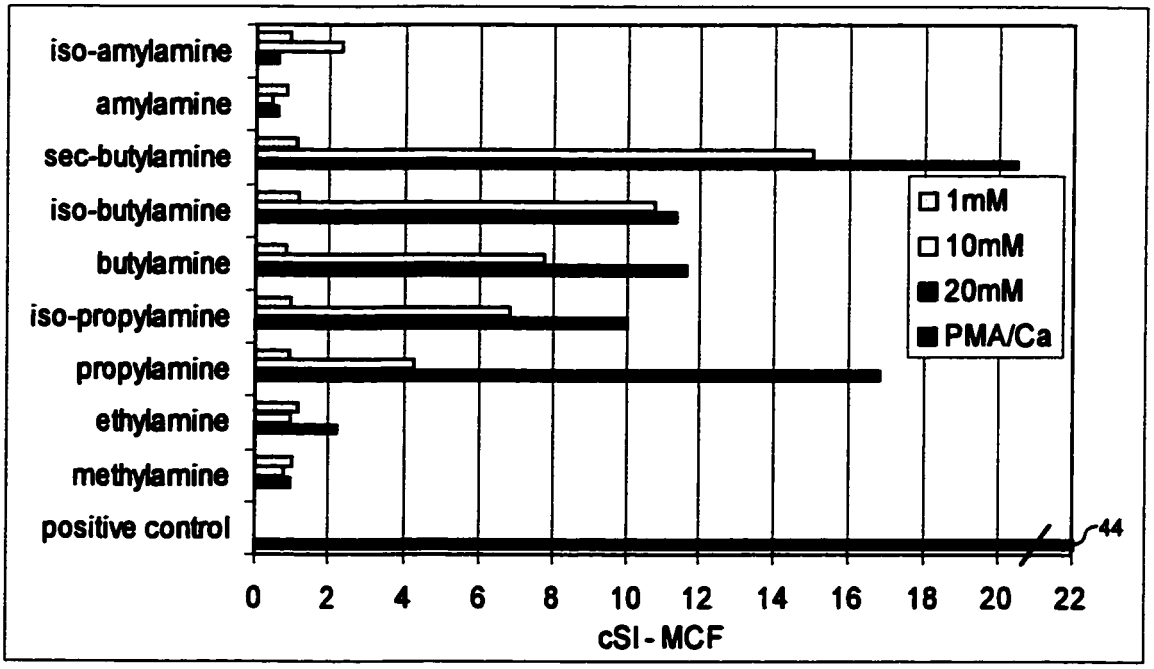
unresponsiveness and consequently cSIs of ≈ 1 (Table 22 and Figure 44-A and B). To probe the responses of the PB *t*-γδ T cell clones to alkylamine antigens in more detail, antigen recognition studies were extended to cover nine different alkylamine antigens (Figure 45). Clone 47-26 (Vγ9Vδ2) differentially responded to the alkylamines in a dose response manner as assessed through TNF-α production, although some toxicity was observed at the highest concentration for some antigens. Clone 47-26 responded to alkylamine at a 10mM concentration with the following pattern recognition based on MCF levels (Figure 45-B): no response to methylamine, ethylamine, and amylamine (cSIs: ~1); increasing from isoamylamine (cSI: 2.3), propylamine (cSI:4.3), isopropylamine (cSI:6.8), butylamine (cSI:7.8), isobutylamine- (cSI:10.7) and peaking with secbutylamine (cSI:15). Similar recognition patterns were observed based on the numbers of responding TNF-α positive 47-26 cells (Figure 45-A). Similar studies with sibling Vγ9Vδ2 clones (47-5 and 47-7) and a Vγ9Vδ-other clone (47-6), yielded essentially the same pattern recognition for the two Vγ9Vδ2 clones (47-5 and 7) but no alkylamine recognition with the Vγ9Vδ-other clone (47-6) (data not shown). These results, together with similar studies with the PB *t*-γδ T cell clone 44-16 (Vγ4Vγ9Vδ1; Figure 36), suggest that the Vγ9Vδ2 γδ TcR pairing is essential for both phosphate containing and alkylamine antigen recognition and that the expression of Vγ9 gene elements alone is not sufficient for γδ T cell responsiveness.

Figure 45. Antigen recognition profiles for a PB *t*-γδ T cell clone from Patient FI to a series of non-peptidic alkylamine antigens assessed through TNF-α production. Clone 47-26 (Vγ9Vδ2) was rested for 48 h in IL-2 free medium prior to a 6 h culture with graded amounts of alkylamine antigens as indicated. Positive controls consisted of treating clone 47-26 with PMA/ Ca^{2+} ionophore. Following γδ TcR surface staining and fixation, permeabilised cells were treated with phycoerythrin labelled TNF-α mAb and enumerated by FACS analysis. Cytokine stimulation indices are based on either the numbers of TNF-α positive cells following alkylamine stimulation relative to spontaneous TNF-α positive cells (Panel A) or on the ratio of TNF-α per γδ T cell after alkylamine treatment to the level of TNF-α occurring spontaneously (Panel B).

A)


iso-amylamine
amylamine
sec-butylamine
iso-butylamine
butylamine
iso-propylamine
propylamine
ethylamine
methylamine
positive control
1mM
10mM
20mM
PMA/Ca
0 1 2 3 4 5 6 7 8 9 10
cSI-% positive

B)


iso-amylamine
amylamine
sec-butylamine
iso-butylamine
butylamine
iso-propylamine
propylamine
ethylamine
methylamine
positive control
1mM
10mM
20mM
PMA/Ca
44
0 2 4 6 8 10 12 14 16 18 20 22
cSI-MCF

4 Discussion

4.1 Short term γδ T cell culture

One of the most difficult and therefore limiting aspects in studying human γδ T cells is the derivation of sufficient cells in which to study, especially cells derived from the tissue of greatest interest, the CNS. For this reason, most studies investigating the role of human γδ T cells in MS pathogenesis are limited to histopathological studies where CNS involvement and biological function are inferred from snapshots of diseased tissue based on their prevalence, location, clonality, and possession of pathogenic mediators such as cytokine or cytolytic molecules (6; 216-218). Alternatively, most studies actively probing γδ T cells and MS have relied on PB derived γδ T cells studied *ex vivo* (139; 150; 219) or after selective expansion to known ligands such as discrete antigen (81; 220), tumour cells (121), immunising agents (77; 147), subtype specific antibody (89) or non-specifically with mitogen (89; 140). Notably, only a few studies have been performed addressing CSF derived γδ T cells and the majority of these were focused on γδ T cell receptor usage (140; 221; 222). One of the major goals of this study was to gain a more complete understanding of the biological functions of MS γδ T cells, specifically comparing PB vs. CSF γδ T cells for potential differences that may relate to MS pathogenesis. To accomplish this, γδ T cells were selectively expanded from blood and CSF using immobilised γδ TcR mAb. That all γδ T cells were polyclonally stimulated was evident from the cross section of γδ T cell subtypes present upon immunophenotyping the expanded cells (Figure 2). PB γδ T cell lines were consistently dominated by the Vγ9Vδ2 subtype (70-90%) with the remaining cells mostly belonging to the Vδ1 subtype; similar to *ex vivo* PB γδ T cell distributions (223; 224). In contrast, expansion of γδ T cells from PB using either mycobacterial extracts or Daudi B lymphoma cells, two common methods employed to generate γδ T cells, results in the outgrowth of Vγ9Vδ2 expressing cells exclusively (58; 66; 121). CSF γδ T cell lines were much more heterogeneous in their subtype distributions and differed considerably among patient samples. Illustrating this point, three random CSF samples displayed markedly different patterns ranging from significant

representation of Vδ1, Vδ2, Vδ3, Vγ4, Vγ9, and Vγ other positive cells (patient C) to samples comprising only limited VγVδ subtypes (patient E). Whether *in vitro* selection for γδ T cell subpopulations is in part responsible for the variation seen in the CSF remains a possibility, however the highly reproducible PB subtype pattern obtained for different patient samples argues against this scenario. In support of the above findings, molecular studies directed at CNS γδ TcR gene repertoires detected Vγ and Vδ transcripts with similar inter-patient variability (138; 225). A more reasonable explanation for the often encountered predominance of 1-2 VγVδ subtypes found in expanded CSF, is that this simply reflects a small number of γδ T cell clones present within the CSF.

This strategy for generating γδ T cells offers certain advantages over methods used by other investigators; contingent with the ever present possible occurrence of *in vitro* selection. Immobilised mAb treatment selectively stimulates γδ T cell expansion and limits the outgrowth of the more abundant αβ T cell populations; a situation often encountered with the use of polyclonal T cell mitogens (138; 140). Furthermore, large numbers of polyclonal γδ T cell lines are generated as opposed to the limited production of time consuming γδ T cell clones; often shown to be problematic as a result of their poor cloning efficiency and generally, only small numbers of cells are available for study (137; 138; 226). PB and CSF γδ T cell production as described in this study yields relatively high numbers of pure γδ T cells in which to study (typically 5-10 x 10^6 cells; >80% pure; Figure 4).

Despite the ability to consistently generate highly pure PB and CSF γδ T cell lines, there were still only sufficient cells for 1-2 small experiments per patient sample. In an effort to circumvent this problem, methods were investigated to prolong the culture life of the expanded γδ T cell lines. An underlying characteristic of both PB and CSF γδ T cells was their propensity for undergoing either stimulation induced or spontaneous death, and was often evident when attempts were made to maintain the short term γδ T cells in cell cycle. Restimulation with anti-γδ TcR mAb with or without accessory cells invariably resulted in widespread γδ T cell death. Interestingly, PHA/ feeder cell stimulation resulted in either the highest expansion index of all

methods studied (Table 3) or in widespread death (Figure 5). Alternatively, IL-2 at a relatively high concentration was an effective stimulus notably with cells that underwent PHA induced cell death in simultaneous assays. Observations recorded from multiple expansion experiments suggested that PHA/ feeder stimulation was most effective with γδ T cell populations containing significant numbers of contaminating αβ T cells, while similar treatment with highly pure γδ T cells (>80%) resulted in death. This phenomenon may be explained in terms of a reduced activation threshold possessed by γδ T cells making them exquisitely sensitive to overstimulation. γδ T cells possess approximately twice the number of TcR/CD3 complexes on their surface and intracellular Ca^{2+} signalling is six times higher upon stimulation compared to αβ T cells (227; 228). Intense stimulation via TcR, as would occur through anti-γδ TcR mAb or PHA treatment on pure γδ T cell populations, could invoke rapid activation induced cell death via Fas-FasL interaction; a phenomenon in which γδ T cells are more susceptible than αβ T cells (171; 172; 229). PHA treatment on semi-pure γδ T cell populations however, would be dilutive as well as invoke the production of feeder cell derived IL-2 and possibly other potent γδ T cell growth factors such as IL-15 (230). This hypothesis is also consistent with the observation that γδ T cell expansion is optimal when IL-2 is added 24 h post stimulation, whereas simultaneous IL-2 addition often results in death. The extensive expansion of γδ T cells in the presence of high IL-2 concentrations may be related to the presence of high affinity IL-2R upregulated upon primary stimulation. This expansion was typically short lived as cells progressively ceased to cycle presumably from IL-2Rα downregulation (Table 5). Despite the ability to keep γδ T cells in cycle for periods significantly longer than that achieved *a priori*, they nonetheless became increasingly resistant to further stimulation and eventually succumbed. This suggests that the *in vitro* culture lifespan of γδ T cells is relatively short and finite and is consistent with *in vivo* tracking studies that highlight a very rapid turnover rate of both naïve and activated γδ T cells (231).

Since conclusive information is lacking regarding the γδ T cell subtypes found within the CSF of MS patients and importantly, whether there exists any biological differences that may have an impact on MS pathogenesis, it was of interest to enumerate the relative Vδ1 and Vδ2

distributions found in blood and CSF of both MS and OND patients. No significant differences were observed between Vδ1 and Vδ2 distributions between MS and OND patients, although there was a trend for increased accumulation of Vδ1 cells both within PB and CSF of MS patients (Figure 7). This latter result is consistent with reports suggesting that the γδ T cell repertoire in MS patients is skewed towards increased Vδ1 cells within the CSF (138; 140; 222). Furthermore, when these cells were tested for cytotoxicity responses against a series of tumour cell targets, no significant differences were found between blood and CSF derived γδ T cells in MS patients (Table 6), consistent with the findings of others (137; 138). There was a significant increase in killing by OND relative to MS γδ T cells towards HSP expressing Daudi and RPMI 8226 cell targets (121). This difference may in part be attributable to the greater numbers of Vδ2Vγ9 positive cells found within OND CSF (Figure 7); the subset of γδ T cells found to be particularly reactive to HSPs (66; 68). The testing of this hypothesis was best achieved using purified γδ T cell subtype populations and will be subsequently discussed. This data suggests, in accord with others (137; 138), that both PB and CSF γδ T cells regardless of disease status, can efficiently kill a series of seemingly unrelated cell targets. This is particularly relevant for MS pathogenesis considering that γδ T cells can induce the most rapid *in vitro* cytolysis of human oligodendrocytes shown to date (127; 151), and further poses the questions as to what ligand(s) they see and in the context of their promiscuous cytotoxic nature, what controls this behaviour in healthy individuals?

These studies, along with those investigating γδ T cell cytotoxicity mechanisms (127), highlighted a number of limitations of short term PB and CSF γδ T cell lines that impeded further, more comprehensive studies. Central to this issue is the relatively short lifespan of cultured γδ T cells which sufficiently provides cell numbers for initial but not for repetitive or further studies with the same patient cells. As a result, there was a substantial increase in interpatient, in addition to the intrapatient variability normally associated with human samples. This situation was highlighted by the variability encountered with the short term γδ T cell subtype and cytotoxicity studies discussed above. Whether there are significant differences between MS or OND patients

or between the PB and the CSF compartments, as the trends suggest, can only be answered with large sample numbers. These were the principal reasons which served as an impetus to find a way to generate consistent long term MS γδ T cells which could be sampled repetitively.

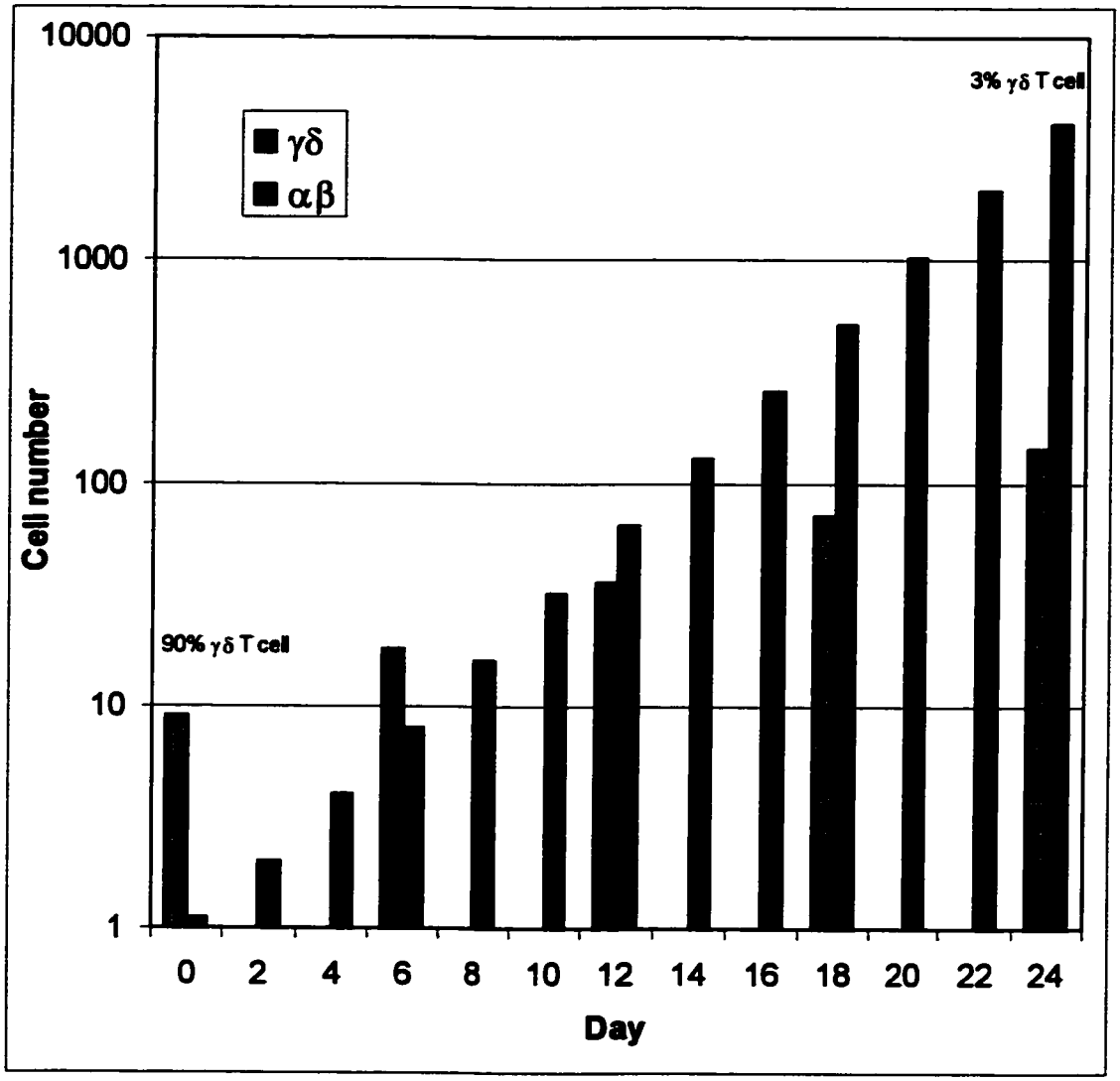
4.2 *Herpesvirus saimiri* immortalisation of MS γδ T cells

The growth transformation of human αβ T cells with *Herpesvirus saimiri* (HVS) was well established at the outset of this work (179), however no reports on the successful immortalisation of γδ T cells were available. Based on the known characteristics of *t*-αβ T cells such as the retention of antigen specificity and function together with continuous and stable growth, it was clear that this was the strategy of choice for generating long lived MS γδ T cells. Due to a lack of precedent in this field, a significant emphasis was placed on developing methodologies to successfully immortalise human PB and CSF γδ T cells, using established conditions for αβ T cells as a guideline (179; 180). Subsequently, two groups have reported success in generating HVS *t*-γδ T cells, although with limited characterisation. Yasukawa and colleagues reported the generation of two *t*-γδ T cell clones from normal PB (184), and Fickenscher and colleagues reported the generation of a number of HVS *t*-γδ T cell clones from human cord blood (185; 186). This work reports for the first time, the successful HVS induced growth transformation of human PB as well as CSF derived γδ T cell lines and clones, along with their extensive characterisation.

HVS growth transformation of human γδ T cells in many ways paralleled methodologies established for αβ T cells, however a number of key determinants were defined that were critical for success. The majority of these determinants centred around two aspects specific to γδ T cells: (1) their precarious culture characteristics, and (2) their relative purity at the time of infection. As with short term γδ T cell growth, γδ T cells required more stringent conditions for optimal HVS transformation relative to those required for αβ T cells and small deviations from these conditions were not tolerated (Table 9). For example, *t*-αβ T cells were reported to ideally grow in 24 well or tissue culture flasks (179; 180), however *t*-γδ T cell growth was only supported

in 96w U-bottom formats and failed to thrive in any other format. This result suggests a greater reliance on CD2-CD58 inter-cell contact to provide one of the necessary growth signals for proliferation, as determined through *t*-αβ T cell studies (196). Moreover, *t*-γδ T cells required large amounts of high activity IL-2 for optimal *t*-γδ T cell growth, however this may simply have been compensatory because *t*-αβ T cells can generate sufficient IL-2 for autocrine growth (196) whereas *t*-γδ T cells are poor IL-2 producers (Figures 19, 23, 37 and (186)). Taken together, these observations suggest proliferation signals are marginally different for *t*-γδ and *t*-αβ T cells, which is supported by recent evidence demonstrating distinct activation induced signalling pathways between the two T cell subsets (228). γδ T cell purity at the time of infection was the single most important determinant for the successful generation of long term γδ T cell lines and hence, also represents the major limitation of this technique. Observations stemming from this work as well as those from others (184; 186) clearly indicated that αβ relative to γδ T cells were more efficiently HVS transformed, the growth transformation occurred earlier, and *t*-αβ T cell growth kinetics were faster. What this simply translates into is an explanation for the heretofore lack of success in generating HVS transformed γδ T cells, and is best explained graphically from a hypothetical growth comparison (Figure 46). Solely based on doubling time differences of 6d vs. 2d for γδ vs. αβ T cells (supported by observations made in this study), it is readily apparent that an initial 90% pure γδ T cell population is reduced to 3% after only 4 cycles. When the issues of greater susceptibility and earlier transformation events are factored in, even highly pure γδ T cell populations can quickly be dominated by contaminating *t*-αβ T cells. Furthermore, attempts to control the expansion of *t*-αβ T cells becomes more problematic with increasing degrees of impurity, and long term culture was only achieved when cell lines were maintained with >85% purity, concomitant with continual monitoring and repurification. For example, a 78% pure PB *t*-γδ T cell line was subjected to two rounds of C' mediated purification to yield a >99.7% pure population. Twenty two days later, the purity of this population was reduced to 96%, followed immediately by repurification to >99.5% which subsequently fell to 90% within the following 2 weeks. This explanation is not without merit as others have encountered a similar phenomenon

Figure 46. Hypothetical growth comparison between *t*-γδ T cells and *t*-αβ T cells. Growth curve is based on a *t*-γδ T cell doubling time of 6d vs. an *t*-αβ T cell doubling time of 2d, which was supported by experimental observations. Day 0: 90% *t*-γδ T cells: 10% *t*-αβ T cells; Day 24 3% *t*-γδ T cells: 97% *t*-αβ T cells.


10000
1000
100
10
1
Cell number
γδ
αβ
3% γδ T cell
90% γδ T cell
0
2
4
6
8
10
12
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16
18
20
22
24
Day

with the poor growth transformation of $CD8^+$ αβ T cells when in the presence of $CD4^+$ αβ T cells (198; 232). Judging from the relative ease and significant numbers of $CD8^+$ *t*-αβ T cells obtained as a by-product in these studies, it appears as though there is a relative gradient for HVS transformation efficiency ranging from low (γδ T cells) to moderate ($CD8^+$ αβ T cells) to high ($CD4^+$ αβ T cells).

γδ T cells successfully growth transformed by HVS behaved in much the same way as described for αβ T cells, with the exception of their culture characteristics and growth kinetics as discussed above. Growth transformation was assessed using similar criteria as for *t*-αβ T cells (179; 180): (1) sustained growth without further mitogen stimulation; (2) blastoid morphologies; and (3) death of non-infected controls. Similar to *t*-αβ T cells (183; 200; 201), established *t*-γδ T cells were never observed to produce infectious HVS as determined in sensitive co-culture assays with the permissive OMK cell line. Over the course of these studies, numerous PB and CSF *t*-γδ T cell lines were generated with culture lives ranging from 71 d to greater than 300 d (Table 10); albeit most exist currently as *t*-αβ T cell contaminated frozen stocks. However, when pure cultures were obtained as in the series of PB and CSF generated *t*-γδ T cell clones (Table 11), stable IL-2 dependent growth has been observed for periods in excess of 2.5 yr. Although some culture related differences were observed between CSF and PB γδ T cells, cells from either compartment were equally susceptible to HVS induced transformation, and the appearance of an over-representation of PB *t*-γδ T cells is explained by their greater availability for methodology development.

One of the long term goals for the application of the HVS technique is to accumulate large numbers of immortalised PB and CSF γδ T cell lines and clones from both MS and OND patients, and to systematically compare each subset for differences that may be relevant to the pathogenesis of the disease. An obvious question regarding this strategy is in what way does HVS growth transformation change the characteristics of the original *in vivo* population? Extensive characterisations to date with *t*-αβ T cells suggest that HVS transformation impacts minimally on the phenotype and function of αβ T cells. They possessed functionally intact TcR

(200; 201; 233) and retained their MHC restricted antigen recognition (201-203), cytokine secretion (200; 202), cytotoxic potential via cytotoxic granules (203) or FasL (192), remained sensitive to activation induced apoptosis (192; 204), and could support B cell activation (205; 206). Results from this study presents evidence for the first time showing that HVS transformed γδ T cells similarly preserves the phenotypic and functional characteristics of their parental cells. A comparison of cell surface molecule expression between parental and their transformed progeny (Table 12, Figure 15) showed little differences, except at the level of activation marker expression such as CD25 and HLA-DR. Similarly, functional properties such as cytokine production (Figure 18 and 19) and cytotoxicity potentials (Figure 22) were virtually indistinguishable between transformed γδ T cells and their parental lines. These results strongly suggest that at the very least, HVS transformed γδ T cells are reflective of the short term expanded lines from which they were generated. As in all studies using cultured cells, there remains the possibility that some degree of *in vitro* selection occurs and hence biases any extrapolation to the *in vivo* setting- however this is a limitation that must be accepted when working with human samples. Direct HVS infection of *ex vivo* cells would in theory minimise any *in vitro* selection events, however all attempts to transform CSF cells directly *ex vivo* were not successful, although these experiments were performed prior to optimisation of the method. This remains a viable and attractive strategy in light of the fact that *t*-αβ T cells can be generated from small numbers of proliferating as well as resting T cell samples (180; 197). Significantly, an *ex vivo* CSF sample was growth transformed by simultaneous HVS infection and stimulation with immobilised γδ TcR mAb, representing one step closer to achieving direct *ex vivo* immortalised γδ T cells.

4.3 Immortalised γδ T cells: Phenotypes

A major concern regarding the utility of the HVS transformation technique was its ability to transform the complete spectrum of γδ T cell subpopulations present within both PB and CSF lines. Indeed from the two groups reporting the derivation of *t*-γδ T cells (184; 186), only one

group addressed this issue in a limited fashion. Fickenscher and colleagues (186) using Vδ1,Vδ2, Vδ3 and Vγ9 specific mAbs, demonstrated that all the *t*-γδ T cell clones generated from the cord blood of two infants were Vδ1 positive and Vγ9 negative, with the exception of one clone that was negative for all mAb tested. Because such a limited subtype distribution was obtained from a large and diverse cord blood subtype population (224), the authors concluded that there appeared to be HVS tropism and/ or increased efficiency for γδ T cell subpopulations. Data obtained in this study clearly indicates that HVS is capable of growth transforming a much larger repertoire than that described. Phenotypic analysis of various PB and CSF *t*-γδ T cells detected significant populations of Vδ1, Vδ2, and Vδ3 as well as Vγ4 and Vγ9 positive cells (Table 13), however conclusive evidence came from the isolation of pure subtype specific *t*-γδ T cell clones (Table 14). Significantly, not only were blood dominant Vγ9Vδ2 transformed clones isolated, but also Vδ1 positive clones more commonly associated with tissue (49; 50), and other rarely described phenotypes such as Vδ3 and non-Vδ1,Vδ2,Vδ3 positive clones or clones with unusual VγVδ chain pair combinations. Furthermore, a *t*-γδ T cell clone was established that possessed dual γδ TcR receptors on its surface; Vδ1 in combination with either Vγ4 or Vγ9. Although this clone was selected at random, γδ T cells expressing dual receptors may be more prevalent than at first thought, as a number of other groups have observed similar dual TcR phenotypes (138; 234-237). Whether there is a unique biological role for these dual TcR γδ T cells is not presently known, however it has been suggested that dual TcR expression may provide an immunological escape pathway for normally controlled autoreactive T cells; as such, they may be particularly relevant to MS pathogenesis (236; 238; 239). Taken together, not only were these rare VγVδ subsets identified in PB and CSF of MS patients, but their isolation and amplification directly as a result of the immortalisation process, now enables their biological study; investigations that have been lacking up till present.

In addition to generating unique TcR subtype clones, both transformed lines and clones possess other phenotypic attributes that also provide clues as to their potential biological role(s); this in a manner analogous to histopathological studies. However, unlike histopathological

studies, hypotheses generated as a result of phenotypic analyses can be directly tested on the relevant *t*-γδ T cell lines or clones. In general, *t*-γδ T cells phenotypically appeared as mature activated lymphocytes similar to that reported for *t*-αβ T cells (182). γδ T cells have been reported to express numerous NK markers and to share similar effector functions; one of the principal reasons why many consider γδ T cells participate primarily in innate vs. adaptive immunity (53; 130; 240). One molecule with important ramifications for MS pathogenesis is the expression of CD16 (FcRIII), which may be involved in ADCC (125) and/ or receptor mediated endocytosis events; the latter linked to the antigen presenting ability of γδ T cells (241). However, contrary to the reported CD16 expression on most circulating γδ T cells (125) and on *t*-γδ T cells (185), the *t*-γδ T cells obtained in this study uniformly lacked surface expression of this molecule, except for one isolated *t*-γδ T cell clone. This was not an artefact of the transformation process, since short term γδ T cell lines also lacked expression of this molecule. Similarly, a recent study demonstrated CD16 expression was virtually absent on synovial fluid γδ T cells (242); a compartment also shown to accumulate γδ T cells in patients with rheumatoid arthritis. In the course of their studies, they showed that CD16 was downregulated in response to γδ T cell activation, which possibly serves as an explanation for this study's observations because *t*-γδ T cells are by definition activated. This explanation is at odds with Klein and colleagues, where they observed constitutive expression of CD16 on the surface of their *t*-γδ T cells (185). This finding may be related to properties of cord blood lymphocytes, the cells of origin in their study, since these cells are typically immunologically naïve and possess different properties relative to mature γδ T cells (224). However consistent with the results of Klein, a single CD16 expressing *t*-γδ T cell clone was isolated in this study from MS PB, despite little evidence for its expression in the parental line and would be useful to probe CD16 mediated events alluded to above. The role if any, for CD4 and CD8 on γδ T cells is not evident considering most γδ T cell interactions are MHC unrestricted and CD8 is composed of homodimeric α chains rather than classical heterodimeric α-β chains (85). The generation of *t*-γδ T cell lines and clones with variably high to no CD8 expression as well as a rare $CD4^+$ *t*-γδ T cell clone, postulated to represent a distinct Th2-

like γδ T cell subset (75; 85), lend themselves for further investigation into their biological roles. Other distinct phenotypes of note expressed by the series of *t*-γδ T cell lines and clones is the strong presence of CD40L shown to be functional with *t*-αβ T cells in providing B cell help (205); the virtual lack of CD28 expression on *t*-γδ T cell clones which correlates with evidence demonstrating no dependence on CD28 costimulation for γδ T cell production of cytokine (228); and the presence of NK killer inhibitory receptors known to modulate the cytolytic activity of γδ T cells (67; 76).

4.4 Immortalised γδ T cells: Function

Data obtained in this study overwhelmingly support the notion that MS patient γδ T cells are primarily proinflammatory in nature similar to non-MS PB (85; 243). Using intracytoplasmic cytokine staining, all *t*-γδ T cell lines, regardless of their compartment of origin, exhibited the production of IFN-γ and TNF-α, with little evidence for IL-2, or the anti-inflammatory cytokines, IL-4 or IL-10. Furthermore, *t*-γδ T cell clones expressed cytokine profiles identical to their parental transformed lines which in turn were similar to the non-transformed γδ T cells from which they were derived (Figures 18-19, 24, 37, 38 and Table 15). Together these results suggest, at least in terms of cytokine profile, that *t*-γδ T cells are representative of the short term polyclonal lines. Furthermore, since all *t*-γδ T cell clones displayed identical proinflammatory cytokine patterns and by virtue of their heterogeneous subtype composition, it follows that cytokine production is not correlative with specific MS γδ TcR subtypes. Cytokine profiles in this study are somewhat in disagreement with those determined by Fickenscher et al. (186) with *t*-γδ T cell clones derived from infant cord blood. Although most of these clones secreted IFN-γ and lacked IL-4 production consistent with this study's results, a significant proportion secreted IL-2 at levels comparable to $CD4^+$ αβ T cells (TNF-α levels were not addressed). This in part also explains the divergent behaviour in their growth conditions since they grew independent of the need for exogenous IL-2, whereas *t*-γδ T cells obtained in this study have an absolute requirement for exogenous IL-2.

Although the patterns of cytokine production remained consistent for all *t*-γδ T cells studied in this work, single cell analysis by flow cytometry identified differences in the levels of IFN-γ or TNF-α produced per cell. These differences were most evident when individual *t*-γδ T cell clones were compared, demonstrating as much as 10-fold differences in the levels of cytokines produced among sibling *t*-γδ T cell clones (Table 15; Figure 37). An interesting example of this phenomenon comes from a *t*-γδ T cell clone expressing dual TcRs on its surface, and producing markedly more TNF-α upon PMA/ Ca^{2+} ionophore stimulation relative to its siblings. This increased TNF-α output may be related to a hypersensitive activation pathway present within this TcR rich cell and is supported by evidence illustrating TNF-α production within γδ T cells is governed solely through heightened TcR mediated signals and independent of CD28 costimulation (228). Taken together, the results of cytokine/ subtype comparison studies are consistent with two opposing viewpoints: (1) overall cytokine expression does not appear to correlate with any particular γδ T cell subtype similar to conclusions reached by Morita and group (85), and (2) despite similar patterns of cytokine production, relative levels of cytokine generated are associated with individual γδ T cell subtype (243). Also of note, some *t*-γδ T cell lines and clones appeared to be primed to secrete IFN-γ as evidenced by their high "spontaneous" production of this cytokine despite being subjected to IL-2 starvation for 48-60 h (Figure 25). Although these observations may be due to a heightened proinflammatory nature occurring in transformed cells as suggested by some (200; 202), the lack of similar findings with TNF-α together with low to no "spontaneous" cytokine production with the majority of the cells studied, argues that these cells at the very least behave differently. Whether these cytokine differences observed between phenotypically distinct sibling clones are related to their TcR subtypes, cannot be determined until further characterisations are performed. Similarly, further studies will be able to shed light on an interesting observed trend found between *t*-γδ T cell clones with high basal IFN-γ production or high levels of induced TNF-α and increased cytotoxicity towards tumour cell targets.

Both *t*-γδ T cell lines and clones were shown in this study to retain their cytotoxic potentials towards tumour cell targets at levels that were comparable to non-transformed short term γδ T cell lines (Figure 22, 26 and unreported data). Furthermore, the *t*-γδ T cells displayed differential cytotoxicity profiles towards the various tumour targets, which again reflected the cytotoxicity patterns exhibited by short term non-transformed γδ T cells (Table 6, Figures 28, 39-41 and (117; 127)). In general, U937 monocytes and Jurkat T cells were killed with the highest efficiency, followed by intermediate killing of KG-1 myeloblasts, and poor killing of Colo-205 colon carcinoma cells. This differential killing pattern suggests three possible explanations that are not mutually exclusive: (1) γδ T cells recognise different substrates displayed by the tumour cells; (2) γδ T cell cytotoxicity is differentially modulated by tumour cell surface molecules; and (3) multiple or different killing pathways are operational. Recognition of differential expression of heat shock proteins has been proposed to explain γδ T cell induced tumour cytotoxicity patterns, however the extensive killing of HSP low U937 and Jurkat cells relative to HSP high RPMI 8226 and Daudi B cells (121) by both short term (Table 6) and *t*-γδ T cells (Figures 39-41 and data not shown), argue against this being the sole mechanism of action. Significant proportions of γδ T cells display NK killer inhibitory or activating receptors which can modulate γδ T cell killing of target cells (67; 76; 244). Preliminary phenotypic evaluation of both *t*-γδ T cell lines and clones showed the strong expression of some KIR such as CD94 but in the few instances addressed, lacked CD158a,b or NKB1P (Table 13-14). Therefore, differential tumour killing may be the result of regulation through these cytotoxicity modulation molecules found on *t*-γδ T cells. Initial studies reported the CD94/NKG2a heterodimeric receptor complex recognised shared motifs within all Class I molecules and negatively regulated γδ T cell cytotoxicity; interference with this receptor-ligand interaction resulted in the restoration of cytotoxicity (245; 246). With this hypothesis in mind, similar cytotoxicity experiments were conducted using a richly expressing CD94 *t*-γδ T cell clone and MHC class I blocked Colo-205 (relatively resistant to *t*-γδ T cell killing) and U937 cell targets to assess for CD94/ NKG2a modulatory effects. The finding that HLA-ABC mAb treatment (clone WT/31) did not affect γδ T cell induced killing of either Colo-205 or U937 (Figure

27) is now not surprising in light of recent evidence demonstrating the true ligand for CD94/ NKG2a is not HLA-A,B,C molecules as originally believed (246; 247) but recognises the non-polymorphic HLA-E molecule (245). Indeed, Braud and colleagues went on to show that HLA-E expression was dependent on leader peptides expressed by some HLA alleles and Class I blocking mAbs (DX15 and DX17) used in the original studies, also cross reacted with HLA-E. Furthermore, it is now recognised that the decision for γδ T cells to kill is more complex than simple receptor ligation and comprises a balance between co-ordinated multiple activation signals (γδ TcR and KARs) and multiple downregulatory signals (KIRs); the mechanism of which is currently being explored using defined KAR and KIR expressing *t*-γδ T cells. Finally, the graded tumour cell killing may be the result of alternate or multiple γδ T cell effector mechanisms operational for some but not all tumour cells. Our studies directed at elucidating the mechanism of target cell killing by short term γδ T cells (127) illustrates this latter view where tumour targets were found to be killed primarily via perforin/ granzyme release, but Fas induced death mediated by FasL expressing γδ T cells was evident and synergistic. The sensitivity of both Jurkat T cells and U937 monocytes to *t*-γδ T cell induced death is consistent with the Fas-FasL death pathway being operational in a similar manner, since both are known Fas sensitive targets (248; 249). Alternatively, Jurkat or U937 cells may be sensitive to the death inducing effects of TNF-α produced by the *t*-γδ T cells. Regardless of the modulating influences, the seemingly promiscuous, MHC unrestricted killing pattern displayed by *t*-γδ T cells towards tumour cells is consistent with the promiscuous killing by two PB *t*-γδ T cell clones (184) as well as MS derived short term γδ T cell lines (138), and is further evidence that some tumour lines may express common γδ T cell recognition elements (114; 117; 129).

Short term γδ T cell cytotoxicity studies suggested both Vδ1 and Vδ2 positive γδ T cells were capable of tumour cell killing, but this hypothesis was inferred from the distribution pattern of these subtypes within PB and CSF of MS and OND patients. With the availability of a panel of pure *t*-γδ T cell clones displaying unique TcR chain combinations, this hypothesis was tested. Ample evidence (Figures 26, 28, 39-41, and Table 19) indicated there were no overt cytotoxicity

differences that correlated with VγVδ subtype. This is especially relevant considering there is conflicting evidence regarding the killing ability of Vδ1 vs. Vδ2 subtypes for some tumour cells especially Daudi B lymphomas. A number of studies suggest Daudi B cell killing is mediated exclusively by Vγ9Vδ2 bearing γδ T cells (109; 115; 119), however others have shown that Daudi lysis is also sensitive to Vδ1 bearing γδ T cells (69; 250). Evidence obtained with defined *t*-γδ T cell clones supports this latter contention because non-Vγ9Vδ2 positive clones were efficient Daudi cell (old lot- see below) killers (Figure 26, 39, and data not shown).

Daudi B cell killing represents somewhat of an enigma in this study, mainly due to a forced change in Daudi lots for technical reasons. It is well established by our group (117; 127) and others (115; 119; 129) that Daudi B cells are well recognised targets for activated γδ T cells and indeed, early studies in this work demonstrated efficient killing of these cells (Table 6, Figure 26, (127) and data not shown), but showed only poor killing of new lot Daudi cells (Figures 28-29, 39-40). Since initial cytotoxicity studies were performed primarily with non-Vγ9Vδ2 *t*-γδ T cells, the subset reported to specifically interact with Daudi B cells (115), this represented a possible explanation. However follow up studies with short term PB cells and *t*-γδ T cell lines (>80% Vγ9Vδ2 positive) as well as with Vγ9Vδ2 *t*-γδ T cell clones similarly were unable to lyse new lot Daudi cells efficiently. While this aberrant cell line obtained from ATCC (perhaps due to extended passages) does pose a problem with sequential interpretations in this study, it also represents a cell target lacking either γδ T cell recognition ligand(s) (119), or possessing γδ T cell cytotoxicity modulator properties such as KIR ligands (76; 129), and hence represents an attractive model for further investigations.

Several other observations were made with *t*-γδ T cell clones expressing unique phenotypes other than those described for TcR subtypes. Although short term studies with PB derived γδ T cell clones suggested γδ T cell cytotoxicity was independent of CD8 expression, these studies used a redirected lysis assay that bypassed the need for appropriate recognition (85). Results from cytotoxicity experiments showing equivalent killing by *t*-γδ T cell clones expressing low to high CD8 (Table 14) as well as in Class I blocking studies (Figure 27 and data

not shown) provided strong evidence that indeed target cell killing operated independently of Class I ligation. Similarly, it has been suggested that the relatively rare $CD4^+$ γδ T cells represent a distinct subset akin to $CD4^+$ Th2-like αβ T cells, although the correlations are rather loose (70; 75; 85). One feature of $CD4^+$ γδ T cells is their relatively poor cytotoxicities displayed towards target cells as determined by Morita et al. (85). Evidence supporting this observation, came from the cytotoxicity comparisons made between a $CD4^+$ *t*-γδ T cell clone with its $CD4^-$ $CD8^+$ siblings. Although not as marked a difference as found by Morita, the $CD4^+$ *t*-γδ T cell clone consistently displayed the poorest killing ability for all tumour cells tested relative to its *t*-γδ T cell clone siblings (Figure 29). Although not performed in this study, it would be of interest to see if the cytokine profile stemming from this *t*-γδ T cell clone differed from $CD8^+$ or DN *t*-γδ T cells as suggested by Wen et al. (75).

Characterisation studies with *t*-αβ T cells have suggested the transformation process confers a non-specific cytotoxic ability on the resultant cells that is due to signals arising from hyperreactive CD2-CD58 triggering upon target cell contact (183; 202). To address whether cytotoxicity obtained in these studies was the result of similar effector: target cell interactions, cytotoxicity studies were conducted in the presence of CD2 and CD58 blocking mAbs using conditions shown to be effective in abrogating non-specific *t*-αβ T cell lysis of targets (196). Both CD2 and CD58 blocking mAbs had no effect on *t*-γδ T cell killing of U937 or Jurkat targets (Figure 30) suggesting the CD2-CD58 pathway was not responsible for the killing observed in this study. Evidence to support this finding comes from the evaluation of CD2 or CD58 expression on the various target cells with the highest expression of CD58 found on Jurkat followed closely by Daudi and KG-1 and lowest for U937 and Colo-205 (Table 16). If cytotoxicity was solely the result of non-specific CD2-CD58 interactions, then *t*-γδ T cell killing of the various tumour targets would be expected to follow their level of CD58 expression and clearly did not. Daudi cells (new lot) were consistently poorly killed by *t*-γδ T cells despite high CD58 expression, and U937 cells were efficiently killed but had among the lowest CD58 expression. Furthermore, the pattern of *t*-

γδ T cell killing of the various tumour targets was reflected by short term non-transformed γδ T cells, where transformation induced CD2 hyperreactivity was not an issue (Table 6).

4.5 Immortalised γδ T cells: Antigen recognition

One of the biggest pieces of the puzzle in trying to understand the biological role of γδ T cells is in the identification of γδ T cell reactive antigens. Indeed up until just recently, specific chemically or biologically defined γδ T cell antigens were not known and the majority of studies centred on γδ T cell reactivity to tumour cells (56; 69; 109; 115; 119; 129) and to mycobacterial extracts (66; 106; 119). Subsequently, it is now understood that most γδ T cell antigens present within mycobacterial extracts are non-peptidic phospholigands (58; 107; 108) rather than HSPs as originally suspected for both the extracts and reactive tumour lines (66-68; 137). It was rationalised that *t*-γδ T cells have great potential in antigen recognition studies due to their stable, functional TcR and regenerative capabilities and would be invaluable in the identification of γδ T cell reactive CNS antigens- of which none have been reported. To determine this functionality, antigen recognition studies were performed with representative antigens from five different antigen classes.

It was intuitive to begin with tumour cell antigens for two reasons: (1) they are recognised γδ T cell reactive ligands but the recognition molecule(s) are not known; and (2) graded cytotoxicity responses obtained in this study suggested differential ligand recognition. Indeed, when the tumour cell panel used throughout this study was screened against MS *t*-γδ T cell lines and clones, a similar pattern of recognition to cytotoxicity responses was observed, using primarily TNF-α output as a measure of recognition (Figures 34-35, 42, Tables 17-18, 20). The non-discriminating nature for *t*-γδ T cell subtypes in their recognition of the tumour ligands fully supports the equivalent recognition and tumour cell killing by all *t*-γδ T cell subtypes observed in the cytotoxicity studies, thus further presenting evidence that all γδ TcR subtypes possess the capacity to recognise a number of tumour cells. These findings are not consistent with several accounts showing that specific γδ T cell subtypes are associated with specific tumour cell

recognition (115; 251) but are supportive of a number of studies indicating a promiscuous recognition and cytotoxic response to tumour ligands irrespective of γδ TcR subtype (69; 117; 250). What is the nature of the tumour ligand(s) is not well understood. Originally much interest was focused on the expression of HSP65 molecules as potential ligands stemming from studies with HSP65 expressing Daudi B cells together with their high sensitivity towards γδ T cell induced cytotoxicity (66; 68). Subsequent studies however, have not been able to conclusively establish a causal link between HSP expression and γδ T cell reactivity (117; 121). Furthermore, the highly recognised U937 and Jurkat targets in this study are poor expressers of HSP65 whereas high expressing HSP65 Daudi B cells were only poorly recognised. It is quite possible that γδ T cells may recognise a number of different HSP families on tumour targets, of which several have been defined (66; 67; 137), that may explain their graded recognition of different tumour cell lines. Alternatively, γδ T cells may recognise a common, but not yet defined, tumour antigen that is differentially expressed. Regardless of the mechanism, it appears from this data and from others, that TcR subtype appears to play no role. One interesting corollary of these antigen recognition studies is the different pattern of cytokine production induced by PMA/ Ca^{2+} ionophore or tumour cell stimulation between the various *t*-γδ T cell lines or clones tested. This is best illustrated with the comparison of three *t*-γδ T cell clones and their reactivity to U937 cells. One clone upon PMA/ Ca^{2+} ionophore stimulation produced ~10x's more TNF-α relative to its siblings while U937 stimulation resulted in almost equivalent TNF-α production by all three clones (Table 17). Similar observations were also noted with other tumour cells and PB vs. CSF *t*-γδ T cell lines (Table 20). It is tempting to speculate that some of the differences observed may be attributed to different signals generated by KAR and KIR γδ T cell- tumour cell interactions, which in addition to modulating cytotoxicity, have also been shown to influence cytokine production (92; 252).

Subsequent studies addressed candidate antigens representing three different classes of the newly discovered non-peptidic small antigens, namely phospholigands (58; 106; 107), alkylamines (110), and mycolic acids (253). Screening both MS *t*-γδ T cell lines and *t*-γδ T cell clones against these non-peptidic small antigens revealed a subtype specific pattern of

recognition for the various antigens (Figures 36, 43-45 and Tables 21-22). The only reactivity observed towards the phospholigands, isopentenyl (IP) and dimethylallyl (DA) pyrophosphate as well as to a series of alkylamine antigens came from a PB *t*-γδ T cell line composed primarily of Vγ9Vδ2 bearing cells (83%) and a number of Vγ9Vδ2 *t*-γδ T cell clones. A *t*-γδ T cell line composed exclusively of Vδ1Vγ9 cells together with a Vγ9Vδ? *t*-γδ T cell clone both showed no reactivity to either the phospholigand or alkylamine antigens. These results are entirely consistent with numerous reports demonstrating phospholigand as well as alkylamine recognition is mediated exclusively by Vγ9Vδ2 positive γδ T cells (58; 107-110). Furthermore, the non-reactivity of the Vγ9Vδ? *t*-γδ T cell clone suggests Vγ9 alone is not sufficient for phospholigand or alkylamine recognition and is in line with recent TcR chain pair switch studies demonstrating an absolute requirement for Vγ9 paired with Vδ2 for phospholigand recognition (113). Notably, both phospholigand and alkylamine recognition studies did not require the addition of accessory presenting cells which were necessary in other described studies (110; 254; 255). The significance of this observation is either *t*-γδ T cells recognise their antigen directly without the need for accessory cells, which is not without precedence (97; 100), or the *t*-γδ T cells themselves can function to stabilise cell surface phospholigand or alkylamine expression and present antigen to one another, in a manner that may be similar to bovine γδ T cell antigen presentation to $CD4^+$ αβ T cells (241). What surface molecules are potentially involved is not known but classic processing and presentation by Class II or cross presentation by Class I molecules is not likely (101; 254; 255), while Class I-like non-polymorphic molecules such as CD1 remain attractive but are as yet not shown to be involved. A proposed role for the apparent constitutive expression of CD16 on γδ T cells (125), though not supported from these studies, is for antigen capture and receptor mediated endocytosis providing antigen processing is a requirement (241). Interestingly, the pattern for Vγ9Vδ2 cell recognition of the various alkylamine antigens tested as assessed by TNF-α output (Figure 45 and data not shown) is strikingly similar to that found with Vγ9Vδ2 TcR transfected Jurkat T cells as determined by IL-2 release (110), and is the first report of this kind to use natural, albeit HVS transformed, γδ T cells. The last type of non-peptide antigen tested was a

candidate mycolic acid (trehalose dimycolate), a powerful trigger of CD1 restricted DN αβ T cells; a T cell subset apparently evolved to recognise glycolipids (253; 256). No reactivity was observed towards this antigen by all *t*-γδ T cells tested suggesting either this class of antigen is not recognised by γδ T cells or as with DN αβ T cells, effective recognition requires antigen presentation by CD1 bearing APCs. Further studies investigating this relationship are required and are particularly relevant since there is an intrinsic, but not yet defined, relationship between γδ T cells and CD1 molecules (105).

Taken together, antigen recognition by MS *t*-γδ T cells to known γδ T cell reactive ligands are entirely consistent with reports using non-transformed γδ T cells and clearly illustrates their usefulness and feasibility for probing MS related antigens in future studies.

4.6 Implications for MS pathogenesis

(A) PB vs. CSF comparisons

The elucidation of significant phenotypic and/ or functional differences between HVS transformed PB vs. CSF *t*-γδ T cells from MS or OND patients requires the comparison of large numbers of immortalised matched samples, and as such, was beyond the scope of this work. To illustrate however, the feasibility of this approach in addressing the role of γδ T cells in MS pathogenesis, a representative MS patient's PB and CSF γδ T cells were HVS immortalised and comprehensively compared. Parenthetically, this intra- as opposed to inter-patient analysis may indeed be more relevant if one views MS as a heterogeneous disease syndrome resulting from a number of possible, but patient specific, pathological processes ending in myelin/ oligodendrocyte loss; a viewpoint currently being debated (6; 9).

A number of significant observations were noted from the PB vs. CSF *t*-γδ T cell comparison studies from this representative MS patient sample, such as differences in the TcR subtype composition within the two subsets. It was somewhat unexpected that the CSF *t*-γδ T cell population was homogeneous in its expression of Vδ1Vγ9 TcRs relative to the typical Vγ9Vδ2 dominant subtype found in matched blood. Upon further analysis, this limited CSF distribution

was not a result of the transformation process but was a reflection of the originating short term CSF line. Although the presence of a single CSF subtype was not previously encountered from the limited CSF subtype analyses conducted, 3 out of 4 samples indicated a high proportion of Vδ1 positive cells present relative to PB, including a CSF sample that was transformed *ex vivo* (Table 13). This trend supports the accumulation of Vδ1 positive cells within the CSF of MS patients as suggested from our short term studies as well as from others (138; 140; 222), but final confirmation of these results awaits further matched PB/CSF comparisons with *t*-γδ T cells from both MS and OND patients. The question of CNS γδ T cell clonality has significant ramifications for MS pathogenesis since many believe γδ T cells with distinct clonal origins may be indicative of recognition and expansion to brain derived antigen. Previous studies addressing this issue have reached mixed conclusions and further clarification is needed. Evidence suggests that oligoclonal γδ T cell expansion occurs in both MS lesions and CSF from the restricted Vγ or Vδ junctional diversity found (139; 225) and from the detection of identical Vγ or Vδ junctional sequences (46; 140; 257). In contrast, others have demonstrated γδ T cells present within the CNS are from diverse clonal origins and are polyclonal in nature suggesting a response to many different antigens (138; 148). Observations made in this study support a limited γδ T cell diversity found within MS CSF as exemplified by the restricted Vδ1Vγ9 CSF population described. That this population is monoclonal and hence may have expanded to local CNS antigen, is further corroborated by the identical patterns obtained from the phenotypic evaluation of a number of *t*-γδ T cell clones derived from this line. However, definitive proof awaits TcR sequencing studies on this and further CSF *t*-γδ T cells, as polyclonal cells may employ the same Vδ1Vγ9 gene elements. Although the significance for Vδ1 positive γδ T cell accumulation within MS CSF may be related to an expansion to brain derived antigen, others speculate their presence may be due to a superantigen induced effect, which can also account for their heterogeneous clonal distributions (137; 226). This latter hypothesis however, is not consistent with the broad Vγ and Vδ TcR distribution found within the CNS as opposed to a more restricted pattern expected from a classical superantigen-like response. Another source of debate stems from conflicting evidence

on γδ T cell subtype distribution and location within the different categories of MS lesions (46; 141; 148; 149) and their relation to their distribution found within CSF. Indeed the frequency of potentially relevant tissue subtypes may be underrepresented in the CSF due to their sequestration within the brain parenchyma (221). As demonstrated in this study, the HVS technique has the ability to amplify relatively rare, and thus perhaps CSF underrepresented, tissue relevant subtypes for further investigation, such as the reported MS brain dominant Vδ2Jδ3 subset (149). Furthermore, most emphasis has been placed on elucidating the role of the major, and therefore most populous γδ T cell subset(s) found within the CNS, under the premise that increased numbers imply functional relevance. While this may rightly be so, less frequent γδ T cell phenotypes may also be instrumental in the course of the disease in a manner analogous to altered peptide reactive αβ T cells, where a small subset of cells was able to control a much larger inflammatory population, mainly by cytokine release (258; 259). More recently, a similar phenomenon was observed with γδ T cells where through the actions of a non-peptidic antagonist, functional inactivation of a whole population of γδ T cells occurred by an as yet unidentified mechanism (260).

There were no observable functional differences found between matched MS PB vs. CSF *t*-γδ T cell lines and clones; this despite significant differences in subtype composition. Both MS PB and CSF *t*-γδ T cells behaved identically by producing the proinflammatory cytokines IFN-γ and TNF-α upon PMA/ Ca^{2+} ionophore stimulation (Figure 37-38) and displaying identical killing patterns towards the tumour cell panel (Figure 39-41 and Table 19); similar to the subtype independent functional profiles previously observed for unmatched MS PB and CSF *t*-γδ T cells and discussed previously. Surprisingly, few reports detailing the cytokine production of CSF derived γδ T cells are available for comparison purposes, owing in part from their inferred proinflammatory nature from repeated studies with PB derived γδ T cells (85; 92; 243; 261). In one such study, Nick et al. (138) looking for cytokine message within two CSF derived clones, detected most cytokine transcripts even in resting cells, suggesting that the expression of the detected cytokine transcripts is probably more restricted. Contrary to the data obtained in this

study, TNF-α transcript was noticeably absent from both and IFN-γ transcript absent from one of the two clones screened. However, immunohistochemical studies (262) suggest that γδ T cells are major sources of proinflammatory IFN-γ and TNF-α consistent with this study's findings. The equivalent PB and CSF *t*-γδ T cell cytotoxicity responses towards the tumour panel is consistent with similar results obtained comparing the killing ability of a series of short term PB and CSF γδ T cell clones towards Daudi B cells (137). Taken together, these results suggest at least in the context of this representative sample, that MS CSF γδ T cells are not intrinsically different from MS PB γδ T cells from the standpoint of PMA/ Ca^{2+} ionophore induced cytokine production and tumour cell cytotoxicity.

Three discernible patterns of antigen recognition were observed between MS PB and CSF *t*-γδ T cells: (1) tumour cell recognition by *t*-γδ T cells from both compartments; (2) non-peptidic phospholigand and alkylamine recognition exclusively by Vγ9Vδ2 PB γδ T cells; (3) no recognition by either subset. Despite small differences in cytokine stimulation indices (Figure 42, Table 20), a similar pattern recognition of the various tumour cells suggests both MS PB and CSF *t*-γδ T cells are responding to either a common tumour associated antigen that is differentially expressed by the various tumour lines, to different individually expressed tumour antigens, or responses (i.e. cytokine production) are modulated by ligands present on the surface of the tumour cells. The latter scenario may also account for the small differences seen between stimulation indices for PB vs. CSF *t*-γδ T cells. The evidence is not in favour of a TcR subtype specific recognition since the CSF was exclusively $V\gamma9V\delta1^+$ and the PB was >82% $V\gamma9V\delta2^+$ and only 13% $V\delta1^+$, but this can be clarified by repeating these experiments using PB and CSF subtype defined *t*-γδ T cell clones. What is the nature of the tumour associated ligand(s)? As discussed previously, it is unlikely that HSP60 related proteins hypothesised to be the reactive ligand on Daudi lymphoma (HSP65) (66; 68) and shown to be present on the surface of oligodendrocytes (46; 141) are solely responsible for the tumour killing observed in this study. Indeed, evidence obtained from a series of cold target competition assays with and without HSP60 expressing tumour cells, suggested at least two distinct ligands were recognised (117).

However of greater relevance to MS, the most effective competitors for HSP60 bearing oligodendrocyte lysis were tumour cells themselves expressing high levels of HSP60. Therefore, although not probably involved in the tumour cell recognition in this study, it remains a potentially important autoantigen. Interestingly, Stinissen and colleagues showed that the majority of their MS derived γδ T cell clones did not react to HSP60 but to HSP70 related proteins, also shown to be induced on oligodendrocytes and consequently killed *in vitro* by γδ T cells (151). Recently, another stress related protein, MICA with similarities to HSP70 and present on a number of epithelial tumours (114) was recognised as a γδ T cell ligand mainly associated with $V\delta1^+$ cells (67) but also shown to bind $V\delta2^+$ γδ T cells (91), and can mediate tumour cytotoxicity through an as yet unclear mechanism. Whether this novel ligand is present within the CNS is not known. Alternatively, a number of studies (109; 119) suggest tumour associated ligands are related to mycobacterial antigens (primarily non-peptidic Ag) that possess structural components that are tantalisingly similar to those found in heavily lipidated myelin (see below).

A second type of antigen recognition was observed that was restricted to the $V\gamma9V\delta2^+$ cells within the MS PB *t*-γδ T cell population and not seen with the CSF derived cells. These cells recognised the candidate non-peptidic phospholigands and alkylamines, and as previously shown are exclusively reactive to this particular γδ T cell subset (110; 263), thus explaining the lack of CSF γδ T cell recognition. This observation has two important ramifications: (1) the skewed presence of CSF Vδ1 γδ T cells may not be as relevant as the high proportion of Vδ2 bearing cells found within MS lesions (148; 149) possibly due to the recognition of non-peptidic myelin antigens such as phosphorylated MBP (264); and (2) there are as yet unidentified non-peptidic antigens that are recognised by $V\delta1^+$ γδ T cells. Evidence to support this latter scenario comes from the recognition and proliferation of lyme arthritis synovial $V\delta1^+$ γδ T cells to lipidated but not delipidated *B. burgdorferi* lipoprotein and hexapeptides (64). Moreover, one of the rare recognition molecules elucidated for $V\delta1^+$ γδ T cells is human CD1 (105; 145); key molecules involved in glycolipid (253; 256) and self-glycolipid (265) antigen presentation to DN αβ T cells. Furthermore, CD1 molecules are expressed on both perivascular inflammatory cells and

endogenous hypertrophic astrocytes in active MS lesions (144) and in peripheral myelinated nerve fibres (266). Additionally in a very recent report, a reference was made to unpublished accounts of significant Vδ1 and Vδ2 γδ T cell responses to myelin glycoconjugates (267). Thus it seems as though CNS γδ T cells, particularly those belonging to the Vδ1 subset, are poised to recognise brain derived glycolipid antigens and illustrates a further important application for *t*-γδ T cells. In an initial attempt, neither CSF or PB γδ T cells responded to a highly active ligand (trehalose dicorynomycolate) for CD1 restricted DN αβ T cells however these studies were not conducted in the presence of CD1 bearing APCs.

A further set of putative MS antigens were also screened against the matched MS PB and CSF γδ T cells as well as to other MS derived PB γδ T cells. These consisted of the prototypical MS autoantigen, myelin basic protein (29); the dominant T cell antigen, αB-crystallin, an HSP shown to be primarily expressed on oligodendrocytes and astrocytes in early MS lesions (103; 142); and *Chlamydia pneumoniae* elementary body and outer membrane protein, suspected to be an etiological agent for MS (215; 268). Both MS PB and CSF *t*-γδ T cells did not respond to any of these putative autoantigens or microbial antigens suggesting they are not γδ T cell targets or these antigens require some form of antigen presentation and are not recognised directly.

Therefore from these comparison studies, it appears as though matched MS PB vs. CSF *t*-γδ T cells both possess similar functional abilities, and the level of difference between the two compartments may be related to their respective subtype composition. Whether CNS γδ T cells exert their effector functions may be related to the availability of autoantigen specifically recognised by the predominant γδ T cell subset found within the CSF or MS lesion.

(B) Other implications

The strong proinflammatory nature observed from both the PB and CSF *t*-γδ T cells has many implications for MS pathogenesis. It is well established that Th1 type cytokines such as IFN-γ worsen the disease when administered systematically (131), and both it and TNF-α increases in MS patients prior to and during exacerbations (269; 270). At an early event, strong

expression of IFN-γ and TNF-α by CSF γδ T cells bearing CD40L as shown in this study, would act to upregulate MHC Class II and adhesion/ costimulatory molecules on resident microglia and astrocytes (271); induce microglia production of IL-12 which along with IFN-γ, would drive the ensuing immune response towards a deleterious, self sustaining Th1 type cellular response (272-274); upregulate adhesion molecules such as VCAM/ VLA-4; stimulate the release of glial cell derived chemokines (31); and increase vascular endothelium permeability. Furthermore, CSF γδ T cell derived IFN-γ and TNF-α could induce iNOS production in astrocytes, microglia, and macrophages, which can result in myelin destruction when in the presence of antibody and complement (216). TNF-α is also implicated as a major mediator of oligodendrocyte injury and demyelination due to the preferential expression of TNF receptors on oligodendrocytes in MS lesions (153) and from TNF-α induced apoptosis of oligodendrocytes *in vitro* (155; 275).

Although not tested in this study, the identical cytolytic behaviour of MS PB and CSF derived γδ T cells and of the many γδ T cell subtypes shown throughout this work, all lend support that MS CSF γδ T cells will be equally as effective killers of human oligodendrocytes as previously seen with MS PB γδ T cells (127; 151). This is particularly relevant to disease pathogenesis since to date, γδ T cells induce the most rapid *in vitro* cytolysis of human oligodendrocytes and as such, may possess the greatest capacity for inflicting oligodendrocyte damage *in vivo*. Although both TNF-α and FasL have been shown to mediate oligodendrocyte apoptosis, this occurred over periods of 24-96 h (154; 155). Promiscuous myelin reactive $CD4^+$ αβ T cells are also able to kill oligodendrocytes *in vitro* through an unclear mechanism possibly involving CD56 homotypic interactions, and likewise requires 18 h to detect significant death (276). Notably, γδ T cells efficiently killed human oligodendrocytes in a 5 h period, primarily mediated through perforin release though Fas/FasL killing was evident and synergistic (127). The high expression of TNF-α and CD56 by *t*-γδ T cells shown in this study, conceivably would also play a minor but synergistic role in oligodendrocyte death during this 5 h period.

The recognition, cytokine release, and cytotoxic response by both MS PB and CSF γδ T cells to the seemingly unrelated tumour cell targets used in this study begs the question as to

what controls this promiscuity in healthy individuals. The answer may rest with the observation that a significant fraction of *t*-γδ T cells express the KIR CD94 and presumably other KIRs as well as KARs (76; 92), though these latter molecules were not addressed in this work. Notably, there are reports indicating KIR expression, primarily CD94, is strong on Vγ9Vδ2$^+$ but low on non-Vγ9Vδ2$^+$ cells (76; 92) however evidence obtained in this study, shows no subtype distinction in CD94 expression (Table 13 and 14). Three scenarios can be envisaged to explain how γδ T cells may lose control in an autoimmune situation: (1) observations indicating patients with MS have reduced levels of CD94/ NKG2a complex on circulating γδ T cells relative to healthy controls (81), suggests a loss of functional control from the lack of downregulating signals; (2) similarly, loss of functional control may be due to the cellular downregulation of natural KIR ligands such as Class I molecules; notably poorly expressed on human oligodendrocytes *in vivo*. An example of this phenomenon was recently demonstrated by the implicated γδ T cell autoimmune response to erythroid progenitor cells lacking Class I expression and resulting in red cell aplasia (128); and (3) upregulation of γδ T cell reactive self-ligands such as HSP in times of stress would serve to tip the normal homeostatic balance towards γδ T cell activation (67).

A recurrent phenotype found on MS *t*-γδ T cells is the prevalent expression of CD40L which has implications in the activation of microglia as previously discussed, but also in the support of B cell generation of antibody. Since γδ T cells appear to be prone to inducing autoantibody (136; 277) and from the recent resurgence in autoantibody mediated demyelination as one of the key mechanisms for myelin loss (160), it is conceivable that γδ T cells may play a key role. This is not without precedence since it was recently demonstrated in Guillain-Barré syndrome (GBS), a demyelinating disease with similarities to MS, that GBS patients had a high frequency of IL-4 producing Vδ1 γδ T cells in their blood, thus lending support for a strong B cell response that is characteristic of the disease (81). Indeed, studies with *t*-αβ T cells have demonstrated polyclonal B cell help was mediated via CD40L interaction with B cells and to a lesser extent by mTNF-α (205). The strong production of IFN-γ as seen in our *t*-γδ T cells, could play a role in inducing isotype switching that can occur in the absence of classical germinal centre formation (134).

Thus it would be of interest to pursue B cell studies with *t*-γδ T cells expressing CD40L derived from MS patients.

4.7 Summary

Results have been presented which in large part addresses the specific goals initially proposed as a means to further our understanding of MS γδ T cells and how they may participate in disease pathogenesis. Specifically, methods were successfully established to consistently generate high purity, polyclonal PB and CSF γδ T cell lines from MS and OND patients. Although efforts to maintain these lines in culture met with limited success, they sufficiently provided the basis for initial comparison and mechanistic studies. Meeting the next objective, methodologies were successfully developed resulting in the *de novo* immortalisation of PB and CSF γδ T cells using *Herpesvirus saimiri*. The resultant HVS growth transformed lines and clones have now been maintained in continuous culture for periods exceeding two years. A series of studies comparing surface molecule expression, PMA/ Ca^{2+} ionophore induced cytokine profiles, and tumour cytotoxicity responses between HVS *t*-γδ T cells and their parental non-transformed lines, established the transformation process impacted marginally on their phenotype and function, thus fulfilling the third objective of this study. Addressing the next objective, extensive characterisation of a number of *t*-γδ T cell lines and clones demonstrated their conserved proinflammatory nature and consistent cytolytic abilities without any apparent correlations to γδ T cell phenotypes. Antigen recognition studies on the other hand, displayed recognition patterns for non-peptidic small antigens that were correlative to Vγ9Vδ2 expression only, in addition to tumour cell recognition that was independent of γδ T cell phenotype. The last major objective of this study was addressed in a limited fashion through the comprehensive comparison of PB vs. CSF *t*-γδ T cells generated from a representative MS patient. The results from these studies were suggestive that no functional differences existed between cells derived from either tissue compartment, but that their distinction may reside in the γδ TcR subtype makeup of the two

compartments, which in turn influences the nature of antigen that they can recognise. Further comparisons of matched *t*-γδ T cells will help to clarify these differences.

The successful implementation of the HVS immortalization strategy on MS PB and CSF γδ T cells represents a significant step forward in our ability to study their role in MS pathogenesis. Their conserved proinflammatory nature and promiscuous cytotoxicity regardless of specific γδ TcR subtype, along with their suggested ability to discriminate CNS specific autoantigen based on γδ TcR subtype, is entirely consistent with the hypothesis that these cells may be instrumental early in the disease process. Whether specific γδ T cell subset functions (Vδ1, Vδ2) are prominent at different disease stages (early vs. late), or indeed whether γδ T cell induced pathogenesis is patient specific, now awaits further study that can be addressed in a unique manner with HVS immortalised cells. In particular, this technology will allow the study of property changes over time within γδ T cells of individual patients, by direct comparison with cells immortalised at different time-points.

4.8 Future directions

Evidence suggests that the level of γδ T cell participation within the CNS may be directly related to the recognition of CNS derived antigen, and that this class of antigen is in all probability unique to γδ T cells, such as danger related stress proteins or non-peptidic small molecules or glycolipids. The availability of renewable, defined, and rarely described CSF *t*-γδ T cell lines and clones along with sensitive and specific antigen recognition techniques, now permit an entry into this area that would otherwise be unfeasible. It would therefore be of great interest to screen CSF *t*-γδ T cells against human myelin extracts, to isolate those reactive fractions, and to characterise and identify the antigenic myelin components.

Additionally, there is now a considerable body of evidence suggesting γδ T cells play a significant role in first line defence against invading microorganisms and cell transformations. A corollary of this early encounter with pathogens or aberrant cell types, is the ability of the γδ T cells to regulate the ensuing immune response. It is therefore a reasonable assumption that γδ T

cells are among the first cell types to respond to a precipitating or cross reactive stimulus present within the CNS of MS patients, and their predominant proinflammatory responses would polarise the ensuing immune response in a deleterious Th1 direction resulting in disease exacerbation. It would therefore be of great therapeutic importance to investigate manners in which to modulate CNS γδ T cell effector functions, specifically those implicated in regulating disease pathogenesis such as proinflammatory cytokine and chemokine secretion, activation of resident glial cells, B cell support for antibody production, and deleterious cytolytic responses. Studies to this effect have historically been hampered by the rare availability of human CNS derived T cells; a limitation now overcome by the generation of immortalised CSF γδ T cells.

Finally, the ability to immortalise and preserve the phenotypic and functional properties of individual patient samples, provides a unique opportunity to study their γδ T cells over time. For instance, comparative studies of an individual's cells prior to and post therapy or in response to changes in disease status (RR to SP MS), may shed light on the role that their γδ T cells play in their disease process.

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Appendix I

Table of antibodies used for flow cytometric analysis

Ab name	Specificity	Source	Characteristics
RPA.2.10	CD2 (LFA-2)	Pharmingen	Recognises a 50 kD transmembrane glycoprotein involved with adhesion and signalling. Ligand is CD58 (LFA-3).
UCHT-1	CD3	Sigma	Recognises the ε chain of the CD3/ TcR complex.
Q4120	CD4	Sigma	Recognises a 59 kD surface glycoprotein. T helper/ inducer phenotype.
SK1	$CD8^{+}$	BD	Recognises the surface glycoprotein associated with the T cytotoxicity/ suppressor T cell subset.
B73.1	CD16	BD	Recognises the transmembrane form of IgG Fc (FcγRIII), which is a NK cell marker.
2A3	CD25	BD	Reacts with IL-2 receptor on activated T cells.
L293	CD28	BD	Recognises the surface molecule on T cells which mediates a second co-stimulatory signal upon activation.
TRAP-1	CD40L (CD154)	Pharmingen	Reacts with the 39 kD glycoprotein on activated T cells. CD40-CD40L interaction involved with cell adhesion and signalling.
F81113	CD45RA	Sigma	Recognises the 220 kD isoform of CD45 associated with naive phenotypes.

Appendix I- Continued

Ab name	Specificity	Source	Characteristics
UCHL1	CD45RO	Sigma	Recognises the 180 kD isoform of CD45 associated with memory phenotypes.
B159	CD56	Pharmingen	Reacts with the glycosylated antigen isoforms found on NK cells. Pan NK cell marker.
BRIC5	CD58 (LFA-3)	Serotech	Reacts with the adhesion molecule LFA-3. Ligand of LFA-3 is CD2 (LFA-2).
HP-3D9	CD94	Pharmingen	Reacts with a 70 kD disulfide-linked heterodimer expressed on NK and γδ T cells.
HP-3B1	CD94	Immunotech	Reacts with a 70 kD disulfide-linked heterodimer expressed on NK and γδ T cells.
UB2	CD95 (FAS)	OnCor	Reacts with the 45 kD transmembrane Fas antigen involved in apoptosis signalling.
NOK-1	CD95L	Pharmingen	Reacts with 40 kD Fas ligand.
BNI3	CD152	Pharmingen	Reacts with the cytolytic T lymphocyte-associated antigen- CTLA-4.
EB6	CD158a	Immunotech	Reacts with a 58 kD type I transmembrane protein belonging to the NK-R family.
GL183	CD158b	Immunotech	Reacts with a 58 kD type I transmembrane protein belonging to the NK-R family.
DX9	NKB1	BD	Reacts with a 70 kD glycoprotein member of the p58/ NK associated transcript family of class I receptors.
B9.12.1	HLA-ABC	Immunotech	Reacts with HLA class I molecules
BF1	HLA-DR	Biosource	Reacts with MHC class II molecules.

Appendix I- Continued

Ab name	Source	Characteristics
WT31	Becton-Dickinson	Reacts with αβ TcR/ CD3ε; pan αβ TcR marker
TCRδ1	T Cell Diagnostics	Reacts with Cδ of γδ TcR ; pan γδ TcR marker.
TCRγδ-1	Becton-Dickinson	Reacts with Cγ of γδ TcR; pan γδ TcR marker.
TCS1	T Cell Diagnostics	Reacts with the Vδ1-Jδ1 subset of γδ TcR .
TS8.2	T Cell Diagnostics	Reacts with the Vδ1 subset of γδ TcR.
15D	T Cell Diagnostics	Reacts with the Vδ2 subset of γδ TcR.
P11.5B	Serotec	Reacts with the Vδ3 subset of γδ TcR.
4A11	T Cell Diagnostics	Reacts with the Vγ4 subset of γδ TcR.
7A5	T Cell Diagnostics	Reacts with the Vγ9 subset of γδ TcR.
MQ1-17H12	Caltag	Intracytoplasmic staining of IL-2
8D4-8	Pharmingen	Intracytoplasmic staining of IL-4
JES3-19F	Pharmingen	Intracytoplasmic staining of IL-10
4S.B3	Pharmingen	Intracytoplasmic staining of IFN-γ
MAB11	Pharmingen	Intracytoplasmic staining of TNF-α
Delta G9	Pharmingen	Intracytoplasmic staining of perforin

Curriculum vitae

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Educational Background

1995-Present University of Ottawa, Ottawa, Ontario.

- Working towards a Ph.D. in Immunology, studying human γδ T cells and their role(s) in the demyelinating autoimmune disease multiple sclerosis.
- Supported by a MRC level scholarship from the Multiple Sclerosis Society of Canada and a University of Ottawa Excellence Scholarship.

1986-1989 University of Ottawa, Ottawa, Ontario.

- Obtained Master's degree in Chemistry, specializing in the isolation, chemical modification, synthesis, and serology of polysialic acid antigens and immunogens. Thesis was recommended for the University Prize.

1980-1985 Carleton University, Ottawa, Ontario.

- Completed a four year Science Honours program, majoring in synthetic organic chemistry.
- Honours project consisted of long chain fatty acid syntheses and their subsequent *in vivo* biological testing.

Employment History

1995 National Research Council, Division of Biological Sciences, 100 Sussex Dr., Ottawa, Ontario.
Position: Technical Officer Level III

- Initiated a study of bacterial heat shock proteins as potential protein carriers in new semi-synthetic conjugate vaccines.

1993-1995 North American Vaccine Inc. at National Research Council, Division of Biological Sciences, 100 Sussex Dr., Ottawa, Ontario.
Position: Research Associate.

- Involved in a number of projects relating to the development of polysaccharide-protein conjugate vaccines against invasive bacterial pathogens. Acquired skills include NMR, HPLC, GC-MS, and animal techniques, immunochemical assays, computers, and lab management. Research carried out was primarily independent without the need of close supervision.

1989-1993

BioChem Pharma at National Research Council, Division of Biological Sciences, 100 Sussex Dr., Ottawa, Ontario.
Position: Research Associate.
-Employment and job description is essentially the same as above. The change from BioChem Pharma to North American Vaccine resulted from a strategic partnership between the two corporations.

Publications

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Contribution of collaborators

This thesis represents original work performed solely by Robert A. Pon and does not represent any combined effort with other investigators.