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The Role of Fcγ Receptor III (CD16) in the Regulation of γδ T Cells in Multiple Sclerosis

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Abstract

Multiple Sclerosis (MS) is an autoimmune central nervous system (CNS) disease characterized by inflammatory infiltrates alongside demyelinated axons, axonal transections and degenerating neurons; the exact mechanism whereby the immune system causes disease is still unclear. This lab has been exploring the possibility that innate immune responses are involved in disease immunopathogenesis, in particular γδ T cells which are concentrated in MS lesions.

The work described in this thesis was undertaken to examine the possibility that γδ T cells, via their expression of CD16, mediate CNS injury by antibody dependent cell cytotoxicity (ADCC) and through the same encounter they release cytokines that regulate the immune responses.

CD16 is a low affinity Fcγ receptor, an activation receptor for γδ T cells, and a mediator of cytotoxicity. In the first part of this study, I found that the number of $CD16^+$ γδ T cells is elevated in MS patients compared with healthy controls. The increase is especially pronounced in patients with a progressive course, and the extent of this elevation shows a positive correlation with severity and duration. Secondly, two *in vitro* experimental systems were established to investigate the hypothesis that these $CD16^+$ γδ T cells are capable of ADCC cytolysis. The results show that the cytotoxicity mediated by γδ T cells is significantly increased by the presence of a bridging antibody, and this effect could be abrogated by removal of Fc portion of antibody or by using $CD16^-$ effector cells, suggesting a CD16-specific antibody-mediated cytolytic contribution. This is the first study of its kind to confirm the ADCC capability of γδ T cells. Lastly, by using intracytoplasmic flow

cytometry, I found that antibody-coated stimulant cells could induce production of cytokines from γδ T cells. Interestingly, the capability of producing Th2 cytokines exclusively resides in the $CD16^+$ population, suggesting a regulative role of γδ T cells in the pathogenesis of MS.

The findings of this work tie together the innate and adaptive immune responses through an interaction that involves the stimulation of antibody production, possibly against CNS molecules such as myelin, via CD16 expressing γδ T cells that could use these antibodies to enact a guided attack on CNS targets via the mechanism of ADCC. This study offers further novel insights into the possible roles of γδ T cells in MS that can involve both potentially pathogenic and immunoregulatory functions.

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I would like to dedicate this work to my loving and beloved families, both here in Canada and back in China. They are the every reason that I could come all this long way to be standing at where I am today.

Table of Contents

List of Abbreviations

%CD16$^+$	percentage of γδ T cells that express CD16
7-AAD	7-Amino-actinomycin D
ACTH	adrenocorticotrophic hormone
ADCC	antibody mediated cellular cytotoxicity
ANOVA	analysis of variance
APC	antigen presenting cell
ATCC	American typical culture collection
BBB	blood-brain-barrier
C'	complement
CCD	charge coupled device
CD	cluster of differentiation
CIS	clinical isolated syndromes
CNS	central nervous system
cRPMI	complete RPMI medium
CSF	cerebrospinal fluid
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescent
EDSS	expanded disability status scale
ELISA	enzyme linked immunosorbant assay
$F(ab')_2$	fragment antigen binding
FACS	fluorescent activated cell sorting
FC_3A	flow cytometric cellular cytotoxicity assay
FcγR	Fc gamma receptor
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FL	fluorescence
Foxp3	forkhead box P3
HC	healthy control
HC/O	healthy control, older group
HC/Y	healthy control, younger group
HLA	human leukocyte antigen
HRP	horse radish peroxidase
ICAM-1	intercellular adhesion molecule-1
IFN	interferon
Ig G	gamma immunoglobulin
IL	interleukin
IP-10	interferon-γ induced protein 10
ITAM	immunoreceptor tyrosine-based activation motif

LFA-1	lymphocyte function-associated antigen-1
LPS	lipopolysaccharide
mAb	monoclonal antibody
MBP	myelin basic protein
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIC	MHC class-I-like molecules
MMP	matrix metalloproteinase
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	multiple sclerosis
MW	molecular weight
NK	natural killer cell
NO	nitric oxide
ns	not significant
OCB	oligoclonal band
OD	optical density
OGC	oligodendrocyte
PAGE	polyacrylamide gel electrophoresis
PAMPs	pathogen associated molecular patterns
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PBS-1	PBS containing 0.1% bovine serum albumin
PC-5	phycoerythrine-cyanine 5
PC-7	phycoerythrine-cyanine 7
PCR	polymerase chain reaction
PE	phycoerythrine
PHA	phytohemagglutinin
PI	propedium iodine
PLP	proteolipid protein
PMA	phorbol myristate acetate
PPMS	primary-progressive MS
PRMS	progressive-relapsing MS
psi	pound per square inch
RA	rheumatoid arthritis
RANTES	regulated upon activation, normal T-cell expressed, and secreted
RRMS	relapsing-remitting MS
RT	room temperature
SD	standard deviation
SDS	sodium docecyle sulfate
SNP	single-nucleotide polymorphisms
SPMS	secondary-progressive MS
STAT	signal transducers and activators of transcription
T-bet	Th1-specific T-box transcription factor

TCR	T cell receptor
TGF	transforming growth factor
Th	helper T cells
TLR	toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TNF	tumour necrosis factor
Treg	regulatory T cells
UV	ultra-violet
VCAM-1	vascular cell adhesion molecule-1
VEPs	visual evoked potentials
VLA-4	very late activation antigen-4

List of Figures

List of Tables

1. Introduction

1.1 Multiple sclerosis

1.1.1 History: The first reported case of multiple sclerosis (MS) could probably date back to the 14th century when a Dutch young woman was recounted as suffering various disabilities that would suggest MS by modern physicians' assessment (1). However, MS was not well recognised by the medical professionals as a specific illness until 1868 when the renowned French neurologist Dr. Jean-Martin Charcot gave the disease its name "sclerose en plaque", after summarizing research of his predecessors and his own (2). As "self-evident" as the name itself implies, MS is a disease characterised by "manifolds (multiple) of hardenings (sclerosis)" – hardenings of scar tissues found in the central nervous system (CNS) of patients, which was first observed and documented some 170 years ago (3). It would have been another half century until 1916, with the development of new microscopic technologies and the discovery and extensive studies on myelin sheath, before Dr. James Dawson was able to give detailed histological descriptions of the myelin damage and perivascular inflammation in the lesions of MS brain (4). The several decades that followed witnessed a host of unsuccessful attempts to the treatment of MS and diligent but fruitless research into the understanding of this elusive and complex disease (5). A breakthrough was seen in 1970 when patients with acute exacerbations (also known as relapses, or neurological attacks) were experimentally injected with the steroid stimulating hormone ACTH (adrenocorticotrophic hormone) which alleviated the symptoms of the attack (6), and is enjoying a bit of a revival today with the availability of a new gel form. Now, instead of using ACTH, steroids in many forms are the mainstay of treating attacks. The introduction

of MRI (magnetic resonance imaging) technique in the mid 1980s remarkably revolutionized the diagnosis of MS (7, 8). This powerful and informative method allowed physicians to "visually" examine the CNS of a patient non-invasively and can be repeated multiple times during a prolonged period. MRI is now an indispensable part of the MS paraclinical tests which also include CSF oligoclonal band (OCB) assays and visual evoked potentials (VEPs) test (9), which help to support the clinical diagnosis.

1.1.2 Symptoms and diagnosis: Because MS lesions are randomly disseminated across the CNS and new lesions that do not necessarily overlap with the old ones occur over time, the clinical symptoms of MS are extremely variable and may include almost all neurological manifestations conceivable. These symptoms range from unilateral or bilateral visual impairment, gait abnormality, numbness and tingling, pain, fatigue, urinary-reproductive system dysfunction, to cognitive and/or psychiatric disturbances, among others (10).

The neurological status of MS patients could be quantified against a numerical rule called Expanded Disability Status Scale (EDSS) (11). This is a 10-point scale with half point increments (1-10), with 0 being neurologically normal and 10 death due to MS. Generally patients scoring less than 3.5 have no obvious disability and are fully ambulatory. This point is also the dividing reference that I adopted in this project to separate the research subjects into groups of different disease severities (see Chapter 3 results). Patients with EDSS between 3.5 and 6 are moderately disabled but able to walk to a limited distance without assistance. Patients with scores over 6 (but less than 8) require walking assistance of various kinds such as a cane, walker, or wheelchair to move around.

1.1.3 Subtypes of MS: Another demonstration that MS is an intricate heterogeneous disease is that it has multiple distinctive clinical patterns that could be categorised into four subgroups (12). Roughly around 80 percent of all MS patients begin with relapsing-remitting MS (RRMS). During this course, patients suffer sudden and acute neurological attacks that could last from a few days to several weeks or, rather rarely, months. In between these discrete attacks, patients take comfort of a period of clinical stability - remission, where some patients will have a full recovery that their disability levels restore to normal and only deteriorate very slowly (benign MS), while others can only recuperate partially. Among these RRMS patients a majority will gradually enter a more progressive phase that tends to be chronic and usually unrelenting (secondary-progressive, SPMS). The tipping point where a patient shifts from RRMS to SPMS is unpredictable and the cause of this transition is largely unknown.

A smaller subset of patients, approximately 15 percent, starts out and continues with a primarily progressive course in the absence of relapses (primary-progressive, PPMS). This subtype differs from the rest in that the patients generally have an older-age onset, non-biased gender distribution (see below "epidemiology"), and less inflammatory lesions.

The last subtype is progressive-relapsing MS (PRMS), which only counts for a very small number of cases. Patients in this category experience gradually progressive disability imitating the course of PPMS, but also sustain superimposed relapses.

Each MS patient falls into one of these subtypes during the course of their disease. However the vast majority of the patients will eventually converge to a point where their symptoms worsen over time. Before a patient who suffers an initial neurological attack can

be diagnosed with definite MS, they are called clinical isolated syndromes (CIS). These patients have an increased risk of developing MS in a period of several years (13).

To offer an accurate prognosis to individual patients can be difficult, and special criteria have now been adopted (14).

1.1.4 Epidemiology and etiology: MS is one of the most common acquired neurological diseases affecting young adults at the prime time of their lives (15). Unlike some other illnesses however, the mortality rate in MS patients is not significantly higher than that of the general population (16) so patients must live out their days with this chronic illness. Gender preference exists in almost all subtypes of MS: a female to male ratio of about 3 to 1, with exception of PPMS which has an equal incidence (15).

Prevalence of MS also shows a geographic distribution pattern around the globe where lower latitude also has lower incidence and vice versa, up to the Arctic/Antarctic Circle, regardless if it is on the north or south hemisphere. Numerous theories have been raised to explain this observation. One of these is that the latitudinal distribution actually coincides with the amount of UV light radiation a person receives, which may in turn translate into how much activated vitamin D that person has (17).

Familial studies have shown that the risks of having MS increased if an individual's first degree family member also has MS. The recurrence rate could be as high as 15 percent in an affected family, compared with 0.1 percent in the general population. This rate is even more striking among monozygotic twins, ranging from 30 to 40 percent (18).

Taken together, data accumulated from epidemiology studies inevitably lead to the conclusion that MS is a disease generated by environmental factors in genetically predisposed individuals.

Despite the intense studies during the past, the aetiology of MS remains unknown. Thus far, no single environmental exposure has been identified as a necessary and sufficient causal factor for MS; however, viral infection is emerging to become a likely candidate (19). A multitude of studies have focused on the association between MS and the detection of various virus strains, with some researchers even extending their quest for a specific "MS virus" (20).

To date, the majority of the extensive genetic studies on MS have pointed to the HLA (human leukocyte antigen) -DR15 locus, where the frequencies of some specific haplotypes are found to be increased in MS patients (21) and some of these alleles are also demonstrated to associate with more severe disease outcome (22). In addition to these "block" allelic polymorphisms, DNA microarrays have revealed dozens of genes with single-nucleotide polymorphisms (SNPs) as heritable risk factors (23). Among them, are genes encoding the Fcγ receptors (24).

1.1.5 Animal model: Experimental autoimmune encephalomyelitis (EAE) is the most widely used inducible animal disease bearing close resemblance to MS (25). Even though this model has its intrinsic restrictions and can not loyally reproduce all the aspects of MS (26), results obtained from EAE studies have lead to the development of several successful drugs now used in MS clinics (27).

EAE can be induced in a host of different mammalian species by injecting emulsified rabbit brain components (28). This inoculation allows encephalitogenic leukocytes to infiltrate the perivenular space in the CNS where they cause inflammation and afflict damage to myelin sheath (29). Animals so treated become blind or paralysed and tend to recover in a few days, reminiscent of MS (30). Of interest, EAE can also be induced by passive transfer of myelin specific $CD4^+$ T cells or anti-myelin antibodies into genetically susceptible naïve recipient animals (31-33). These observations set a solid ground for the establishment of the "autoimmune" paradigm of EAE and its human counterpart - MS.

1.2 Pathology of multiple sclerosis

1.2.1 Oligodendrocyte: The primary pathological targets in MS are the myelin sheath and the cell that gives rise to it – the oligodendrocyte (OGC). OGCs are highly differentiated glia cells only residing in the CNS. A single OGC can extend multiple processes (cytoplasmic extensions) from its cell body to form membranous myelin segments, each of which wraps up a portion of a different neuronal axon. This segmental myelination along axon fibres constitutes the anatomical basis for the saltatory conduction of action potentials, whereby the electrical signals are rapidly propagated down the axon in discrete jumps along the interspaces between adjacent segments - the nodes of Ranvier. Because of its critical role in the conduction velocity of nervous sparks, it is not unfathomable that myelin/OGCs damage has a profound impact on the MS patient, producing neurological deficits.

Several myelin proteins that are shielded from immune cells under normal conditions could become potentially antigenic in MS patients. Among them are: MBP (myelin basic protein), MOG (myelin oligodendrocyte glycoprotein), and PLP (proteolipid protein).

1.2.3 Demyelination: is believed to be the major histopathological abnormality in MS observed primarily in white matter, though recent studies reveal that lesions approximating gray matter are not unusual (34). The demyelinated areas, ranging from 1 mm to several centimetres in size, appear slightly darker than the surrounding normal tissue on an autopsy specimen and can be clearly distinguished by a circumscribed demarcation.

In early, active demyelinating lesions inflammation is pronounced. Infiltrating leukocytes, mostly $CD8^+$, γδ T lymphocytes, and macrophages, can be detected around blood vessels in the periventricular regions. Myelin damage is obvious and degraded myelin debris can be seen microscopically being engulfed in macrophages and microglial cells. In some lesions deposition of immunoglobulin and complement is also prominent (35).

A chronic inactive plaque is less inflammatory and lacks evidence of active myelin breakdown. Astroglial scarring becomes evident and mature OGCs are remarkably diminished, resulting in hypocellularity within the boundary of the plaque. Around the border of the plaque, remyelination could sometimes develop from the pool of OGC progenitor cells. However, due possibly to the unfavourable lesional microenvironment, remyelination is seldom complete (36).

1.2.4 Neurodegeneration: In the past it was believed that MS was principally a demyelinating disease with neuronal axons relatively spared of damage. However recent

studies have shown that axonal injury exists in MS patients in both acute and chronic lesions (37). Large terminal ovoids indicating axonal transection can be visualized by confocal microscopy in all lesions in post-mortem MS brains (38). The correlation between inflammation and axonal dysmorphia may imply that neuronal damage is a direct result of inflammatory insult (39). In keeping with this argument, association between the numbers of macrophages and $CD8^+$ T cells and the degree of axonal loss has been reported (40). It has also been observed that axon-specific antibodies are significantly increased in MS patients (41). Nonetheless, this does not exclude the possibility that axonal loss could be secondary to inflammatory demyelination which may lead to the demise of trophic support that is vital for neurons to survive (42, 43). Furthermore, naked axons are more easily accessible to destructive immune components (44).

1.3 Immunology and multiple sclerosis

Though the aetiology of MS remains to be fully elucidated, a plethora of evidence has accumulated demonstrating immune element involvement in this disease. Previously considered an immunological privileged site, the CNS is actually permeable to leukocytes in both physiological and pathological conditions, though with extra restrictions enhanced by the blood-brain-barrier (BBB) (45). Under normal condition a scant amount of immune cells patrol the CNS by extravasation through the choroid plexus or subarachnoid space and migrate back to systemic circulation with the flow of CSF that ends at venous sinuses (46). But in an MS brain, the tight junction of BBB is ruptured so that the activated leukocytes can conveniently enter the CNS in exodus through these breaches (47). Another major source of immune cells in the CNS may come from the ectopic lymphoid follicles that have

been seen in MS brains (48). This structure of aggregated B cells, T cells, and dendritic cells that imitate germinal centres of the secondary lymphoid organs could be an explanation for the continuous local activation and proliferation of lymphocytes in MS (48).

1.3.1 Trafficking: Myelin-specific leukocytes can be isolated from blood and CSF of MS patients (49). These cells may have increased expression of adhesion molecules such as very late activation antigen-4 (VLA-4), lymphocyte function-associated antigen-1 (LFA-1), or CD6 once they are activated in periphery (50-52). These adhesion molecules recognise their corresponding ligands (vascular cell adhesion molecule-1, VCAM-1 or intercellular adhesion molecule-1, ICAM-1) which are also found upregulated on the endothelial cells of BBB in the inflamed MS brains (50, 51, 53, 54). Such recognition and pairing between adhesion molecules allow the circulating leukocytes to slow down in the blood stream, roll along the vessels, halt at endothelium surface, and finally extravasate through the interendothelial junctions into the perivascular space (55). Extravsating cells, obstructed by the basement membrane of the *glia limitans*, secrete matrix metalloproteinases (MMP) that would dissolve the extracellular matrix, and ultimately enter the brain parenchyma (56-58). The fact that blocking of adhesion mediated leukocyte transmigration by anti-VLA-4 mAb (natalizumab) could ameliorate MS symptoms lends support for the significance of leukocyte trafficking in the pathology of this disease and underlies the importance of cellular immunity (59, 60). γδ T cells use different sets of adhesion molecules for tranendothelial migration, as discussed in detail below.

1.3.2 αβ T lymphocytes: Among the inflammatory infiltrates into the CNS, $CD4^+$ αβ T cells have been a prominent focus, following the profound findings that adoptive transfer of encephalitogenic $CD4^+$ T cells alone could introduce EAE in disease prone animals (61). In order for naïve $CD4^+$ T cells to get activated and become functional, two signals are required: one from a cognate peptide presented by a MHC-II molecule on an antigen presenting cell (APC); and meanwhile a secondary co-stimulation with molecules such as CD28 that interacts with the members of B7 family on the APCs. However the observation that MBP-specific T cells recovered from MS patients require relatively low intensity of CD28 co-stimulation to proliferate suggests that these cells are previously activated and indicates their involvement in MS pathogenesis (62, 63).

One of the major functions of $CD4^+$ αβ T cells is to "help" other immune cells where they exert their functions either by cell-cell direct contact offering co-stimulations, or by secreting soluble messengers such as cytokines that will act on recipient cells. Based on the types of cytokines produced, Th cells can be roughly divided into mutually exclusive and suppressive subgroups: Th1 cells secrete pro-inflammatory cytokines such as interferon-γ (IFN-γ), interleukin-2 (IL-2), or tumour necrosis factor-α (TNF-α), which facilitate the removal of intracellular pathogens; while Th2 cells produce IL-4, IL-5, IL-10, that are capable of inducing B-cell differentiation, isotype switch, and clearing of extracellular pathogens. Recently a third subgroup of Th cells has been identified and designated Th17 cells. These cells produce their signatory cytokine IL-17 and actively participate in inflammation or autoimmunity. How cytokines are involved in MS is discussed in details below in the section of "Cytokines and chemokines".

The major function of $CD8^+$ αβ T cells is cytotoxicity, whereby they kill the cells whose MHC-I molecules present an antigen-derived or self peptide that matches their TCR. An indirect proof of their involvement in MS comes from the observations that the numbers of $CD8^+$ αβ T cells are increased in the peripheral blood and CSF of MS patients compared to controls; moreover these cells display an activated memory phenotype (64-66). A close look at this subtype of T cells by means of laser microdissection and single-cell PCR has revealed that they are clonotypically expanded at the site of brain lesions, demonstrating a direct interaction with particular antigens in the CNS (67). Though $CD8^+$ T cells have been suggested to participate in inflammatory demyelination and axonal damage, direct evidence is still missing and their functional roles remain to be elucidated (68-70).

Regulatory T cells (Treg) are a specialized subtype of αβ T cells existing in small quantity. They act to maintain immune homeostasis following a bout of foreign pathogen attack and contribute to peripheral tolerance to self-antigens. Treg cells bear the hallmark surface markers of CD4 and CD25, as well as intracellular transcription factor Foxp3, which is crucial for their development (71). This subtype of T cells becomes of particular interest in the pathogenesis of MS in light of recent studies showing deranged suppression of self-reactive T cells leading to autoimmunity (72). In fact, even though the frequency of $CD4^+CD25^{high}$ T cells are found increased in the CNS of MS patients (73, 74) , their Foxp3 expression is actually decreased and their suppressive functions compromised (73, 75, 76), consequently leaving the destructive autoimmune cells unconstrained. A complimentary regulatory T cell subtype in the CD8 compartment has recently been identified, namely $CD8^+CD122^+$ T cells (77). These cells are shown to contribute to EAE recovery (78) and their suppressive functions in MS patients are impaired during exacerbation (79). Though

lacking the title of Treg, under certain circumstances γδ T cells and NK cells have demonstrated immune regulatory functions and participate in tissue repair (80, 81).

1.3.3 Humoral immunity: Humoral immunity and αβ-T cellular immunity together constitute the adaptive immune system. The major players in the humoral immune system are B cells and the immunoglobulins they produce. It has been known for years that the expansion of a few clones of B cells in the intrathecal space results in detectable oligoclonal IgG band in CSF in over 90 percent of MS patients (82, 83). However these antibodies are highly variable from patient to patient and only a small proportion of the total Igs are MS-related (84), thus limiting their significance only to the diagnosis of MS, but not the pathogenic relevance.

The interest in humoral immunity in MS is recently renewed after an immunopathological study has found that antibodies and complement present in over half of the active lesions studied (35, 85). That treating acute demyelinating by plasma exchange could ameliorate MS also lends indirect support on a causative role of humoral immunity (86). The specificities of the lesional auto-antibodies and the antibodies isolated from sera and CSF of MS patients have been gradually identified to react to auto-antigens such as MBP (87), PLP (88), MOG (89, 90), and neurofascin-186 (91). Most of these autoantibodies mediate target cytolysis through activation of complement or antibody mediated cellular cytotoxicity (ADCC) (35), however a special anti-MBP autoantibody has been reported to possess intrinsic proteolytic activity, which enables it to cause myelin injury directly (87). Aside from the large body of evidence suggesting a pathologic role,

some special autoantibodies partaking beneficial remyelination process have also been proposed (92, 93).

In addition to producing antibodies, B cells also participate in immune regulation by presenting processed antigens and providing co-stimulatory signals to T cells, and secreting cytokines. Circulating B cells that express CD80 co-stimulatory molecule are significantly increased in the course of relapse, but their number returns to normal during remission (94). Th1/Th2 balance of the B cell cytokine network is dysregulated in MS patients, whereas these B cells exhibit a "memory" phenotype and have decreased production of down-regulatory cytokine IL-10 (95). An animal study implies that this transfer of balance could also be due to the dysfunction of native B cell suppressive molecules, a potential candidate of such is CD19 (96).

Given the potential role of humoral immunity in the pathogenesis of MS, eliminating mature B cells from systemic circulation of MS patients by administrating a humanised anti-CD20 mAb (rituximab) is currently under clinical investigation (97). The variable region of rituximab is cloned from a mouse anti-human CD20 antibody (clone 2B8) and the constant regions of both κ light chain and γ1 heavy chain are copied from human sequences (98). The mechanisms of actions of rituximab in MS are still unknown, but ADCC, complement mediated cell lysis, inhibition of growth, and induction of apoptosis have been suggested (99). (In my work however, rituximab is used to form part of the components of an in-house developed flow cytometry cytotoxicity assay to examine the ADCC functionality of γδ T cells.)

1.3.4 Innate immunity: In contrast to adaptive immunity, which is highly variable, specific, and maintains a long-lasting memory, the innate immunity provides immediate host defence via a more conservative "pathogen associated molecular patterns" (PAMPs) recognition mechanism; e.g. TLR-4 (toll-like receptor-4) reacts with lipopolysaccharides (LPS) of gram-negative bacteria origin and CD94/NKG2 recognises infected cells that have reduced expression of HLA-E molecules (missing self) (100).

Macrophages are one of the most prevalent innate immune cells in the acute lesions of MS (101). Their functions contributing to CNS destruction may include: [a] antibody mediated oligodendrocyte damage (102, 103) and [b] endocytosis (104), [c] production of nitric oxide (NO) that is directly toxic to OGC or neurons (105), [d] release of proinflammatory cytokines and chemokines (106), and [e] antigen presentation to T cells (107).

Microglia are endogenous CNS phagocytic cells of bone marrow origin (108). Many of their detrimental functions mirror those of the macrophages listed above.

The role of NK cells remains controversial, despite the fact that most of the human studies are in favour of an immunoregulatory function of this cell type in the chronic course of MS (81).

1.3.5 Cytokines and chemokines: Cytokines are a family of soluble peptidic hormones produced *de novo* mostly by activated immune cells. The primary effects of cytokines consist of [a] controlling proliferation and differentiation of hematopoietic cells, [b] regulating activation and maturation of lymphocytes, and e mediating natural immunity and inflammation (109). Cytokines exert their functions through binding to their individual

receptors, which, not unusual, share at least one common subunit between their hetero-polymeric structures (109).

The polarization of Th cells into pro- (Th1) and anti- (Th2) inflammatory subsets was first established in mouse models (110). Though it has never been as clear-cut in human system, this paradigm is gradually being accepted with caution (111). With the recent identification of Th17 cells, it might be more appropriate to extend the spectrum of pro-inflammatory cytokines to incorporate both Th1 and Th17 subpopulations.

Inflammation in the CNS of MS patients is obvious; nonetheless, the profile of cytokine network in this disease is by no means as apparent. To further complicate this issue, both pro- and anti- inflammatory cytokines are found elevated, sometimes even in parallel, in MS lesions (112).

Transcripts and production of typical proinflammatory cytokine TNF-α, IFN-γ, or IL-1 have been reported increased in both systemic circulation and active MS lesions (113, 114). This may bring up the proposal that eliminating these cytokines could ameliorate the disease. Surprisingly, therapeutic attempts trying to neutralize TNF-α actually leads to prolonged exacerbations or new onset of MS (115, 116). In a similar scenario, IFN-γ, which has been shown to attenuate disease of EAE in mice (117), causes exacerbations when administered to patients (118). Taken together, these proinflammatory cytokines may not only exist to fire up the inflammations in MS, but also exhibit complex functions depending on the timing and location of their appearance, and the activation state of their putative targets as well (119).

Anti-inflammatory cytokines (IL-4, IL-10 and TGF-β) and their receptors have also been reported to be unexpectedly upregulated; their preferred localization in and around chronic

active lesions may suggest that they are important for disease progression and/or resolution (120, 121).

"Chemokine" - the chemo-attractant (or chemotactic) cytokine - is a subpopulation of small molecule cytokines that are mainly responsible for chemotaxis of leukocytes, inflammation, and angiogenesis (122). In EAE, chemokine (e.g. macrophage chemotactic factor-1) expressions are found to follow the clinical disability curve of disease, and coincide with the extensive perivascular accumulation of monocytes at the height of disease (123). A number of chemokines and their receptors are also found in brain lesions of MS patients, rendering interruption of the interaction between the pairs a potential therapeutic choice (124).

1.4 γδ T cells and multiple sclerosis

1.4.1 Biology: γδ T cells were discovered in the mid 1980's (125), making them the newest to join, together with αβ T cells, B cells, and NK cells, the tri-partitioned lymphocytes system. They are a minor subset of the T cells representing a mere 0.5 to 5 percent of the total $CD3^+$ population in circulation in adult mice or human. Though theoretically γδ T cells can express TCRs with enormous varieties in the order of billions by somatic recombination of different V-(D)-J gene segments, along with junctional diversification, in reality they utilize a very limited number of genes (preferentially Vγ9 and Vδ2 gene in adult human and Vδ1 in postnatal mouse) under yet unidentified selective pressures (126). In contrast to αβ T cells, γδ T cells are able to recognize their antigens devoid of the restrictions of MHC molecules, in a way similar to antibodies or B cell receptors. Because of the lack of requirement for antigen presentation, notwithstanding the

relatively limited TCR repertoires, γδ T cells can interact with a broad spectrum of antigens of various biochemistry compositions, such as hsp, non-classic MHC class-I-like molecules A or B (MICA or MICB), small molecular-weight non-peptidic pyrophosphate or alkylamines of mycobacterium or eukaryote metabolites (the mevalonate pathway), or glycolipids presented by non-polymorphic CD1 molecules (126). It is noteworthy that even though the majority of these antigens are cellular manifestations of "environmental stress" or transformation, the fact that they are of endogenous origin may well imply a prospective tendency of γδ T cells to participate in self-reactivity, once the well balanced tolerance machinery is overturned by yet undetermined factors.

γδ T cells' capacity of immune response consists of eliminating infections, malignancy surveillance, and immune regulation (127). Their ability of prompt response to initial antigenic challenge and the subsequent release of immune-shaping cytokines have positioned them in the strategic frontier that cross-links the innate and the adaptive immunity systems. γδ T cells are capable of produce both types of Th cytokines, however, seemingly that the arm of proinflammatory cytokines predominates the dimension (128). Strikingly though it has been shown that in a monophasic EAE model a transition of cytokine production by γδ T cell may occur following a temporal curve as disease develops, whereas at the onset these cells produce proinflammatory cytokines IL-2 and IFN-γ to flare-up the immune response but at the peak and later stage they release IL-4 to contain the inflammation and retain immune homeostasis (129). It is also well demonstrated that γδ T cells are cytotoxic towards a variety of tumour cell lines and freshly isolated human OGCs, possibly via release of granzyme/perforin or through direct intercellular contact of FasL/Fas pathways (130).

Whether γδ T cells are able to develop immunological memory is not well established. However, recent studies have demonstrated that, by phenotypical and functional analogy to αβ T cells, some Vδ2 cells could be further subdivided into highly differentiated subsets of central memory, effector memory, and terminal effector cells based on their physiobiological functions, which coincide with the expression of several surface molecules (131, 132). CD16, in combination with CD45RA and CD27, identifies a terminally differentiated population that is highly cytotoxic and could readily deliver attacks against malignant cell lines (133).

Another feature that makes γδ T cells closer akin to innate immune components is that they have been recently found expressing a host of germ line gene encoded NK receptors, including CD16, CD56, CD161, NKG2D, and Killer Ig-like receptors such as CD158 and CD94 (134).

1.4.2 Oligoclonal proliferation: γδ T cells are found disproportionably represented in the perivenular infalmatory cuffs of MS patients as compared to the periphey, whereas upto 30 percent of the $CD3^+$ cells in MS lesions bearing TCR δ chain as opposed to about one tenth of this number in periphery circulation (135). Moreover, immunohistological evidence has shown that infiltrating γδ T cells are enriched in and around both active demyelinating lesions and chronic plaques, and co-localize with OGCs – the primary pathological targets in MS (136). Numerous studies examining the clonotypic expansions of γδ T cells in the CNS have converged to one conclusion that these cells are essentially oligoclonal, likely being the result from the recognition of, and reaction to a few specific CNS ligands (135, 137). The search for the potential CNS antigen (or antigens) is not straightforward given that γδ T cells

react with a variety of ligands, yet early studies carried out at the turn of last decade focus on the family of heat shock proteins with various molecular weights in light that hsp expression is elevated on OGCs of MS patients and that γδ T cells are reactive to these proteins. Indeed, it has been shown that excessive hsp-60/65 expression could be induced on OGCs *in vitro*, but not on astrocytes, upon heat shock stimulations (138, 139), and these hsp^+ OGCs preferentially stimulate the proliferation of γδ T cells from the mixed culture of PBMC, suggesting that OGC may contribute to the local expansion of γδ T cells within MS plaques (140). A "cold target competition assay" that concurrently mixes two hsp^+ target populations (Daudi and OGC) with effector γδ T cells has suggested that the susceptibility of OGCs to γδ T cell cytotoxicity is, at least in part, due to the presence of hsps (141).

Alternatively, γδ TCR could also be charged through the engagement to CD1 molecules of antigen presenting cells, which are the non-polymorphic counterpart of MHC molecules capable of presenting lipids or glycol-lipids in its hydrophobic groove (142). The abundant presence of lipid (80% by weight) within myelin and the unique CD1 recognition pattern of γδ T cells may emphasize the potential that lipids are a pathological source (143).

1.4.3 Transmigration: The observed intrathecal clonal expansion of γδ T cells does not, however, exclude the possibility that these cells are actually transmigrants from the peripheral compartment. Even though there is no significant numeration difference of γδ T cells in the blood circulation between MS patients and healthy controls (144), the fact that these cells derived from MS patients, but not control individuals, are readily responsive to IL-2 induced proliferation indicates that γδ T cells in MS are somewhat previously activated (145). Activated γδ T cells could upregulate their adhesion molecules and chemokine

receptors which work in concert to facilitate the trans-endothelial migration of these cells into the CNS. Indeed, adhesion molecules unique to γδ T cells such as NKR-P1A (CD161) or beta 2-integrin have both been shown to be important for the infiltration process (146, 147). Interestingly, post-migrated γδ T cells derived from CSF of MS patients show decreased expression of chemokine receptors (CXCR1, CCR5) but elevated intracellular level of RANTES and IP-10 (148). This apparent auto-regulatory loop suggests that homing γδ T cells in the CNS could potentially modulate disease by mediating subsequent leukocyte chemotaxis to the loci of inflammation. Alternatively, lower chemokine receptor expression could also reflect the result of repeated *in vivo* stimulation of γδ T cells by autoantigens or local inflammatory micro-environment (148).

1.4.4 NKR mediated cytotoxicity: Besides γδ TCR, cytotoxicity mediated by a couple of NK receptors has also been demonstrated. NKG2D is an activating or co-activating receptor for γδ T cells (149). Its ligands MICA/B are detected on OGCs in MS lesions but not control brains (150). Co-culturing NKG2D$^+$ γδ T cells with MICA/B$^+$ OGCs leads to the demise of the latter, moreover, this killing could be at least partially inhibited by disrupting the NKG2D-NKG2D ligand interaction using blocking antibodies, suggesting a myelin injurious role of γδ T cells in MS (150).

CD16 is another activating NK receptor found on γδ T cells. Its involvement in γδ T cells mediated ADCC cytotoxicity will be one of the major foci in my work.

1.4.5 Neurotoxicity: As aforementioned, neuronal degeneration has increasingly been appreciated as one of the underlying causes of the clinical deficits in MS, but the direct

evidence that γδ T cells are toxic towards neurons is still absent. Nevertheless, studies have shown that axonal injury is spared in perforin-deficient mice in an MS model induced by Theiler's murine encephalomyelitis virus, reflecting the requirement of perforin producing cells, that include γδ T cells, to participate in the process of axonal damage (151). Because of the unique biological properties of neurons that render them able to elude direct immune attacks, it is, therefore, reasonable to propose that the observed neuronal damage is secondary to demyelination. In line with this hypothesis, Darlington et al. have observed that neurons, otherwise resistant to direct killing, are lost as a consequence of γδ T cells mediated injury towards the supporting glial cells (152). In addition, not dissimilar to the proposed mechanism of ADCC against OGCs in this study (see below), γδ T cells may cause neuronal death via interaction with neuron-specific autoantibodies such as anti-neurofascin 186 that has been identified in MS patients (91).

1.4.6 Immuno-regulation and disease recovery: Most of the perception that γδ T cells play an immune regulatory role in MS comes from animal studies, however the exact role that γδ T cells hold is controversial. Different research groups have reported varied, sometimes contradictory, roles that γδ T cells undertake, from pathogenesis (153, 154), to disease amelioration (155), to merely irrelevant (156) to the observed CNS damage. These apparent paradoxes may be explained in view of the irreconcilable differences existing in the experimental designs or genetic backgrounds of animals among these studies. In summarizing the studies, a possible scenario will likely come to light that: γδ T cells contribute to disease development in genetically predisposed animals at onset by secreting pro-inflammatory cytokines such as IL-12, IFN-γ, TNF-α, and chemokines (153, 157-159);

at height of disease these cells may switch their role to induce apoptosis of encepholitigenic αβ T cells (160); and at the later stage they release IL-4 or TGF-β to help resolve the inflammation (129, 161). However, what the factors are triggering the switch and whether these multi-faceted roles are undertaken by different subsets of γδ T cells or by the same cells at different time point of disease needs further clarification. Furthermore, the complexity of the immune network that obviates the importance of interaction between γδ T cells and other immune components commands us to jump out of the box and to examine it with a global view (162).

That γδ T cells assume regulatory functions in MS is highlighted in a limited number of therapeutic studies in human subjects. A pilot study using attenuated MBP-specific T cell to vaccinate MS patients in an effort to induce immune tolerance has found that γδ T cells are expanded upon vaccine stimulation and these cells produce high levels of IL-2, TNF-α, and IL-10 (163). In another study on understanding the mechanism of IFN-β1a treatment, the perceived benefit is largely attributed to the reversed Th1/Th2 cytokine balance of γδ T cells, but not other cell types (164).

1.5 Fcγ receptor III (CD16)

Fcγ receptors are members of the Ig superfamily. They play a unique and integral role in host immunity in that these receptors, by binding to the Fc portion of IgG, mediate the communications between humoral and cellular immunity and thus orchestrate the functions of the entire immune system (165, 166). Three types of Fcγ receptors have been identified in humans. FcγRI (CD64) is the high affinity Fcγ receptor predominantly found on monocytes or macrophages. FcγRII (CD32) is an inhibitory receptor mainly expressed by B

cells that could down-regulate the production of antibodies by suppressing the other activating FcγRs (167). FcγRIII (CD16) is the low affinity receptor that preferentially binds immune complexes but only weakly monomeric IgG (168).

Two homologous genes encode for nearly two identical transcripts of CD16s: FcγRIIIa and FcγRIIIb (169). CD16a is expressed by various types of leukocytes (macrophages, NK cells, and γδ T cells) at various levels. Its cytoplasmic tail associates with FcRγ chian which contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain, suggesting a role in immune cell regulation. On the other hand, CD16b is exclusively expressed by neutrophils and does not contain the intracytoplasmic tail, functioning as a decoy receptor (169).

Engagement of CD16 on the effector cells by cross-linking or by immune complexes leads to one or more of the biological functions, including phagocytosis, ADCC, and release of cytokines (170). CD16 can be upregulated on cultured resting γδ T cells upon activation through their TCR-CD3 complex (171) or by the presence of proinflammatory cytokines such as IL-15 (172). These $CD16^+$ γδ T cells are highly cytotoxic (133) and they can be re-activated through their acquired CD16 (171). The fact that γδ T cells contain a subset of $CD16^+$ population raises the possibility that these cells might be able to cause cytotoxic damage indirectly via ADCC through CD16 binding and activation.

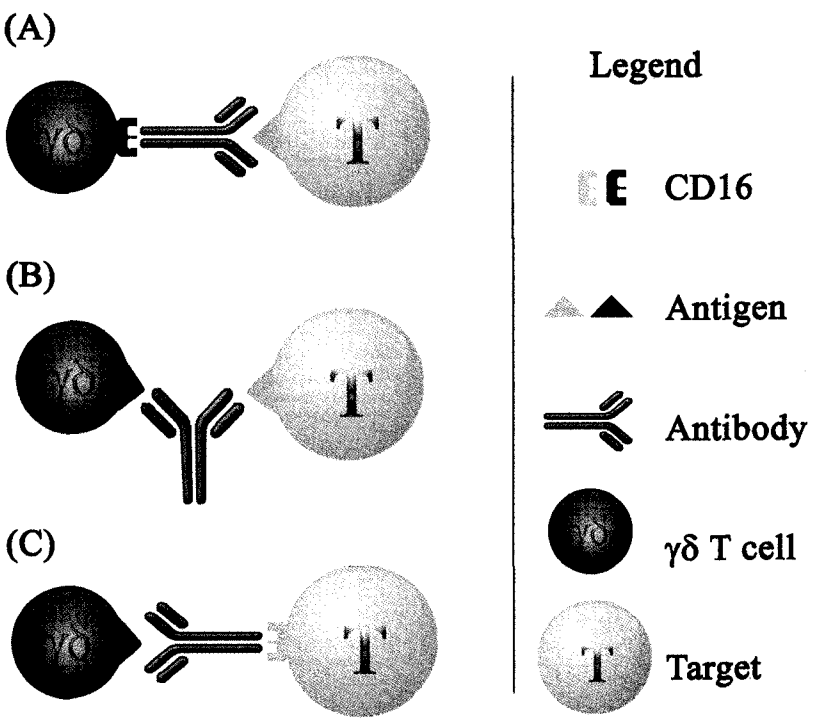
ADCC is a unique cell-mediated immunological process in which $CD16^+$ effector cells actively lyse target cells that have been bound by specific antibodies. Several previous studies suggest that γδ T cells lyse target cells via ADCC mechanism involving surface expression of CD16 molecules, but this observation lacks confirmatory research. Moreover, the majority of the studies failed to comply with the conventional concept of ADCC, with

some of the studies using antibodies in opposite orientation (reverse-ADCC) (173, 174) and others using a bi-specific antibody which mediates a CD16-independent ADCC (175, 176) (Fig. 1). One other drawback of most of these studies is that the antibodies used to bridge the target and effector cells are xenogeneic (177-179). Therefore it will be of great interest to examine if the these $CD16^+$ γδ T cells could, in the presence of a human or humanized bridging antibodies, mediate ADCC in a physiological configuration, as illustrated in Fig. 1A.

Given the particular functions attributed to CD16 expressing γδ T cells and their implication in various autoimmune diseases (180, 181), I investigated this subset in MS.

Figure 1. Illustration of ADCC mediated by γδ T cells.

(A) Conventional concept: γδ T cells capture and cytolyse antibody-coated target cells via their CD16 molecules on their surface. (B) Bi-specific antibody mediated ADCC: CD16 is not necessarily involved in killing. γδ T cells and targets are brought together by an engineered antibody that recognizes different antigens on either of them. (C) Reverse ADCC: it is the target that expresses CD16; the antigen on γδ T cells could be CD16 or other molecules specifically recognized by the antibody.



1.6 Rationale and hypothesis

A wealth of evidence has accumulated that MS exhibits an autoimmune pathology characterized by the infiltration or local production of inflammatory cells and soluble factors that work jointly towards the destruction of myelin and its cells-of-origin OGCs within the CNS. Among the effectors, γδ T cells have been found to have increased presence around the multi-focal brain lesions, giving rise to the possibility that they may actively participate in the disease. On another note, several intrathecal myelin antigens have been proposed and auto-antibodies against these antigens have been identified and shown to selectively injure myelin by the mechanism of complement fixing or complement independent processes.

My preliminary data have shown that some of our cultured γδ T cells derived from MS patients express CD16, the low affinity activating Fcγ receptor. These receptors on γδ T cells are believed to act not only as a bridge linking humoral and cellular immunity but also the innate and adaptive immune response, by orchestrating the interaction between antibodies and the γδ T cells.

Therefore, I hypothesize that γδ T cells, in the pro-inflammation milieu in MS patients, could up-regulate the expression of their CD16 receptors. Auto-antibodies generated in response to myelin auto-antigens will lead to the recognition of CNS targets by γδ T cells through CD16, which subsequently deliver lethal attacks on myelin-bearing elements by mechanism of ADCC. In addition, consequence to CD16 engagement, γδ T cells could further fire up the CNS inflammation by secreting pro-inflammatory cytokines.

1.7 Specific aims

1. Determine the expression of CD16 on γδ T cells and correlate it with disease severity.

The percentage of CD16 expressing γδ T cells (%$CD16^+$) derived from peripheral blood of MS patients or healthy controls are assessed on immediately *ex vivo* cells using multiple parameter flow cytometry. The relationships between the %$CD16^+$ and the clinical characteristics of the patients will be investigated.

2. Evaluate %$CD16^+$ change in response to cytokine stimulations.

Purified γδ T cells are cultured in the presence or absence of proinflammatory cytokines such as IL-2, 12, and 15, those found increased in the milieu of MS lesions. %$CD16^+$ is then measured with flow cytometry and the responsiveness is analysed.

3. Confirm the role of CD16 in γδ T cells-mediated ADCC.

In vitro experimental systems are to be established with target cells expressing known antigens that will be bound by specific human or humanized antibodies. Target cells are first coated with these antibodies followed by co-culture with γδ T cells derived from patients with MS. Specific lysis of target cells will be determined by a developed ELISA or flow cytometry based cytotoxicity assay.

4. Measure the cytokine production of γδ T cells upon CD16 engagement.

Purified γδ T cells are cultured in the presence of antibody coated target cells. The production of pro- or anti- inflammatory cytokines are measured intracytoplasmically by

flow cytometry. The cytokine production preference may shed light on immuno-regulatory functions of γδ T cells.

2. Materials and Methods

2.1 Patients and healthy controls

Patients undergoing diagnostic assessment and clinical follow-up at the MS Centre of the Ottawa Hospital General Campus were included in this project. Table 1 shows the demographic profiles of the patients and healthy controls recruited for the γδ T cell CD16 expression study. Among the patients, 49 were diagnosed with MS according to the "revised McDonald Criteria" (14); the other 15 patients, suffering initial neurological attacks, were diagnosed with Clinical Isolated Syndromes (CIS) (182). None of the patients had experienced a recent exacerbation or received any disease modifying drugs (IFN-β or glatiramer acetate) (183) or steroids at least three months before the time of blood sampling, nor did they have active infections of any kind. Age and gender matched healthy controls were recruited from lab staff or hospital personnel.

This study was approved by the Research Ethics Board of the Ottawa Health Research Institute. The nature of this study was explained to all patients and written informed consents were obtained from all the individuals.

2.2 Reagents

Fluorochrome conjugated or non-conjugated monoclonal/polyclonal antibodies used in this study are listed in Table 2.

Table 1.
Patient demographics.

Table 2.
Antibodies.

Specificity	Host	Clone	Conjugate	Supplier
CD16	mouse	LNK16	FITC	AbD Serotec, Oxford, UK
CD16	mouse	3G8	PE/PC7	Beckman Coulter, Mississauga, ON
CD16	mouse	3G8	N/A	BD Biosciences
CD19	mouse	J4.119	PE	Beckman Coulter
CD20	mouse	B1	PE	Beckman Coulter
CD3	mouse	UCHT-1	PC5	Beckman Coulter
CD3	mouse	UCHT-1	Quantum Red	Sigma
CD32	mouse	FLI8.26	R-PE	BD Biosciences
CD4	mouse	S3.5	N/A	Caltag, Cedarlane Lab. Ltd.
CD64	mouse	22	PE	Beckman Coulter
CD8	mouse	3B5	N/A	Caltag, Cedarlane Lab. Ltd.
Murine IgG	sheep	AFF	N/A	Bindingsite, Birmingham, UK
γδ TCR	mouse	TS8.2	FITC	Endogen
IL-4	mouse	4D9	PE	Beckman Coulter
IL-10	rat	JES3-19F1	PE	BD Biosciences
TNF-α	mouse	IPM2(188)	PE	Beckman Coulter
IFN-γ	mouse	45.15	PE	Beckman Coulter
Human IgG	goat	polyclonal	PE	BD Biosciences
Murine IgG	Rabbit	polyclonal	HRP	Sigma
Human IgG (Fc specific)	goat	polyclonal	PE	Sigma
Human IgG (Fab specific)	goat	polyclonal	PE	Sigma
Mouse Isotype Control	Rat	IgG1	PE	Beckman Coulter
Mouse Isotype Cotrol	Rat	IgG2a	PE	BD Biosciences

2.3 Blood samples and γδ T cells

2.3.1 Isolation of peripheral blood mononuclear cells (PBMCs)

Fresh blood samples were collected by venipuncture from patients and healthy controls (HC) using heparin as an anticoagulant. PBMCs were isolated by density gradient centrifugation according to standard procedures. Briefly, 15 ml of blood was diluted with equal volume of PBS in a 50 ml conical tube. 15 ml Ficoll-Hypaque (Pharmacia Biotech AB, Uppsala, Sweden) was carefully underlayered beneath the diluted blood. The tube was centrifuged at 300xg for 30 min at room temperature (RT) without setting on the brake on the centrifuge. PBMC enriched layer at the interface was carefully removed, washed twice with PBS, and resuspended in cRPMI (complete RPMI, consists of RPMI-1640 supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin, Life Technologies, Burlington, ON) at 1 x 10^6 cells/ml.

2.3.2 Expansion of γδ T cells

γδ T cells were generated as previously described (130). Briefly, 24-well culture plates (Costar, Corning, NY) were sequentially treated with 300 μl (5 μg/ml) sheep anti-mouse IgG antibody (The Binding Site, Birmingham, UK) in PBS for 1 h at 37 °C, 300 μl cRPMI for 5 min at RT, and monoclonal anti-TCRγδ antibody (aka MB antibody, courtesy of Dr. M. Brenner, Harvard University, titrated to yield maximal stimulation) for 30 min at 37 °C. PBMCs isolated as described above were seeded in such treated plates at a density of 3 x 10^6 cells per well in total of 3 ml cRPMI. Cells were kept at 37 °C in humidified atmosphere supplied with 5% CO_2. 50 U/ml human IL-2 (a gift from Hoffmann-La Roche, Nutley, NJ)

was added the next day. On day 5, cRPMI was replaced with serum free AIM-V media (Life Technologies, Burlington, ON) and cultured for another 3 or 5 days with fresh IL-2. Normally the yield of γδ T cells at this stage is 40 to 60% of total cells in the wells.

2.3.3 Purification of γδ T cells by complement lysis

γδ T cells were further purified by elimination of the αβ T cells with complement lysis technique. Cell cultures were collected after the period of expansion and washed and resuspended in 100 μl of PBS buffer. The number of contaminating αβ T cells was calculated by multiplying the total number of collected cells (enumerated with haemacytometer) with the percentage of this population as determined by flow cytometry. For every 1 x 10^6 αβ cells, 4 μg (4 μl from stock) of each of anti-CD4 and anti-CD8 mAbs were added and incubated at 4 °C for 30 min. Cells were washed with ice cold PBS and resuspended in PBS pre-warmed to RT. 200 to 300 μl of sterile baby rabbit complement (C') was added to the cells which were kept in a 37 °C water bath for 30 min with occasional shaking. The cells were washed and resuspended in 3 to 5 ml cRPMI depending on the final count of cell numbers. Cytolysed cellular debris form large fabric clumps were removed by passing the cell suspension through a 40 μm sterile nylon strainer (Falcon). Purified γδ T cells were then seeded and cultured for future use under the same culturing condition described above.

2.3.4 Purification of γδ T cells by positive selection

An alternative method was developed to purify γδ T cells from cultures that contain relatively high percentage (>15%) of NK cells. This was done by positive selection with CELLection Pan Mouse IgG kit (Dynal, Oslo, Norway) following manufacturer's instructions. Briefly, cells were collected, washed with PBS-1 (PBS containing 0.1% bovine serum albumin), and co-incubated with anti-TCR γδ for 30 min at 4 °C. Unbound antibodies were removed by washing with PBS-1. Cells were mixed with human anti-mouse IgG coated paramagnetic polystyrene Dynabeads at target to beads ratio of 1 to 5 and incubated for another 30 min at 4 °C with gentle tilting and rotation on a mixer. The sample was then placed in a magnetic particle concentrator for 3 to 6 min to allow the rosetted cells to attach to the tube wall. Supernatant that contains unbound cells was discarded. Pelleted beads were resuspended and washed with PBS-1 twice to remove residual unwanted cells before they were transferred to a new tube containing 200 μl cRPMI pre-warmed to 37 °C. γδ T cells were then released from the beads by enzymatic cleavage with 4 μl DNase supplied in the kit for 15 min at RT. Purified γδ T cells were incubated overnight in the same media. Cells were then checked by flow cytometry using FITC-TCRγδ and PE-CD3 to verify over 95% purity.

2.3.5 Separation of $CD16^+$ and $CD16^-$ γδ T cells

Purified γδ T cells were further separated into two populations according to their CD16 expression. 15 x 10^6 cells were washed and resuspended in 100 μl PBS-1 in a 5 ml flow tube. 50 μl (0.1 mg/ml) of FITC-CD16 (LNK16, Serotec, Oxford, UK) were added to the cells, which were subsequently kept at 4 °C in the dark for 30 min. Cells were washed with

PBS-1 to remove unbound antibodies and resuspended in 1 ml sterile FACS buffer (PBS containing 1% BSA and 0.1% sodium azide). The tube was loaded to a high speed MoFlo cell sorter (Dako cytomation, Denmark) and $CD16^+$ and $CD16^-$ populations were separately directed into two 15 ml conical tubes containing 2 ml RPMI with 50% (v/v) human serum. The sample pressure was set at 31 psi. The sorting was monitored and analyzed with Summit software (Dako, Denmark).

Sorted cells were plated and cultured overnight as described above. The purity of the preparation was confirmed with flow cytometry by double-staining with PE-TCRγδ and FITC-CD16. Artificial CD16 gradients were achieved by mixing $CD16^-$ and $CD16^+$ populations at various proportions and confirmed by flow cytometry (see 3.6.5).

2.4 Flow cytometric analysis

2.4.1 Extracellular staining for flow cytometry

γδ T cell Fcγ receptor expression was studied on immediate *ex vivo* PBMCs isolated from each of the individuals or on long term *in vitro* cultured cells. For *ex vivo* study, 1 x 10^6 freshly isolated PBMCs were distributed into several Eppendoff tubes and washed once with FACS buffer (5% BSA in PBS with 0.1% (w/v) sodium azide). Cells in the first tube were not stained with any mAb and were used as autofluorescence control. Cells in the other tubes were stained with FITC-TCRγδ, PE-CD16 (or -CD32, -CD64), and PC5-CD3, or iso-type controls respectively, and a cocktail of various conbitnations of the above mAbs as indicated. The cells were kept on ice for 30 min before they were washed and suspended in 500 μl FACS buffer for analysis. Flow cytometry was carried out on a Coulter FC500 flow

cytometer (Beckman Coulter) equipped with one Argon laser. The first tubes with single fluorochroms staining were used to set up the voltage and compensation values for each photomultiplier channel. Typically 2 x 10^5 events were acquired for the multiple staining tubes. Data were analyzed with Coulter CXP software.

For all the washing steps, cells are centrifuged at 2500 rpm for 4 min on a desktop centrifuge (Eppendorf, Hamburg, Germany). The supernatant was then decanted gently.

CD16 expression on *in vitro* cultured γδ T cells was measured in the same way as described above.

2.4.2 Intracytoplasmic staining for cytokine production assay

Cytokine production stimulated by CD16 engagement on γδ T cells was tested with an intracytoplasmic flow cytometry assay. 4 hr before the procedure of intracellular staining, PMA/Ionomycin at the final concentrations of 10 ng/ml and 0.2 μg/ml (Both from Sigma) respectively were added to the positive control wells, and intracellular protein transport inhibitor Monensin (3 μM, Sigma) was added to all the wells.

Cells were collected into Eppendorf tubes at the end of culturing and washed with FACS buffer. Surface staining with FITC-TCRγδ and PC7-CD16 was performed as described above. After washing off unbound antibodies with FACS buffer, cells were fixed and permeablized in 250 μl fixing/permeablization buffer (4% paraformaldehyde and 0.1% saponin in PBS, Sigma) at RT for 25 min in the dark. Fixed cells were washed twice with intracellular washing buffer (1% BSA, 0.1% saponin, and 0.1% NaN_3 in PBS). 100 μl of blocking buffer (5% skim milk, nestle, in washing buffer) was added to the tubes, which were kept in the dark at RT for 10 min to block any unspecific intracytoplasmic bindings.

Cells were washed again and divided equally into four Eppendorf tubes. 2.5 μl of PE conjugated anti-INF-γ, TNF-α, IL-4, or IL-10 antibodies and isotype controls were added to each individual tube, which were then incubated on ice for 30 min. After washing another time with intracellular washing buffer the cells were resuspended in FACS buffer and analyzed on the FC500 flow cytometer.

2.5 Cytokine responsive assay

Purified γδ T cells were harvested, washed, and re-plated for another 24 hours without IL-2. Cells were then seeded in U-bottomed 96-well Petri-dish at 2×10^5 cells per well. Interleukins-2, -12, and -15 were then added to the wells at various concentrations as indicated. cRPMI was added to each well to fill them up to 200 μl. The cells were cultured at 37 °C with 5% CO_2 for 72 hours before their surface expression of CD16 was measured with flow cytometry as described above.

2.6 Intracellular cytokine examination upon CD16 engagement

Purified γδ T cells were transferred to 96-well U-bottomed culture plate (some wells were pre-treated as described below) in duplicate at the density of 2×10^5 cells per well in total of 200 μl cRPMI. Cells were then cultured under various stimulation conditions detailed below for a period of 18 hr before they were subjected to intracellular staining.

The wells assigned for the purpose of cross-linking TCR were first coated with goat anti mouse IgG (5 μg/ml in PBS) for 1 hr at 37 °C. Wells were washed with PBS to remove unbound antibodies before anti-TCRγδ antibody was added to the wells and incubated for 30

min at 37 °C. Unbound antibodies were removed by washing and the wells were ready to receive the responder γδ T cells.

The wells assigned for cross-linking of CD16 were treated the same as above, only that anti-TCRγδ antibody was substituted with mouse anti-human CD16 antibody.

For engagement of CD16 with immune complex, Raji cells were first coated with $F(ab')_2$, rituximab, or remained uncoated. These stimulant cells were then washed and fixed with 4% paraformaldehyde for 30 min at RT. After fixation, they were washed two more times and transferred to corresponding wells that contained γδ T cells. For comparison, $F(ab')_2$ or rituximab alone was used as negative controls.

2.7 Target cell lines

Burkett's B cell lymphoma Daudi and Raji cells were used as target cells in FC_3A assay. They are both CD19 and CD20 positive as tested by flow cytometry using PE-CD19 or PE-CD20 mAbs. Cells were cultured in cRPMI-1640 medium and passaged regularly according to ATCC's recommendations. Only cells with over 95% viability (tested by Trypan Blue exclusion) were used in experiments.

2.8 Generation of $F(ab')_2$ fragment from rituximab

2.8.1 Preparation of $F(ab')_2$ fragment

$F(ab')_2$ fragment of rituximab was generated by using a commercial kit - ImmunoPure $F(ab')_2$ Preparation Kit (Peirce, IL) - following manufacturer's instructions. Briefly, 1.0 ml of rituximab (10 mg/ml, gift from Genentech, CA) was transferred to dialysis tubing and

dialyzed against Digestion buffer (20 mM Sodium Acetate, pH 4.5) over night at 4 °C. Rituximab was mixed with Immobilized Pepsin and the mixture shaken in a temperature controlled shaker at 37 °C for 18hrs. The crude digest was separated from the Pepsin by using a resin separator, passed through an Immobilized Protein A Column. The column was washed with Binding buffer and the flow-through fraction collected, containing the $F(ab')_2$ fragments. Small Fc debris contaminant was removed with a Microsep Centrifugal Device (OD0304C, Pall, Mississauga, ON) and the concentration of final $F(ab')_2$ preparation was determined by spectrometry with Bio-Rad Protein Assay Dye Reagent (Bio-Rad, CA).

2.8.2 Test for purity of $F(ab')_2$ fragment

The relative purity of the $F(ab')_2$ preparation was determined by "dot blot" assay coupled with densitometry. Two pieces of nitrocellulose membrane were cut out at the size of 2 x 4 cm. On each of the membranes, one dot (2 µl) of rituximab (10 µg/ml) and one dot of $F(ab')_2$ (10 µg/ml) were placed. The membranes were submerged into blocking buffer (5% skim milk, 0.1% Tween 20 in PBS) and shaken for 30 min at RT on an orbiter shaker (Bellco Biotech, NJ). Blocking buffer was discarded and the membranes were rinsed with washing buffer (0.1% Tween 20 in PBS). The two membranes were placed into two separate Petri dishes, one containing primary antibody against Fc portion of human IgG and the other against Fab domain (both 1:100,000 dilution in blocking buffer). After allowing the reaction to last 30 min to 1 hr the membranes were washed twice on the shaker, 20 min each time. A secondary detection antibody rabbit anti-mouse IgG (100,000 dilution in blocking buffer) was added to both dishes, which were then shaken for another hour. After repeating the

washing twice, equal volumes of the two ECL (enhanced chemiluminescent) substrates (A: 4 μM p-Coumaric Acid and 0.25 μM Luminol in 0.1M Tris/NCl, pH 8.5; B: 0.4% H_2O_2 in 0.1M Tris/HCl, pH 8.5) were mixed immediately before applying to the membranes. The fluoreluminescent images on the membranes were collected by a cooled CCD camera (HD2, Alpha Innotech, CA). Densitometry analysis was done with AlphaEaseFC software (Alpha Innotech).

The purity of $F(ab')_2$ preparation was further confirmed with western blot and flow cytometry. For the western blot assay, rituximab or the $F(ab')_2$ preparation was first resolved with SDS-PAGE and then transferred to a nitrocellulose membrane as per standard procedure. The membrane was sequentially probed with a primary mouse anti-human IgG (Fc specific) antibody and a secondary HRP-conjugated anti-mouse antibody, following the procedure similar to that of the dot blot assay described above. The image of the protein bands was finally exposed and captured on a photographic film.

For the flow cytometry assay, rituximab and $F(ab')_2$ preparation were used to stain Raji cells, which were then examined with PE conjugated antibody against either Fc portion or Fab portion of human IgG.

2.9 ELISA-based ADCC assay

γδ T cells ADCC was determined by using Rh_o^+ red blood cells as targets and human anti-Rh_o IgG as bridging antibody. Briefly, target cells were labeled with human polyclonal anti-Rh_o antibody before they were co-incubated with purified γδ T cells. Following co-culturing, cell mixtures were spun down and the supernatant was analyzed with ELISA for hemoglobin released from cytolysed red cells.

2.9.1 Labeling target cells with antibody

Rh_o^+ blood was obtained from the Blood Bank at the Ottawa Hospital General Campus. 10 ml of whole blood was centrifuged at 300g for 15 min. The plasma and buffy coat (containing leukocytes) were carefully removed from the top of the pact red blood cells. The erythrocytes were resuspended in PBS and spun down again for washing. 2×10^6 cells were transferred to an Eppendoff tube in 100 µl of PBS. Polyclonal human anti-Rh_o IgG (Cangene, Winnipeg, MB) was added to the tube at the final concentration of 20 µg/ml. The cells were incubated at 4 °C for 30 min with occasional shaking. Following incubation, cells were washed twice with PBS to remove unbound antibodies. After the last wash, the supernatant was carefully removed and the cells were resuspended in 1 ml of cRPMI. The efficacy of antibody binding was confirmed with flow cytometry by using a secondary PE-conjugated goat anti-human IgG antibody.

2.9.2 Co-culturing of γδ T cells with antibody coated erythrocytes

Antibody coated and un-coated erythrocytes were seeded in 96-well U-bottomed plate in duplicates at a constant number of 1×10^5 cells/well. Purified γδ T cells were added to the wells at various E:T ratios as indicated. The total volume of each well was brought up to 200 µl with cRPMI. The plate was placed in a 37 °C, 5% CO_2 incubator for 3 hrs. After incubation, the plate was gently centrifuged at 100g for 2 min. 150 µl of supernatant from each well was carefully aspirated and transferred to an Eppendoff tube, and stored for ELISA test. Erythrocytes that were completely lysed by the addition of lysing solution (BD

Bioscience) were used as positive control and the absorbance readings from these wells were used in the calculation of specific lysis as detailed in 2.9.3.

2.9.3 Measurement of hemoglobin by ELISA

Hemoglobin released from ruptured erythrocytes was measured with a commercial Human Hemoglobin ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, TX). 96-well C bottomed Immunoplate was coated with capture antibody at 1:100 dilution in Coating Buffer (0.05M Carbonate-Bicarbonate, pH 9.6) for 60 min at RT. After incubation, capture antibody solutions were removed from each well, which were subsequently washed with Washing Solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) for 3 times. The remaining active binding plastic surface of the wells was blocked with Blocking Solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) for 30 min at RT and washed three times. 100 µl of each sample were transferred to assigned wells and the plate was incubated for 60 min at RT. After incubation, samples were removed and the plate washed for five times. 1:100,000 diluted HRP-conjugated detection antibody was then added to each well for incubation for 60 min. Wells were washed five times. Equal volumes of the two-substrate reagents (TMB and hydrogen peroxide) were mixed and 100 µl of this substrate solution was transferred to each well. The plate was protected from light and kept for incubation for 10 to 20 min at RT. Enzymatic reaction was then stopped by the addition of 100 µl of 2 M H_2SO_4 to each well. The plate was detected with a microtiter plate reader (Multiskan Ascent, Thermo Labsystems, MA) at the wavelength of 450 nm. Specific lysis was calculated as:

$$\text{Specific lysis \%} = \frac{A_{450}\text{ sample lysis} - A_{450}\text{ blank}}{A_{450}\text{ complete lysis} - A_{450}\text{ blank}} \text{ X } 100$$

2.10 Flow cytometric cellular cytotoxicity assay

An in-house developed flow cytometry based cytotoxicity assay was established with an "all human" components to check the ADCC capability of human γδ T cells *in vitro,* which was named FC_3A (flow cytometric cellular cytotoxicity assay). Briefly, target lymphoma cells Daudi or Raji were labeled with a humanized anti-CD20 mAb, rituximab, before they were co-incubated with purified γδ T cells. Following co-culturing, cell mixtures were stained with a cocktail of PE-CD19 mAb and 7-AAD (or another combination of FITC-CD19 and PI) to distinguish targets from effectors and analyzed for specific lyses of target cells caused by $CD16^+$ γδ T cells.

2.10.1 Labeling target cells with rituximab

1 x 10^6 viable target cells were transferred to a sterile flow tube, washed with PBS-1, and resuspended in 90 μl of PBS-1. 10 μl of serial diluted rituximab was added to the cells to reach various final concentrations as indicated. The same volume of human IgG (100 μg/ml, Sigma, Saint Louis, MI) or buffer alone were also used as negative controls. Where mentioned, $F(ab')_2$ fragment of rituximab (10 μg/ml) was also used to label the target cells. Cells were incubated at 4 °C for 30 min with occasional shaking. Following incubation, cells were washed twice with PBS-1 to remove unbound antibodies. After the last wash, the supernatant was carefully removed and the cells were resuspended in 1 ml of cRPMI. The efficacy of rituximab binding was confirmed with flow cytometry by using a secondary PE-conjugated goat anti-human IgG antibody.

2.10.2 Co-culturing of γδ T cells and rituximab coated target cells

Rituximab coated Daudi or Raji cells were seeded in a 96-well U bottomed plate in duplicates at a constant count of 2×10^4 cells/well. Purified γδ T cells were added to the wells at various E:T ratios as indicated. The total volume of each well was brought up to 200 µl with cRPMI. The plate was placed in a 37 °C, 5% CO_2 incubator for either 4 hr or 18 hr. Target cells that were not labeled with rituximab were cultured in parallel in the absence of γδ T cells to measure spontaneous cell death.

2.10.3 Flow cytometric analysis of ADCC

After co-culturing cell mixtures from each well were collected into a 1.5 ml Eppendorf tube, washed with PBS-1, and resuspended in 100 µl of the same buffer. A cocktail of PE-CD19 and 7-AAD (3 µl of each from stock) was added to the tubes, which were kept at 4 °C in the dark for 30 min. Samples were then washed and resuspended in 250 µl of FACS buffer and transferred to flow tubes.

Acquisition was performed on the FC500 flow cytometer. The emissions of PE and 7-AAD fluorescence were redirected through specific band-pass optical filters (575 nm for PE and 675 nm for 7-AAD) and detected in channels FL-2 and FL-4 respectively. Proper compensations for fluorescence overlap between these two detectors were adjusted by running target cells stained with either single fluorescent dye. Cell mixtures were then loaded to the equipment and 5,000 target events from each tube were acquired. Data were analyzed with Coulter CXP software. Percent Specific lysis was defined as:

$$\text{Specific lysis \%} = \frac{\text{\% sample lysis - \% spontaneous lysis}}{\text{100 - \% spontaneous lysis}} \text{ X 100}$$

2.11 Statistical analysis

All data are presented as mean±SD. Differences between two groups were compared with paired or non-paired two-tailed *student's t* test, where appropriate. Differences between multiple groups (three or more) were evaluated with one-way ANOVA. If statistical significance was noted in ANOVA, *Tukey's* post-hoc test was applied for inter-group comparisons. Normal distribution of the variables was assessed with the D'Agostino and Pearson test. Disease duration and EDSS scores were not following a normal distribution, therefore non-parametric Spearman's rank correlation was used for the correlation analyses. In all assays, $p < 0.05$ was considered statistically significant. Statistical analyses or graph making were performed with Prism (GraphPad Inc., San Diego, CA), WinSTAT (A-Prompt Corp., PA, USA), or Kyplot (KyensLab Inc. Japan) software.

3. Results

3.1 Fcγ Receptor expression on γδ T cells

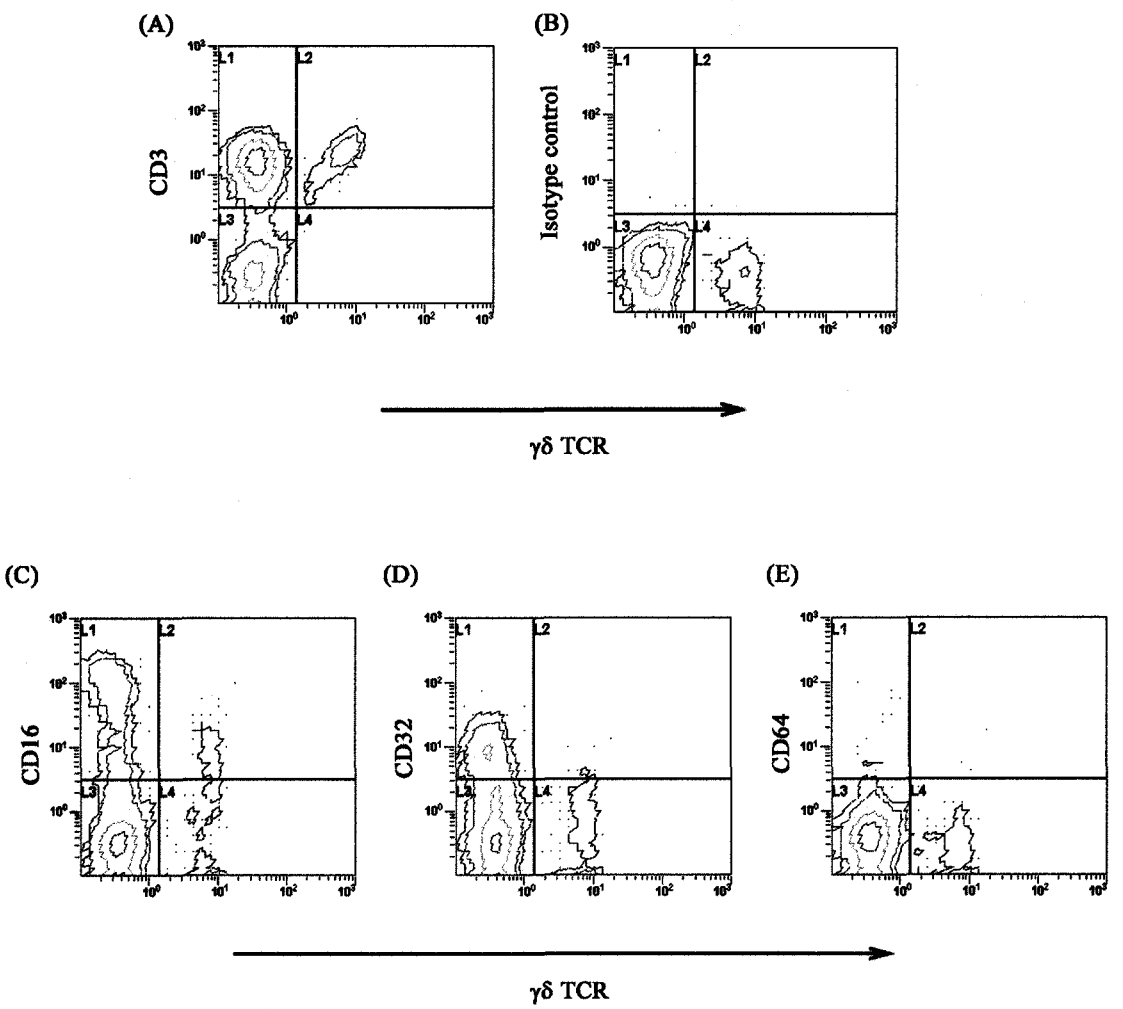
3.1.1 CD16 is the only Fcγ receptor detected on γδ T cells

PBMCs isolated from either MS patients or HCs were stained immediately *ex vivo* with PC5-CD3, FITC-TCRγδ, and one of the following PE conjugated mAbs directed to: CD64, CD32, or CD16. The cells were analyzed with flow cytometry for their expressions of these three Fcγ receptors in separate acquisitions respectively (Fig. 2). In comparison with isotype controls, no CD64 and CD32 could be detected on γδ T cells, irrespective they are cells originated from patients or HCs. That this is due to defects of staining is, however, not likely because using the same fluorochrome conjugated antibodies, CD32 can be detected on other lymphocytes (Fig. 2 D) and B cell lines (not shown), and CD64 on monocytes (not shown). In contrast to these two types of Fcγ receptors, CD16 could be seen constitutively expressed by γδ T cells derived from either MS patients or HCs, but the percentage of the $CD16^+$ cells varied greatly among individuals (Fig. 2). A general trend was observed that patients demonstrated higher percentages of $CD16^+$ cells than HCs; this observation was statistically confirmed in the next section below.

To exclude the possibility that long term culturing of γδ T cells in the presence of initial TCR stimulation in combination with exogenous cytokine IL-2 would cause the *de novo* expression of CD64 and CD32, the same analysis protocol was repeated on several γδ T cell cultures after they were expanded and purified *in vitro*. This confirmation is necessary

Figure 2. Fcγ receptor expression on γδ T cells *ex vivo* derived from patients or HCs.

Fresh PBMCs isolated from either MS patients or HCs were stained immediately with PC5-CD3, FITC-TCRγδ, and one of the following PE conjugated mAbs directed to: CD64, CD32, or CD16. γδ T cells were shown in upper right quadrant in A. In comparison with isotype controls (B), no CD64 (C) or CD32 (D) could be detected on γδ T cells, irrespective they are cells originated from patients or HCs. but CD16 could be seen constitutively expressed by γδ T cells derived from either MS patients or HCs (E), but the percentage of the $CD16^+$ cells varied greatly among individuals (see Fig 5). (Figure is representative of at least six experiments.)



because the presence of Fcγ receptors other than CD16 could gravely interfere with the interpretation of the results of the functional studies (sections 3.5 and 3.6). Similar to the *ex vivo* observation that γδ T cells do not express CD64 or CD32, Figure 3 confirmed that neither CD64 nor CD32 were detectable on γδ T cells by flow cytometry after the period of the culturing. The cells remained $CD16^+$ in all the cultures tested, though the percentages of $CD16^+$ γδ T cells changed when compared with *ex vivo* prepared γδ T cells.

Overall, γδ T cells selectively express CD16, the low affinity Fcγ receptor on their surfaces, but not the other two Fcγ receptors CD64 or CD32, as determined by flow cytometry. The expressions of CD16 were constitutive in all individuals studied, including both patients and HCs, but the percentages of positivity were considerably different and may change over time under the condition of culturing (see section 3.4).

3.1.2 Percentage of $CD16^+$ γδ T cells is elevated in patients compared with HCs

Could the observed differential expression of CD16 be related to the disease state of MS? To answer this question, a larger group of MS patients and HCs were recruited, as detailed in section 2.1. The percentage of CD16 expressing γδ T cells (%$CD16^+$) circulating in peripheral blood of these individuals was assessed on immediately *ex vivo* isolated cells using multiple parameter flow cytometry. γδ T cells were identified and gated by double positive staining of CD3 and TCRγδ. The gated cells were further analyzed for surface expression of CD16 and the data were expressed as percentage of CD16 positive γδ T cells over total γδ T cells (Fig. 4).

CIS tends to present in younger patients compared to those already with a confirmed diagnosis of MS ($p<0.05$ by *t*-test). In order to insure that the %$CD16^+$ is not just an

Figure 3. Fcγ receptor expression on γδ T cells cultured for an extended period in vitro.

Neither CD64 nor CD32 were detectable by flow cytometry after a period of culturing *in vitro*. However, the expression of CD16 remained in all the cultures tested, though the percentages of $CD16^+$ γδ T cells changed when compared with *ex vivo* prepared γδ T cells. (Figure is a representative of at least four experiments.)

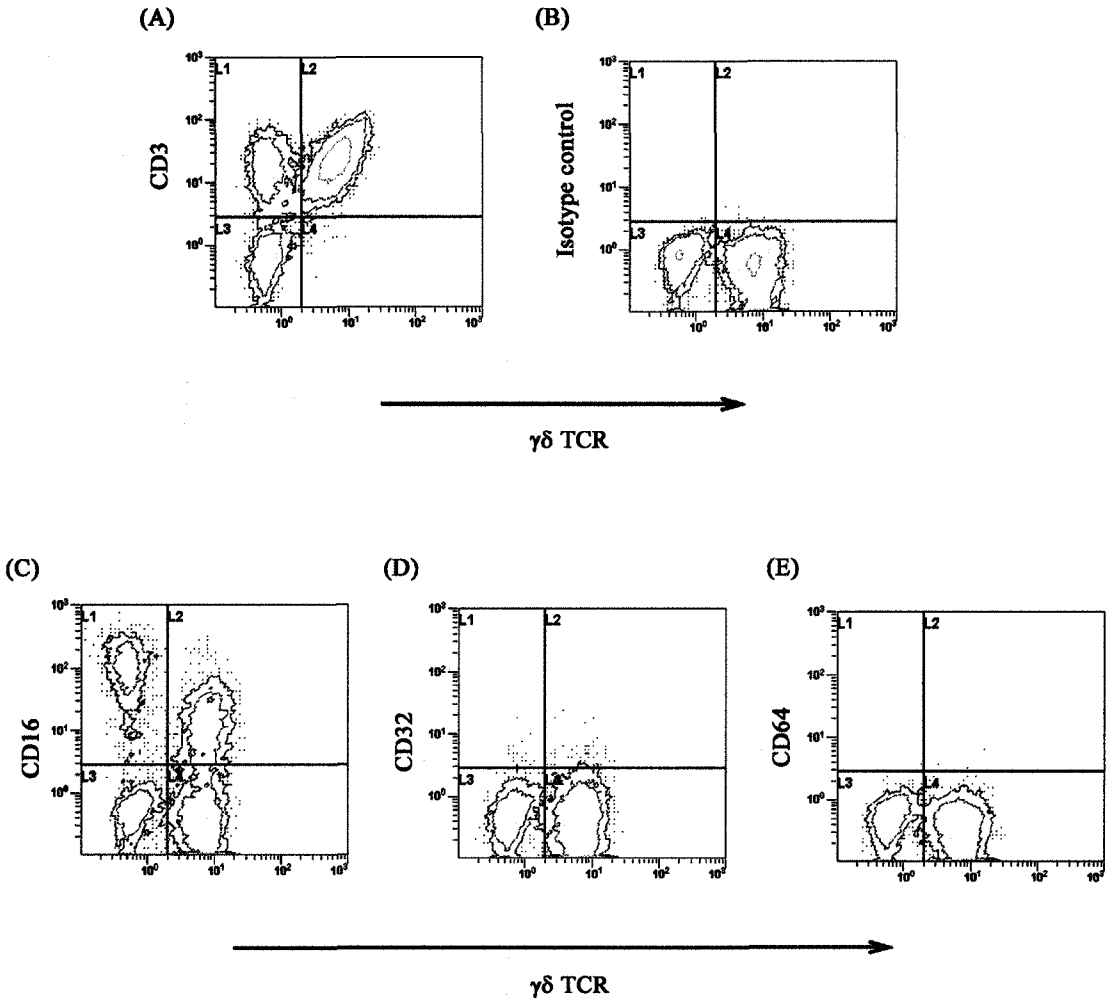
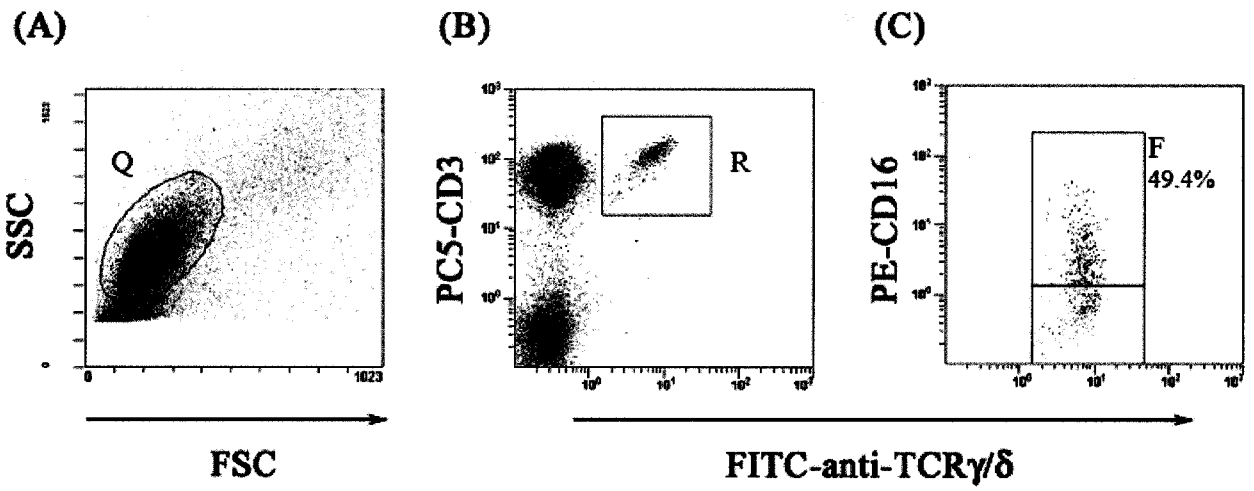


Figure 4. Representative flow cytometric analysis of percentage of $CD16^+$ γδ T cells derived from peripheral blood of MS patients and HCs.

PBMCs were stained with a mixture of FITC-TCRγδ, PE-CD16, and PC5-CD3. Lymphocytes were gated out by their light scatter properties (A); γδ T cells were further separated by positive staining of CD3 and TCRγδ (B). $CD16^+$ γδ T cells were gated in F and their percentages were calculated (C). $CD16^-$ gate was set with reference to PE-labelled isotype control antibody.



age-related phenomenon, the healthy controls were divided into two age groups with the younger groups (HC/Y) matching the age of CIS and the older (HC/O) matching the rest MS groups. The mean and SD of %$CD16^+$ were calculated for each of the groups of MS, CIS, HC/Y, and HC/O and the results were illustrated in figure 5. When compared with their respective age matched healthy controls, the percentage of $CD16^+$ γδ T cells was significantly elevated in patients diagnosed with MS (35.6±24.0% vs. 24.3±14.2% of HC/O, p=0.0429) and similar results were seen in CIS compared with HC/Y's (30.1±12.6% vs. 21.0±10.2%, p=0.0387) (Fig. 5).

Looking particularly within MS subgroups (PP-, RR-, and SP-MS) the variations of the means of %$CD16^+$ were compared across each subgroup as well as the HC/O using one-way ANOVA test. A significant difference (p=0.0132) was noticed among the groups (Fig. 6). Tukey's *post-hoc* inter-group comparison identified that it was the SPMS group that had significantly higher $CD16^+$ γδ T cells than the healthy controls, while the differences between other patient groups and the healthy controls did not reach statistical significance.

A cut-off value was defined as (mean %$CD16^+$+2SD) observed in the HC/O, which worked out to 52.8%; values higher than this were considered to be elevated. It was found that more SPMS patients had elevated %$CD16^+$, with 7 out of the 19 (36.8%) exceeding the upper limit. About 17.6% of PPMS patients were found with elevated percentage of $CD16^+$ γδ T cells; while only 1 in the 13 patients with RRMS (7.7%) had high %$CD16^+$ (Fig. 6).

Taken together, the %$CD16^+$ was increased in disease status when compared with age matched HCs. The elevated %$CD16^+$ was especially pronounced in patients with a secondary progressive course. Not only did this subgroup have a higher average % $CD16^+$, but also more patients in this group demonstrated higher %$CD16^+$ than the cut-off value.

Figure 5. CD16 expressing γδ T cells are elevated in patients compared with HCs.

Healthy controls were divided into two age groups, old and young (HC/O and HC/Y). The mean %$CD16^+$ was significantly increased in MS patients when compared with HC/O and in CIS compared with HC/Y. Box and whiskers indicated the median, 25^{th} percentile range, and highest/lowest values. The means were shown with a cross.

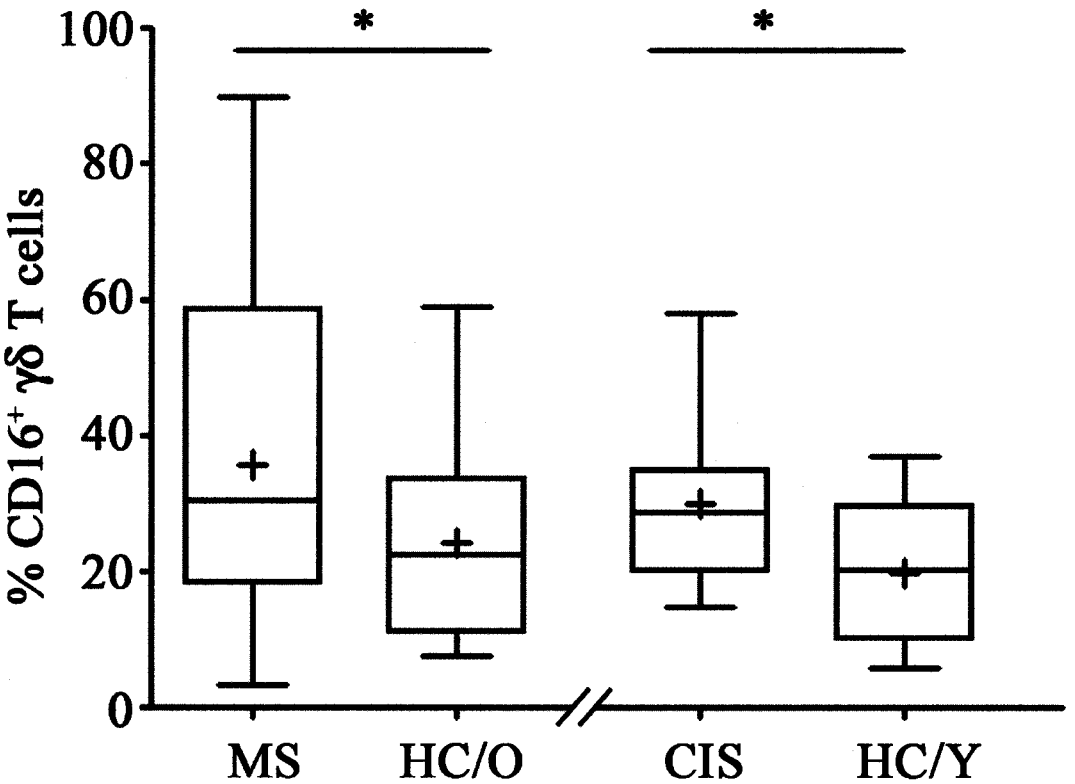
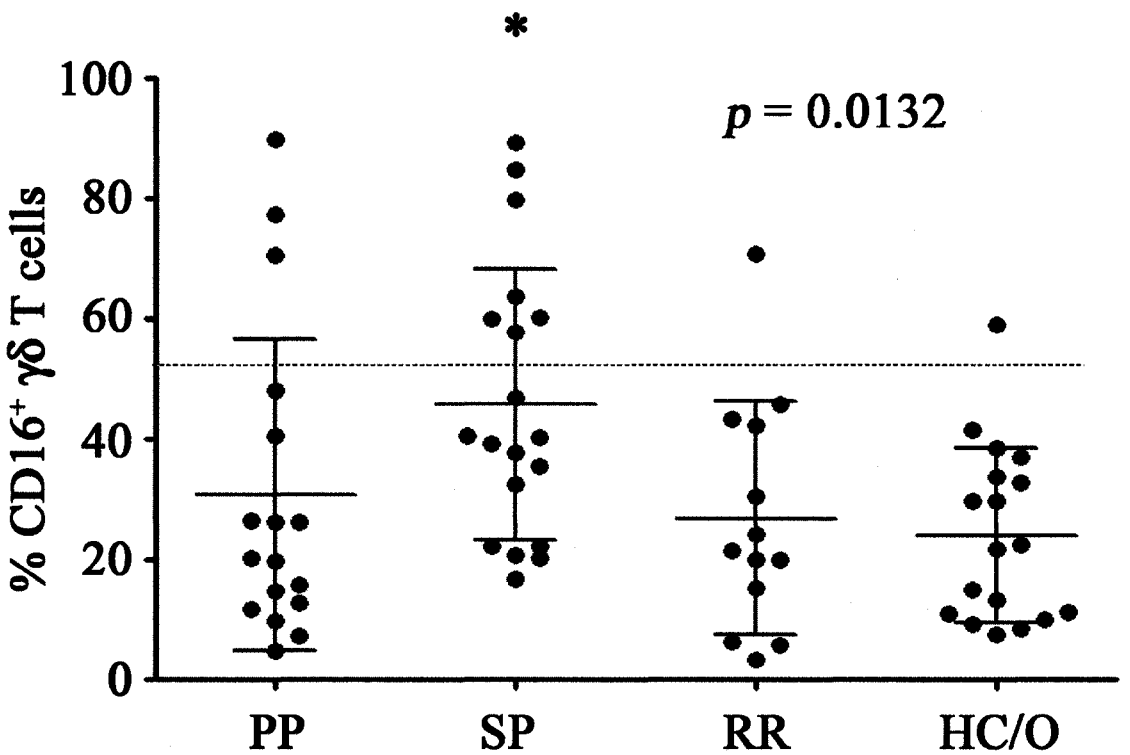


Figure 6. CD16 expression elevation is particularly pronounced in patients with progressive MS.

Means of %$CD16^+$ of the subgroups of MS were significantly different from that of the healthy controls (p=0.0132). SPMS patients had significantly higher %$CD16^+$ than the HC/Os. A cut-off value (dotted line) indicating the mean of the HC/O+2SD was shown, above which %$CD16^+$ was considered high. The SPMS group also had the highest percentage (36.8%) of high $CD16^+$ γδ T cells. The percentages of patients with high values in RRMS and PPMS were 7.7% and 17.6% respectively. Closed dots represented %$CD16^+$ value of each individual and the means and SD were shown with bars. * Denotes statistical significance.



3.2 %CD16$^+$ correlates with disease progression and disability

The relationship between the %CD16$^+$ and the clinical characteristics of the patients were next examined. Though a general trend could be noticed that older individuals showed higher %CD16$^+$, there was no correlation found between %CD16$^+$ and ages for HCs (p=0.196, Fig. 7). This was in line with the result of others who studied the relationship between %CD16$^+$ and ages in healthy subjects that included octogenarians (184). There was no significant correlation found for MS patients either (p=0.055), however this p value suggested a trend towards higher %CD16$^+$ with the age in MS patient group.

There was also no gender bias observed for CD16 expression on γδ T cells derived from MS patients (p=0.56 by t-test) (Fig. 8).

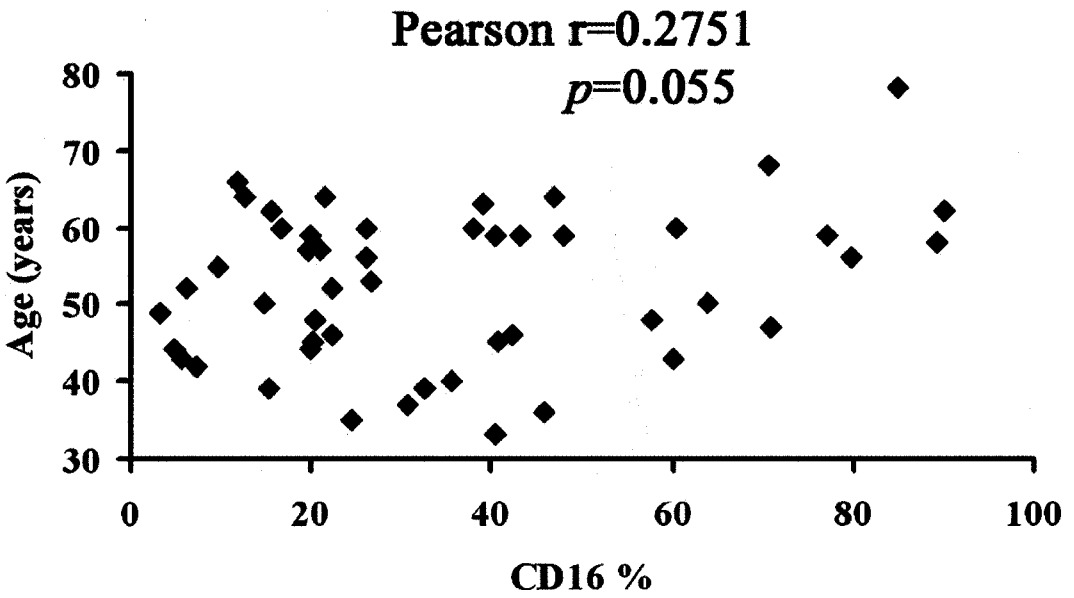
However, a significant correlation was noted between %CD16$^+$ and either disability (EDSS) (r=0.31, p=0.031) or disease duration (r=0.40, p=0.0042) when calculated with Spearman's test (Fig. 9 A and B). These correlations remained true when patients were subdivided into four groups (four quadrants in Fig. 10) according to their disease duration and EDSS scores: patients with short disease duration (≤10 years) and low EDSS (≤3.5) had the lowest average %CD16$^+$ (25.5±15.5%); patients with both long duration (>10 years) and high EDSS (>3.5) showed the highest percentage of CD16 (45.6±26.4%); whilst the other two groups with different combinations (i.e. short disease duration/high EDSS or long disease duration/low EDSS) demonstrated %CD16$^+$ in between (26.7±18.8% and 27.9±21.6% respectively) (Fig. 10).

In conclusion, %CD16$^+$ was increased in patients with longer natural history of disease, as well as those who had greater disability. But this elevated presence of CD16$^+$ γδ T cells in patients' circulation is not merely due to older age, nor is it related to gender.

Figure 7. Relationship between %$CD16^+$ and patients' ages.

There was no correlation found between %$CD16^+$ and ages of MS patients (p=0.055, A), nor those of HCs (p=0.196 by Pearson test, B), however the p value for MS does suggest a trend towards higher %$CD16^+$ with age.

(A)



(B)

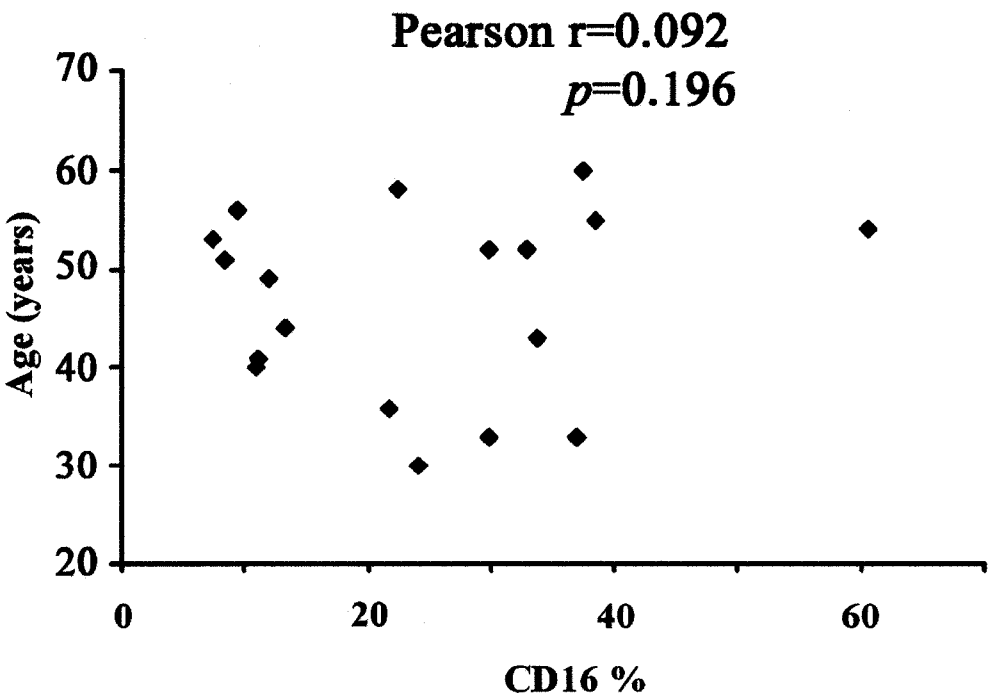


Figure 8. Relationship between %CD16^{+} and patients' gender.

No gender bias was observed for CD16 expression on γδ T cells derived from MS patients (p=0.56 by t-test). Total n=49, male= 21; female= 28.

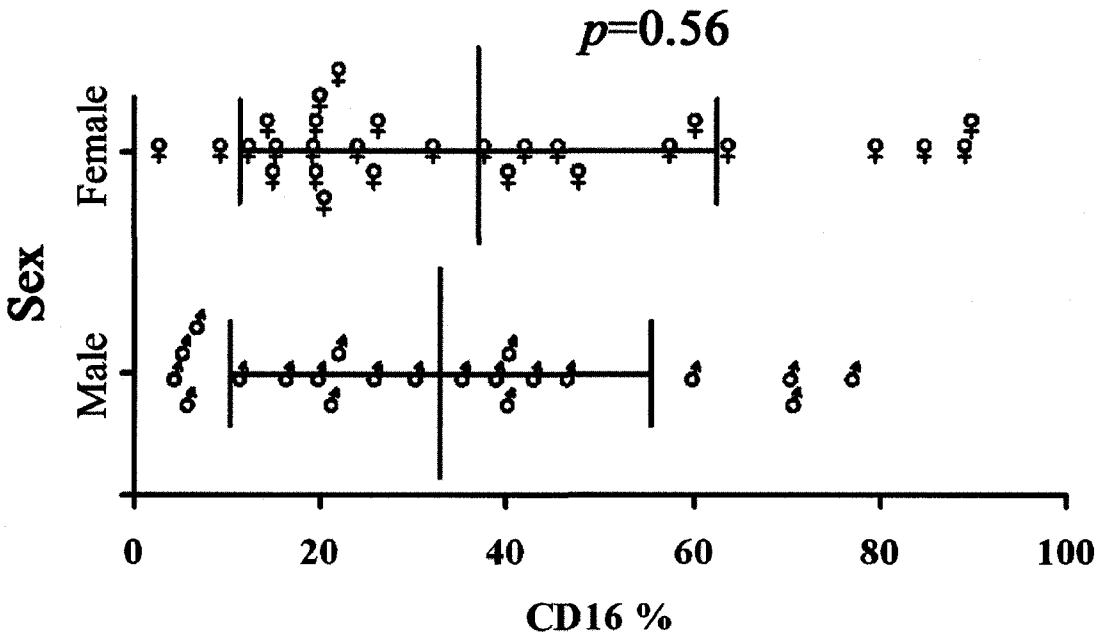
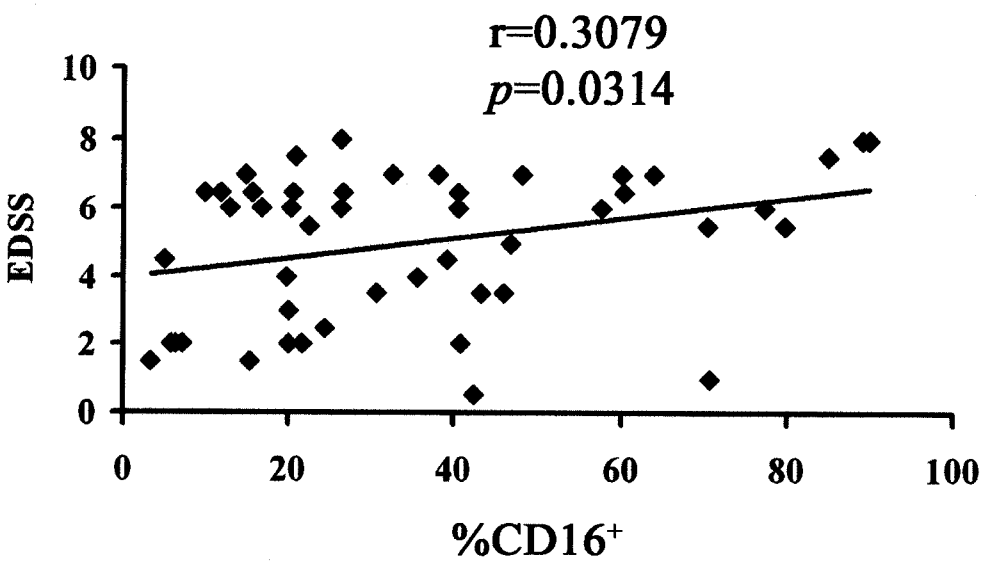


Figure 9. Relationship of %CD16$^+$ to patient clinical characteristics.

%CD16$^+$ correlated significantly with both disease severity (expressed by EDSS, in A) and disease duration (years since onset of MS, in B). Total n=49, $p<0.05$, Spearman correlation test.

(A)



(B)

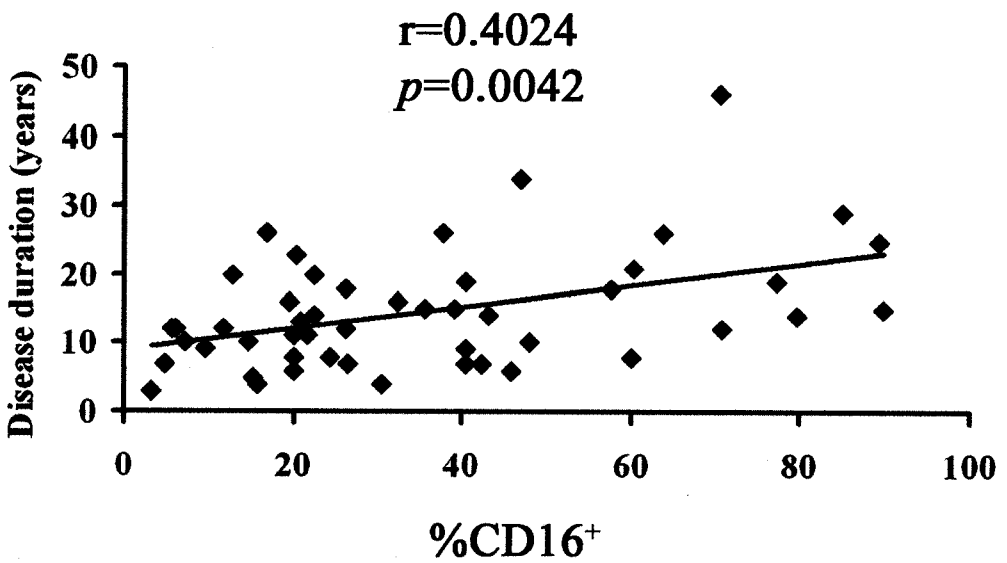
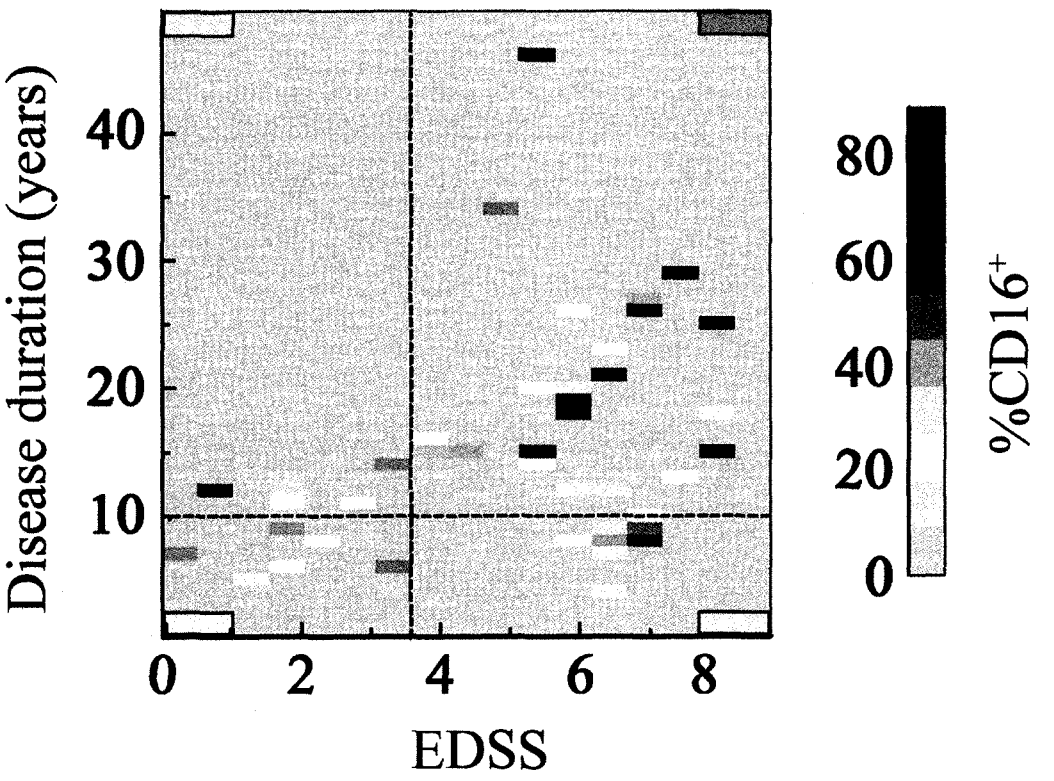


Figure 10. %CD16$^+$ corresponds to disease duration and EDSS.

MS patients were subdivided into 4 quadrants according to their disease duration (separated by 10 years) and EDSS scores (separated by 3.5 points), indicated by the dotted lines. Each rectangle represented one patient and was coloured based on their %CD16$^+$ value converted against the colour map on the right. The mean %CD16$^+$ of each quadrant was shown at the corners in a bigger closed rectangle.



3.3 Expansion and purification of γδ T cells from PBMCs

γδ T cells only account for a very limited number of the peripheral blood lymphocytes, which poses a hindrance for further functional analysis of this population and therefore demands a procedure of expanding them *in vitro*. Such a protocol has been developed in our lab and was used throughout, as described in section 2.3.2. PBMCs isolated from either MS patients or HCs were seeded into anti-TCRγδ pre-coated plates and cultured for a period of 7 to 10 days. Plate bound anti-TCRγδ antibody, functioned as a mitogen, stimulated multiple-clonal proliferation of γδ T cells in the mixed culture, but not other types of cells. Clumps of proliferating γδ T cells can be easily observed with a light microscope, or even by gross examinations with unaided eyes. For most of the cultures thus generated, γδ T cells could increase their amount to about 40 to 70 percent of the total cells at the end of the culturing period, compared to only 1 to 5 percent in PBMCs at the beginning (Fig. 11).

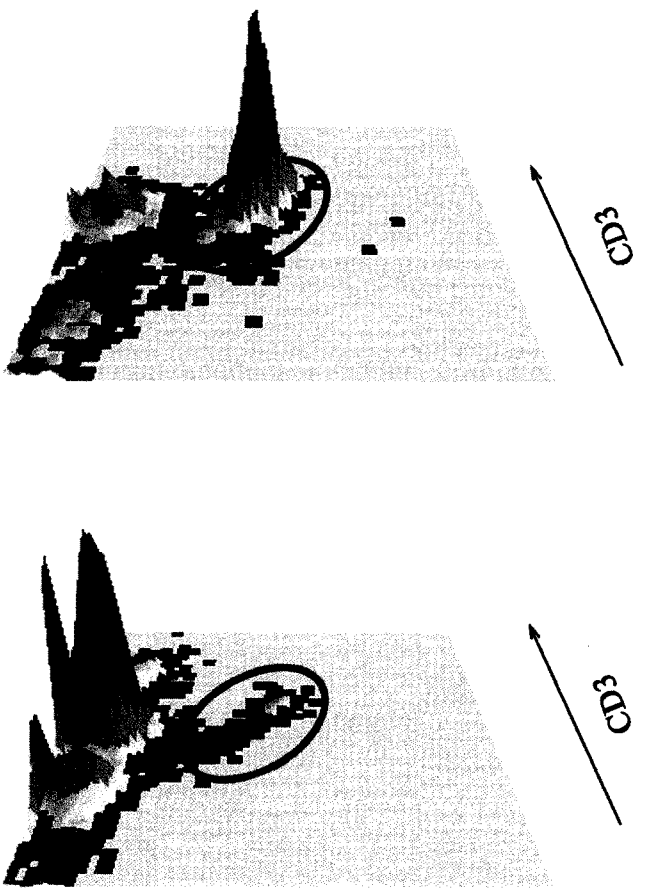
The contaminating populations are predominantly αβ T cell, together with small amount of monocytes or NK cells. Monocytes could be easily removed, taking advantage of their adherent property, by simply transferring the non-adherent cells into a fresh plate. To further eliminate the other contaminating cells and enrich the γδ T cell in culture, some specific procedures were needed, as described in details below.

3.3.1 Purification of γδ T cells by C'-lysis

γδ T cells expanded *in vitro* were further purified by complement (C') lysis to remove contaminating cells. Fresh prepared rabbit complements could be activated by IgGs fixed on

Figure 11. Enrichment of γδ T cells *in vitro*.

In vitro expanded γδ T cells could increase their amount to about 40 to 70% of the total cells at the end of the culturing period (upper topograph), compared to only 1 to 5% in PBMCs at the beginning (lower topograph). Areas of interests that correspond to γδ T cells were circled in black. Figure is a representative of a typical culture.



target (αβ T) cells. A subsequent cascade of enzymatic reactions of C' results in the ultimate assemble of many membrane-penetrating pores, which cause the breakdown of the targeted cells (185). Because the attempts of using a panel of anti-TCRαβ mAb in combination with C' were unsuccessful and resulted in unwanted stimulation and expansion of residual αβ T cells, a pair of surrogate antibodies that specifically targeted at CD4 or CD8 antigens were used. This C' mediated purging of $CD4^+$ or $CD8^+$ αβ T cells from the culture normally leads to improved purity of γδ T cells to about 90 percent of the total cell population, as illustrated in figure 12. Occasionally a second round of C'-lysis would be performed should the purity failed to reach this desired level.

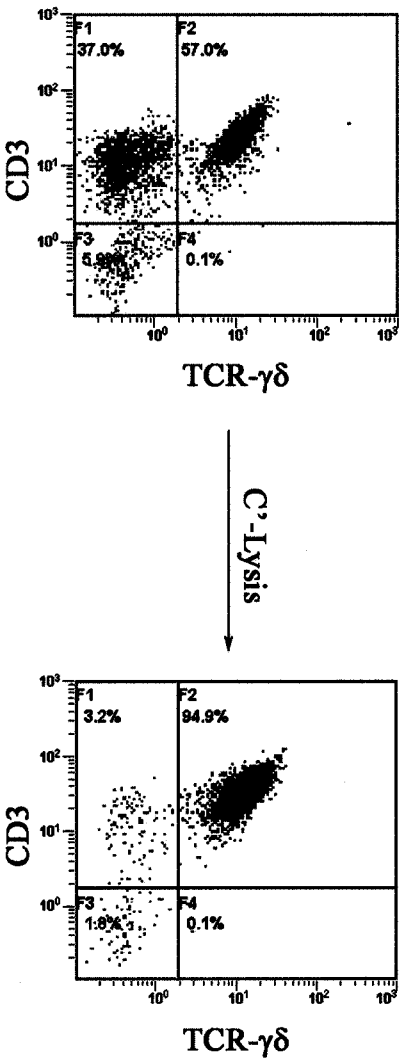
3.3.2 Purification of γδ T cells by positive selection

Alternatively, in some situations where NK cells are an obvious contaminant in the culture, a positive selection (Dynal CELLection) method would be preferred. This is considered crucial because it is known that the majority of NK cells are CD16 positive, and this contaminating population could falsely increase the readout of ADCC should they be indiscreetly used as effector cells together with γδ T cells.

During this positive selection process, only cells that are $TCR\gamma\delta^+$ would be specifically captured by the magnetic beads, which has been attached to the anti-TCRγδ antibody, and thus isolated from the culture. Cells of types other than γδ T cells, i.e. αβ T or NK cells, would be left behind untouched. Constant and gentle rotation of the tube that keeps the beads in suspension is critical for optimal capturing, because Dynabead has a considerably large size and heavy density, and tends to settle easily thus reduces the chance of capturing

Figure 12. Purification of γδ T cells by C'-lysis

Comparison of purity of γδ T cells as percentage of total cells before (top) and after (bottom) C'-lysis. It could be seen that 90% purity was achievable by this method.



the target cells. Optimized beads to target cell ratio such as 5 to 1 used in this study is also important in increasing the efficiency of bead-cell interaction.

Figure 13 depicts the typical results of positive selection with CELLection beads. This method has an edge over C'-lysis in that it shows improved specificity and purity. However this improvement may be at the expense of a decreased yield, about 20 to30 percent of the γδ T cells population were lost during the handling, possibly due to the weak linkage between cells and the beads, and multiple steps of the procedure as well.

Conclusively, *in vitro* expansion of γδ T cells could selectively expand their population from approximately 1 to 1.5 x 10^6 in PBMC to about 30 to 40 x 10^6 in the culture, which makes the following functional studies more feasible. Both purification techniques, C'-lysis or beads selection, could achieve γδ T cell purities over 90% or higher, which is believed to be sufficient for further functional studies.

3.3.3 FACS sorting of $CD16^+$ and $CD16^-$ γδ T cells

Purified γδ T cells were stained with FITC-CD16 and sorted on MoFlo flow cytometer to separate $CD16^+$ and $CD16^-$ populations. Unlike NK cells, a dichotomous distribution of CD16 has never been observed in cultured γδ T cells. CD16 expression on cultured γδ T cells shows a continuum pattern and the positive population normally express low levels of CD16, demonstrated by the dim signal on the flow cytometry plots (Fig. 14 C or D). In contrast to this low level expression, a bright CD16 population could also sometimes be observed. This population is presumed to be NK cells, because they were in fact CD3 negative (see Fig. 13). Keeping this in mind, two gates were placed on the dot plot and histogram in order to achieve the cell sorting (Fig. 14C; D): Gate 1 (R3), circumscribed the

Figure 13. Purification of γδ T cells by magnetic selection.

Comparison of purity of γδ T cells as percentage of total cells before (top) and after (bottom) positive magnetic selection. γδ T cell could increase their amount to about 40 to 70 percent of the total cells at the end of the culturing (A), but in some cultures $CD16^{+}CD3^{-}$ NK cells is a contaminating population (B). After magnetic selection, >95% purity can be achieved by this method (C), and importantly, NK cells could almost be eliminated (D).

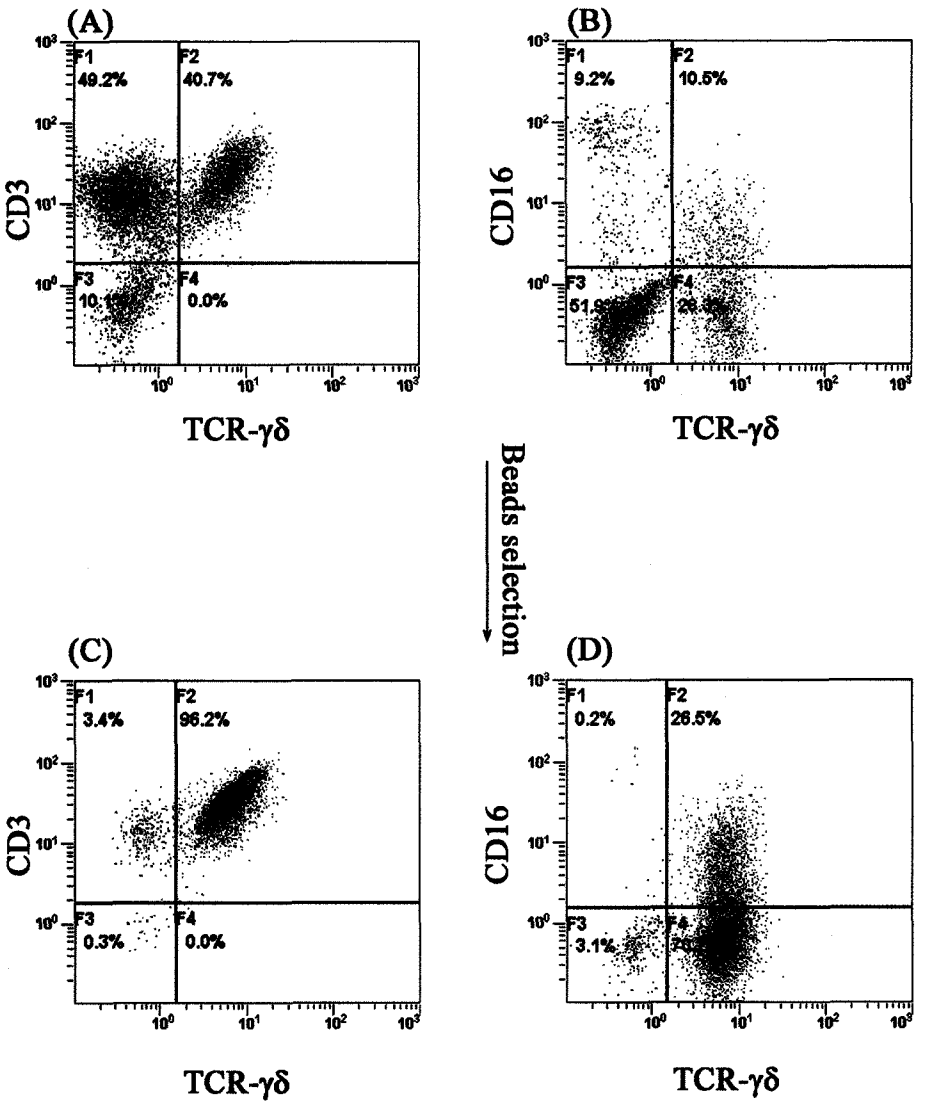
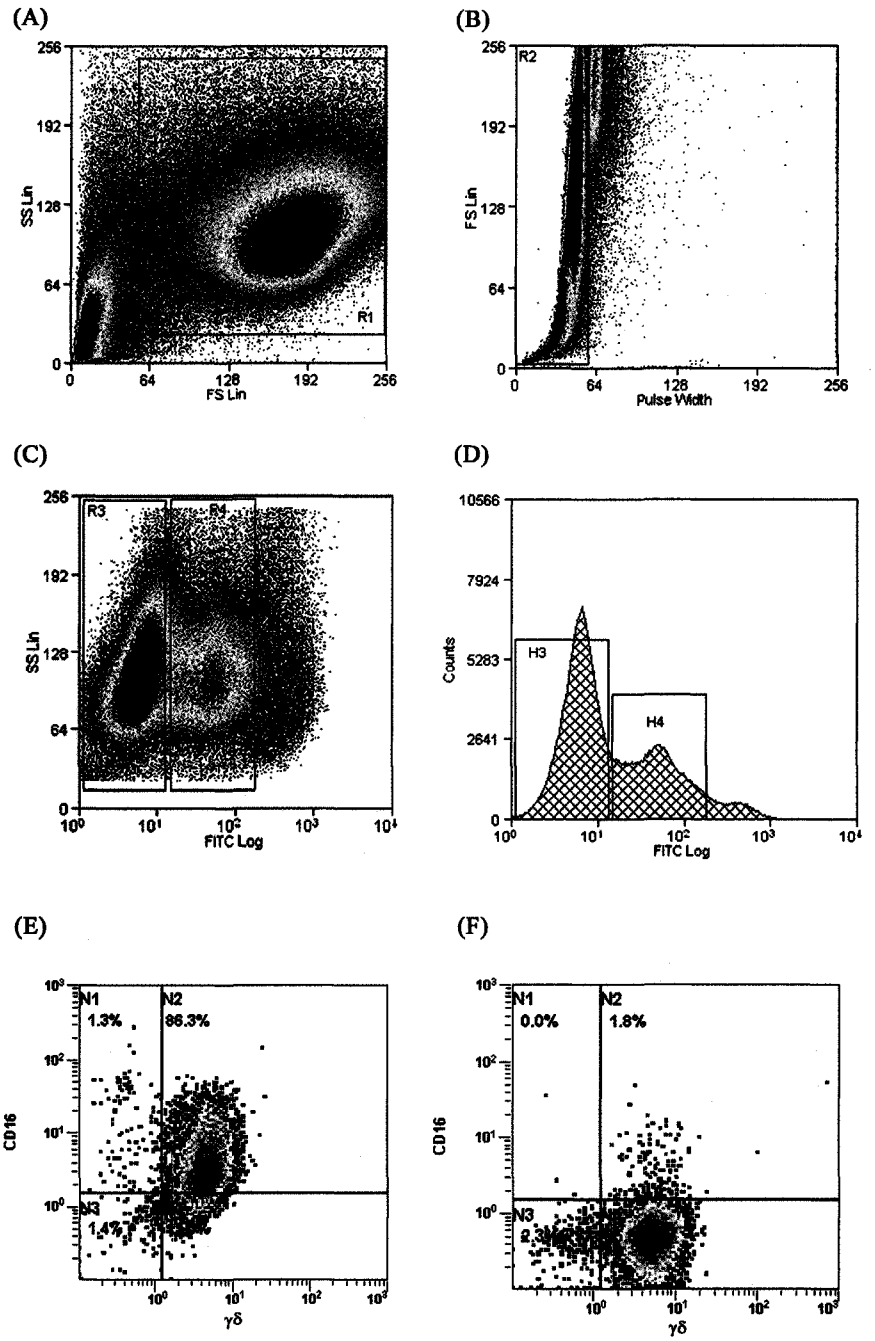


Figure 14. $CD16^+$ and $CD16^-$ γδ T cells were sorted by MoFlo high speed cytometer.

Live (R1 in A) and singlet (R2 in B) purified γδ T cells were chosen to sort CD16 positive or negative populations. CD16 expression on cultured γδ T cells shows a continuum pattern and the positive population normally express low levels of CD16, demonstrated by the dim signal on the flow cytometry plots (C and D) Therefore, two gates were placed on the dot plot or histogram for the cell sorting: Gate 1 (R3 on C or H3 on D), circumscribing the population in the negative field, was used to isolate the $CD16^-$ γδ T cells; while gate 2, set above the negative gate and enclosing the homogenous dim population (R4 on C or H4 on D), was used to collect the $CD16^+$ γδ T cells. Cells that located outside of these two gates were discarded. Purities of positively (E) and negatively (F) selected cells were shown. A-D were acquired on MoFlo and analyzed with DAKO Summit software and E, F were acquired on FC500 and analysed with CXP software.



population in the negative field, was used to isolate the $CD16^-$ γδ T cells; while gate 2 (R4), set in between the negative and bright populations detected in channel FL-1, was used to collect the $CD16^+$ γδ T cells. Cells that located outside of these two gates were discarded.

Thirty one (31) psi of sheath pressure normally yields a sorting speed at 10×10^3 events per second. Higher pressure reduces the duration of sorting, but resulted in poor viability of isolated cells.

FITC-CD16 was chosen to label the purified γδ T cells for FACS sorting for the purpose of avoiding any interference with the subsequent FC_3A assay, which would use at least two fluorochromes (PE and 7-AAD, in this case) that were detected in FL-2 and FL-4 respectively.

Sorted cells were either used immediately or cultured over night as described before. Their purity was confirmed by flow cytometry prior to being used in cytotoxicity assays. In a typical sorting, over 85% of the γδ T cells in the positively sorted population were $CD16^+$ (Fig. 14E); while less than 2% were $CD16^+$ in the negatively sorted population (Fig. 14F).

3.3.4 Reconstitution of %$CD16^+$ gradient

Because there are no known anti-CD16 blocking antibodies capable of blocking CD16 on γδ T cells, I applied a strategy whereby $CD16^+$ cells were either nearly completely removed or titered back to examine their contribution to ADCC. To achieve this goal, both positively and negatively sorted γδ T cells were resuspended at the same density of 2×10^6 /ml in cRPMI. Different volumes of cell suspension from either tube were calculated in a way that, when mixed together, a serial CD16 positivity of γδ T cells could be obtained

(section 2.3.5). When these cells were used as effectors in ADCC assay, an increase in specific lysis along with the increase of CD16 gradient would be expected.

Table 3 is an example of this procedure showing both calculated %$CD16^+$ and the actual percentages of $CD16^+$ cells as measured by flow cytometry.

Table 3.
The constitution of the artificial CD16 percentage gradient.

Gradient step	1	2	3	4	5
Proportions of negative[a] and positive[b] sorted cells	1:0	3:1	1:1	1:3	0:1
Calculated %CD16	1.6	22.6	42.8	64.1	85.5
Actual %CD16[c]	1.6	20.9	43.8	69.9	85.5

[a] %CD16 of negative sorted cells is 1.6%
[b] %CD16 of positive sorted cells is 85.5%
[c] Measured by flow cytometry

3.4 CD16 expression in response to cytokines

In an effort to explain the elevated %CD16$^+$ in MS patients, especially those with progressive MS, I postulated that chronic inflammation may be the driving force. In particular, cytokines such as IL-2, IL-12, and IL-15 have been found to be elevated in the blood of SPMS patients (186-188). To address this possibility, purified γδ T cells were cultured in the presence of cytokines IL-2, -12, and -15 and the %CD16$^+$ determined.

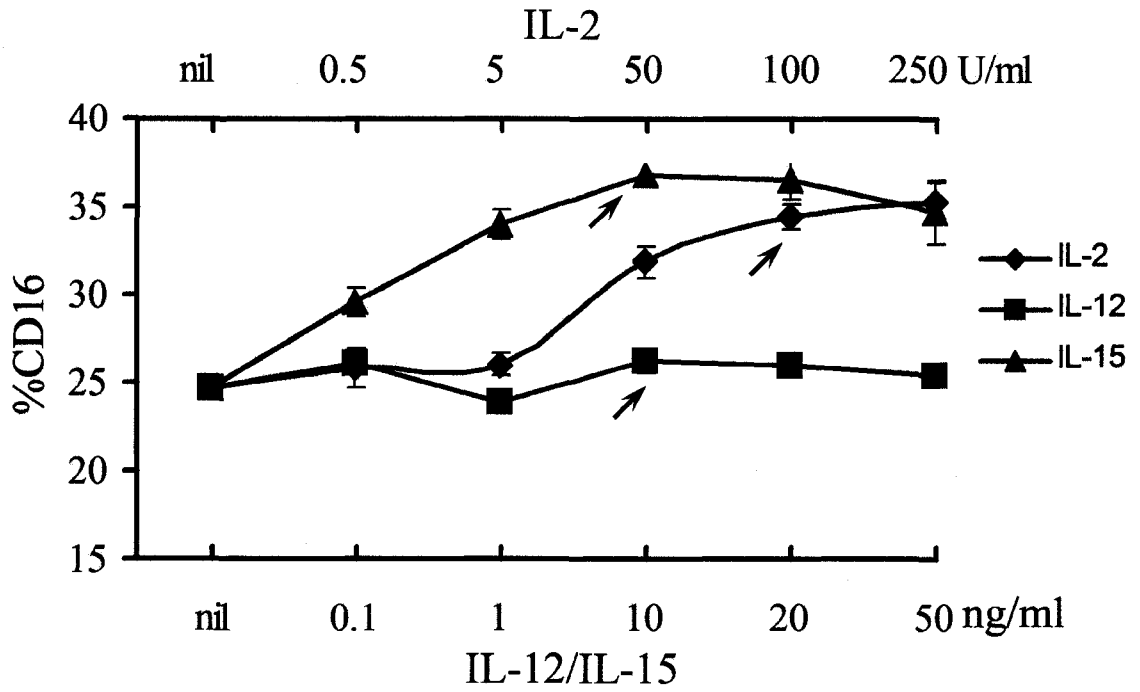
3.4.1 Optimisation of cytokine concentrations

Purified γδ T cells were cultured with serial dilutions of each of these cytokines as indicated. CD16 expression was measured with flow cytometry after culturing for a period of 72 hr for all of the cell cultures studied (Fig. 15). All these three cytokines could increase CD16 expression to different extents when compared with medium alone, though that of the IL-12 is only minimal. The expression of CD16 peaked at 100 U/ml IL-2, 10 ng/ml IL-12, and 10 ng/ml IL-15 respectively, and these concentrations were considered optimal.

This procedure was performed on γδ T cells derived from 5 MS patients and 2 HCs and similar results were obtained. Figure 15 is a representative of these experiments.

Figure 15. Optimization of interleukin concentrations on the expression of CD16 on γδ T cells.

Purified γδ T cells were cultured with the serial dilutions of IL-2 (upper x-axis), IL-12, and -15 (lower x-axis), and their CD16 expressions were measured with flow cytometry after a period of 72 hr. All these three cytokines could increase CD16 expression, but to different extents. The expression of CD16 peaked at 100 U/ml IL-2, 10 ng/ml IL-12, and 10 ng/ml IL-15 respectively (indicated with arrows), and these concentrations were determined as optimal. Figure is representative of seven different experiments.



3.4.2 CD16 is upregulated by proinflammatory cytokines

Purified γδ T cells from either MS patients or HC were cultured with or without IL-2, -12, and -15 at previously optimised concentrations of 100 U/ml, 10 ng/ml, and 10 ng/ml respectively for 72 hours. %$CD16^+$ was measured with flow cytometry as described above. Culturing in the presence of all 3 cytokines significantly elevated %$CD16^+$ derived from MS patients, the most robust stimulation seen with IL-15 (Fig. 16). These three cytokines also increased %$CD16^+$ derived from HCs, though to a lesser extent and IL-12 failed to show a statistically significant difference. Moreover, for each of the cytokines, there was no statistical difference noticed of their ability to increase %$CD16^+$ between healthy controls or MS patients as tested with *t*-test.

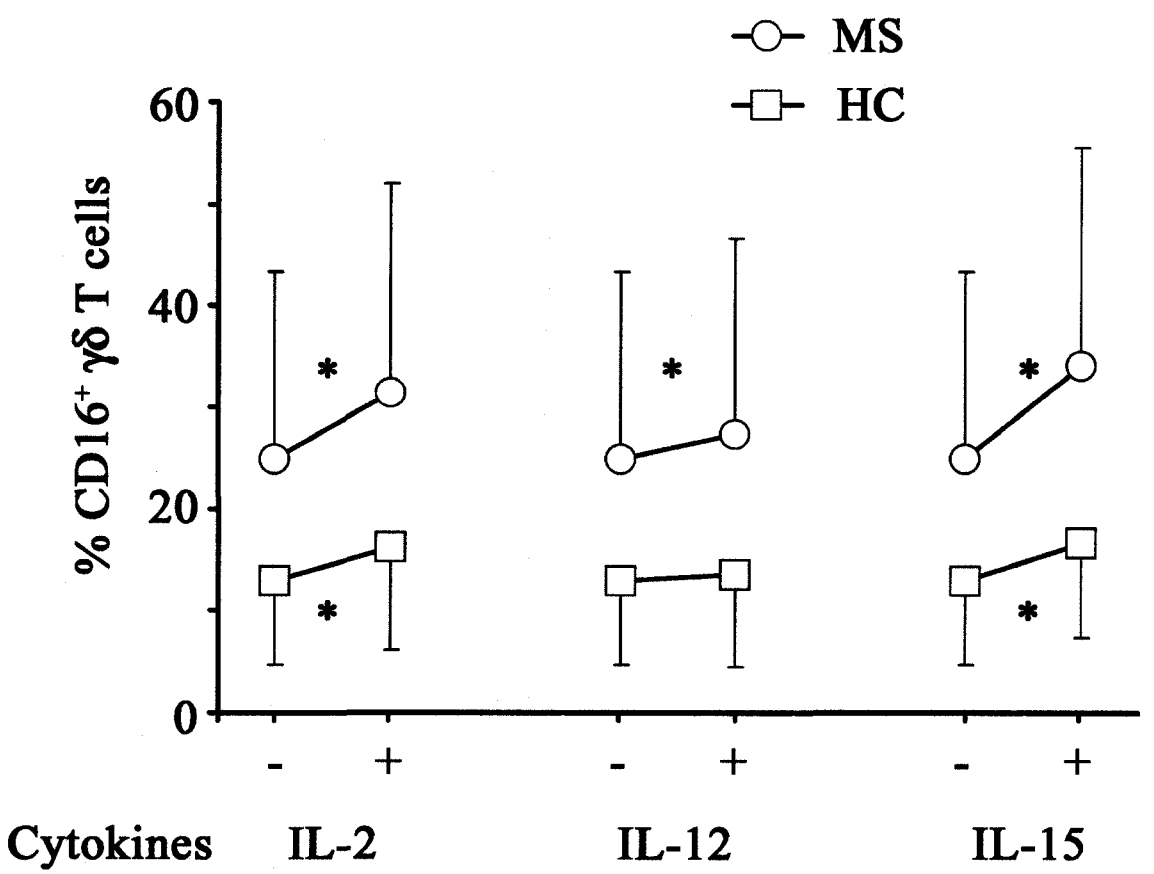
These results confirmed that proinflammatory cytokines such as IL-2, -12, or -15 were effective in increasing the portion of $CD16^+$ γδ T cell *in vitro* in the absence of antigenic stimulations. The increase was more pronounced in γδ T cells from patients than from HCs, though the differences did not reach statistical significance. IL-15 was the most robust cytokine among the three studied, and increase caused by IL-12 was almost negligible.

3. 5 Quantitation of γδ T cell ADCC by ELISA

Three key components are indispensable in an ADCC system: [a] target cells that are relatively resistant to cytotoxicity caused by γδ T cells when the antibodies are absent; [b] bridging antibodies that recognize the specific antigens present on the target cells and are reactive to CD16 on γδ T cells, and [c] CD16 positive effector cells, which are γδ T cells in this study (Fig. 1A).

Figure 16. Increase in %CD16$^+$ in response to *in vitro* cytokine stimulation.

Pro-inflammatory cytokines IL-2, -12 and -15 significantly elevated the %CD16$^+$ γδ T cells derived from MS patients (n=10), the most robust stimulation seen with IL-15. IL-2 and IL-15 also increased %CD16$^+$ γδ T cells in healthy controls (n=6). For each of the cytokines, there was no difference noticed in their ability to increase the %CD16$^+$ γδ T cells between healthy controls and MS patients. * $p<0.05$ by paired student's *t*-test.



The first such system established in my work contains Rh_o^+ red blood cells serving as the targets and polyclonal human anti-Rh_o^+ IgG as the bridging antibody. Normally erythrocytes are resistant to γδ T cell killing. But when these cells were coated with anti-Rh_o^+ IgG, the Fc portion of the antibodies would undergo conformational change, which leads to high affinity interactions between the Fc domains of IgG and the CD16 molecules on γδ T cells (170). Once this target-antibody-effector complex was assembled, the erythrocytes were lysed and the intracellular haemoglobin was released into the supernatant from the ruptured erythrocytes. The amount of soluble haemoglobin was then quantified with ELISA, whose results reflected the extent of erythrocyte lysis, and consequently γδ T cell ADCC capability.

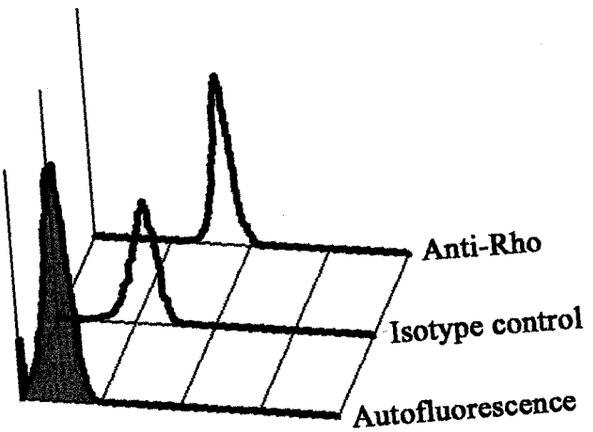
In most of ADCC experiments (other than those specified), the effector γδ T cell lines comprised a high percentage of CD16 with on average 50 to 60% of the cells expressing this surface antigen. But they did not express FcγRs of other kinds as has been shown above (see Fig. 3).

3.5.1 Antibody labelling of red blood cells

Anti-Rh_o IgG coating of erythrocytes was confirmed by flow cytometry with PE conjugated anti-human secondary antibody. Red blood cell coated with anti-Rh_o demonstrated a significant shift of fluorescent intensity when compared with isotype matched antibodies or un-coated cells (Fig. 17). The histogram suggested that the staining was complete and homogeneous because 99 percent of the PE^+ cells located within a narrow range in the second decade on the graph.

Figure 17. Coating red blood cells with polyclonal anti-Rho antibody.

Anti-Rh_o IgG coating of Rh_o^+ erythrocytes was confirmed by flow cytometry with PE conjugated anti-human IgG secondary antibody. Red blood cells coated with anti-Rh_o demonstrated a significant shift of fluorescent intensity when compared with isotype matched antibodies or un-coated cells.



3.5.2 Haemoglobin determination by ELISA

The amount of erythrocytes and the duration of incubation were optimized in a way that the readout of the OD values of all the wells would fall in a linear range allowed by the ELISA kit. It was critical to gently centrifuge the plate before aspirating the supernatant because red blood cells were easily stirred up by handling thus accidentally taken into samples, yet over-spinning was not recommended since this may cause erythrocytes lysis under mechanical forces.

Because the specific lysis was a relative value, it was not necessary to run a haemoglobin standard in the assay to determine the actual concentrations. Rather, calculation with only OD values sufficed.

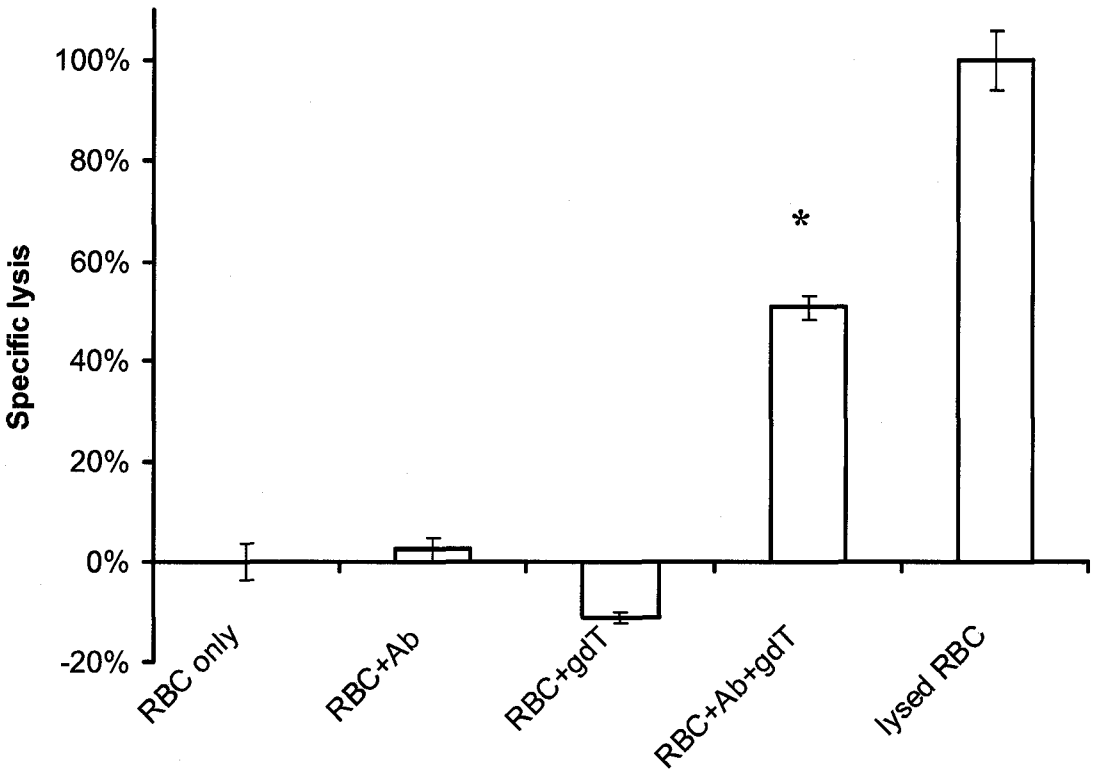
3.5.3 Erythrocyte lysis caused by γδ T cells via ADCC

Figure 18 representatively depicts the ADCC lysis of erythrocytes induced by γδ T cells. γδ T cells alone did not cause red blood cell death in the absence of anti-Rh_0 antibody, nor did the bridging Ig by itself. However when anti-Rh_0 coated red cells were used as targets, the lysis caused by γδ T cells became obvious. ANOVA test confirmed that this increase is statistically significant, $p<0.05$.

Overall, results obtained from this erythrocyte-anti-Rh_0 system suggested that γδ T cells were able to mediate ADCC cytotoxicity. Based on this proof-of-principle, I moved on to establish another system that would use nucleated cells as targets and a humanized mAb to bridge the E:T populations.

Figure 18. Representative depiction of the ADCC lysis of erythrocytes induced by γδ T cells.

γδ T cells alone did not cause red blood cell damage in the absence of anti-Rh_o antibody, nor did the bridging antibody do the same. When anti-Rh_o coated red blood cells were used as targets, the result was γδ T cell mediated lysis. E:T=10:1. 100% specific lysis was achieved by lysing the RBC with commercial lysis buffer. * $p<0.05$.



3.6 Quantitation of γδ T cell ADCC by flow cytometric cytolytic assay

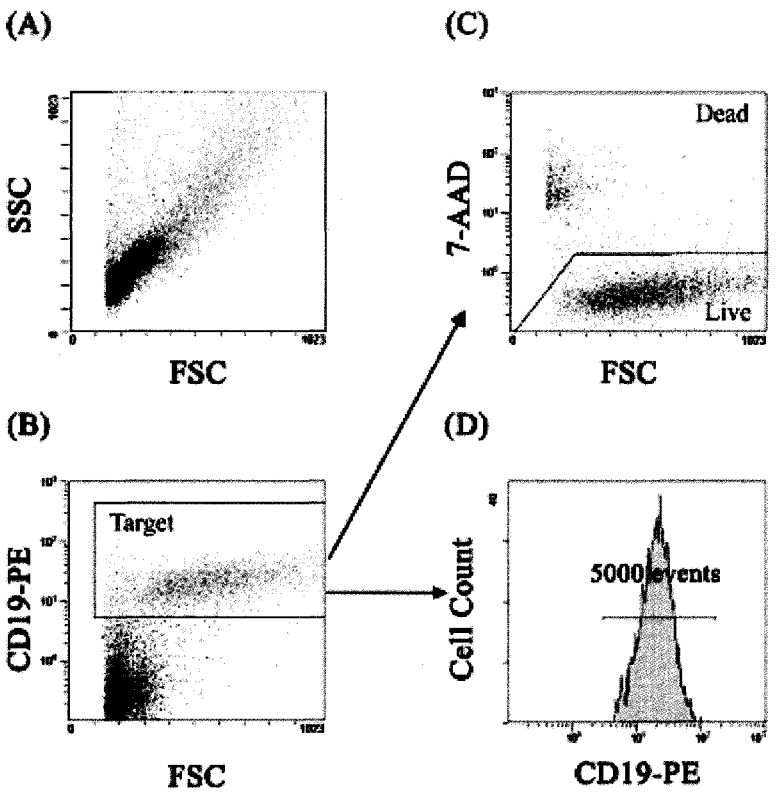
In a second system that I designed to investigate the ADCC hypothesis, B cell lymphomas that express CD20 antigen were used as targets; and a humanized mAb that specifically target CD20 – rituximab – was used as the bridging antibody. This system is one step closer to the actual pathophysiological situation than the red blood cell system in that the targets are not only subjected to death resulted from cell membrane penetration by pore-forming proteins, but also other means that γδ T cells may use, such as granzymes or FasL effected attacks. However, the detailed apoptotic pathways of target cells' death and their crosstalk with CD16 signaling could be of great interest of future work but were not studied in this project.

3.6.1 Development of flow cytometric cellular cytotoxicity assay (FC_3A)

Rituximab coated or non-coated Raji or Daudi cells were co-incubated with purified human γδ T cells for 18 hr or 4 hr before the cell mixtures were stained with PE-CD19 and 7-AAD, and immediately analyzed with flow cytometry. Figure 19 is a representative dot plot showing an experiment with E:T ratio of 4:1. The larger target cells were morphologically distinguished from smaller γδ T lymphocytes on forward and sideward light scatter plot (Fig. 19A), however in addition, a special gating strategy was applied which allowed the resolution of these two populations, where the PE positive population was the target and the PE negative was the effector γδ T cells (Fig. 19B). The target population was gated in "Target" and further analyzed for their percentage of lysis,

Figure 19. Quantitative analysis of γδ T cell ADCC by flow cytometric cellular cytotoxic assay (FC_3A).

This figure represents dot plots corresponding to an experiment with E:T ratio of 4:1. Rituximab coated or non-coated Raji cells were co-incubated with purified human γδ T cells for 18h before the cell mixtures were stained with PE-CD19 and 7-AAD, and immediately analyzed with flow cytometry. (A) Morphological features of effector and targets as distinguished by forward light scatter (FSC) and sideward light scatter (SSC) properties. Smaller and less granular γδ T cells are concentrated on the lower left corner on the plot, while Raji cells spread through from the center to the upper right of the plot diagonally. (B) PE positive target cells can be distinctively separated from PE negative effector cells by their strong fluorescence detected in FL-2. This target population is gated in "Target" and further analyzed for their percentage of lyses in: (C) Percentage of dead cells can be obtained from the "Dead" gate by dividing the number of $7\text{-}AAD^+$ population (lysed cells) over the total number of target cells. Specific lysis could subsequently be calculated according to the formula described in section 2.10.3. (D) A histogram of the PE^+ target cells is used as a "counter", which would automatically terminate the acquisition when the event number reaches 5,000.



which was calculated by dividing the number of 7-AAD$^+$ population over the total number of target cells, as shown in gate "Dead" of Figure 19C.

A histogram of the PE$^+$ target cells was used as a "counter" (Fig. 19D), which would automatically terminate the acquisition when the target event number reached 5,000. This was done by checking on the "Stop and save" option in the "Plots" settings of the CXP software. Of note, this strategic setting is particularly useful when one wants to automatically stop the acquisition according to a specific parameter of a specific population, but not a general population.

To determine spontaneous lysis, target cells that had not been labeled with rituximab were cultured in parallel in the absence of γδ T cell. The percentage lysis of this spontaneous cell death was used to calculate % specific lysis as shown in section 2.10.3.

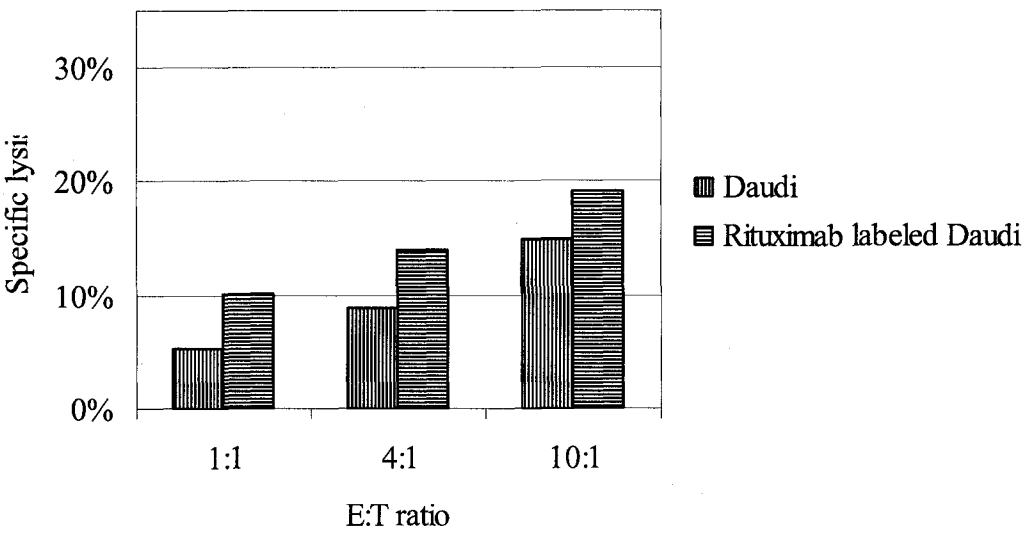
Both Daudi and Raji cells are non-Hodgkin's lymphoma cell lines. Though they were both subjected to γδ T cells mediated ADCC killing, Daudi cells were less susceptible than Raji under the same conditions (Fig. 20).

Durations of co-culturing also resulted in noticeable differences on cell deaths. The lysis of target cells after 18 hrs co-culturing was evidently higher than 4 hrs while the spontaneous lysis remained nearly no change. This improved signal-to-baseline ratio therefore conferred improved results for this assay (Fig. 21).

As such, Raji cell was chosen as the target cell line and 18 hr as the standard co-culturing time throughout the rest of this study.

Figure 20. Daudi cells are relatively resistant to γδ T cells killing compared with Raji cells.

ADCC killing of Daudi and Raji were compared under the same conditions. Specific lyses by γδ T cells against Daudi cells were consistently lower than those against Raji cells.



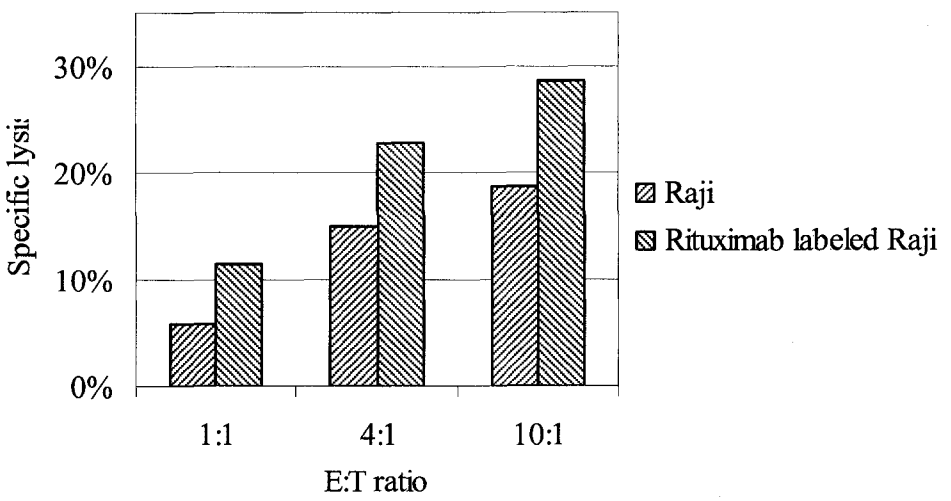
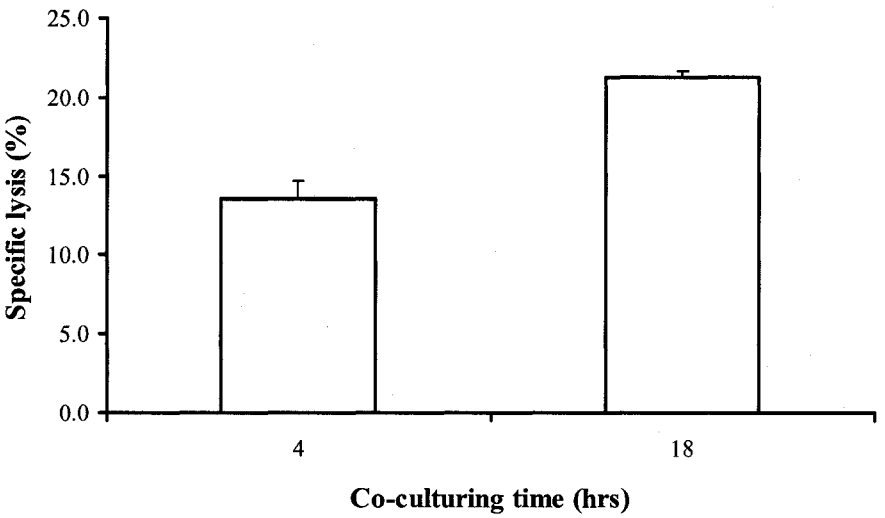


Figure 21. 18 hour co-culturing of E:T yields better readout than the 4 hour protocol.

Duration of co-culturing also resulted in noticeable differences on cell deaths. The lysis of target cells after 18 hr co-culturing was higher compared to 4 hr with improved signal-to-baseline ratio. Therefore the 18 hr co-culturing scheme was used as a standard protocol for this study.



3.6.2 Optimization of bridging antibody concentration

Serially diluted (final concentrations 0.01~100 μg/ml) rituximab and an irrelevant human IgG (10 μg/ml) were used to label Raji cells, which were subsequently stained with PE conjugated goat-anti-human IgG mAb and tested with flow cytometry to confirm the labeling efficiency (Fig. 22). More rituximab bound to Raji cells with the increase of its concentrations, as evidenced by the shift of mean fluorescence intensity (MFI), until it could almost saturate the antigen binding sites on the surface of Raji cells at 10 μg/ml, at which concentration the MFI curve almost overlap with that of 100 μg/ml. As a result, 10 μg/ml of rituximab was used as the optimal staining concentration in the following cytotoxic assays. In contrast, background staining of Raji cells using purified human IgG was minimal at this same concentration (Fig. 22).

To confirm that the abundance of bridging antibodies attached to target cells could essentially have an effect on the ensuing ADCC lysis, such differentially labeled Raji cells were used as targets and the ADCC assays were repeated following the protocol described above. The percent lysis of 10 μg/ml human IgG labeled Raji did not differ from the non-labeled control at the E:T ratio studied (10:1), nor did the two groups of rituximab at lower concentrations (0.01 and 0.1 μg/ml). However, when rituximab concentrations reached 1 μg/ml or above, the ADCC lysis was significantly elevated compared with either non-labeled control or irrelevant-IgG-labeled target cells (Fig. 23).

Taken together, evidence suggested that 10 μg/ml rituximab was the most economical and effective concentration under which the ADCC assay yielded the optimal results.

Figure 22. Rituximab coating of target cells.

Rituximab was serially diluted to reach final concentrations ranging from 0.01 to 100 μg/ml (shown in different colors) and were used to label Raji cells. Human IgG were used as a control (orange). Such labelled Raji cells were subsequently stained with secondary PE-conjugated-mouse-anti-human IgG mAb and tested with flow cytometry to confirm the labelling efficiency. More rituximab bound to Raji cells with the increase of the concentrations, as evidenced by the shift of mean fluorescence intensity (MFI), until it could almost saturate the binding sites on the surface of Raji cells at 10 μg/ml (overlapping of the last two peaks). In contrast, purified human IgG only marginally labelled Raji at this concentration. Yellow curve represents Raji cells stained only with the secondary antibody.

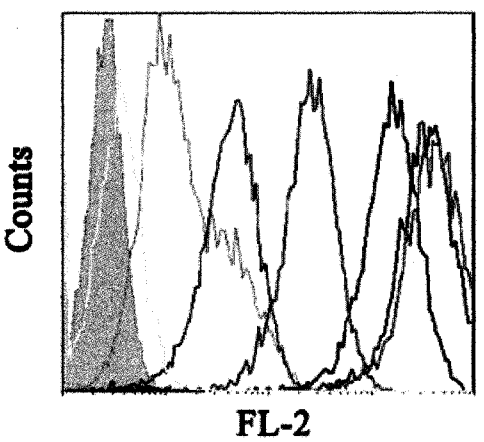
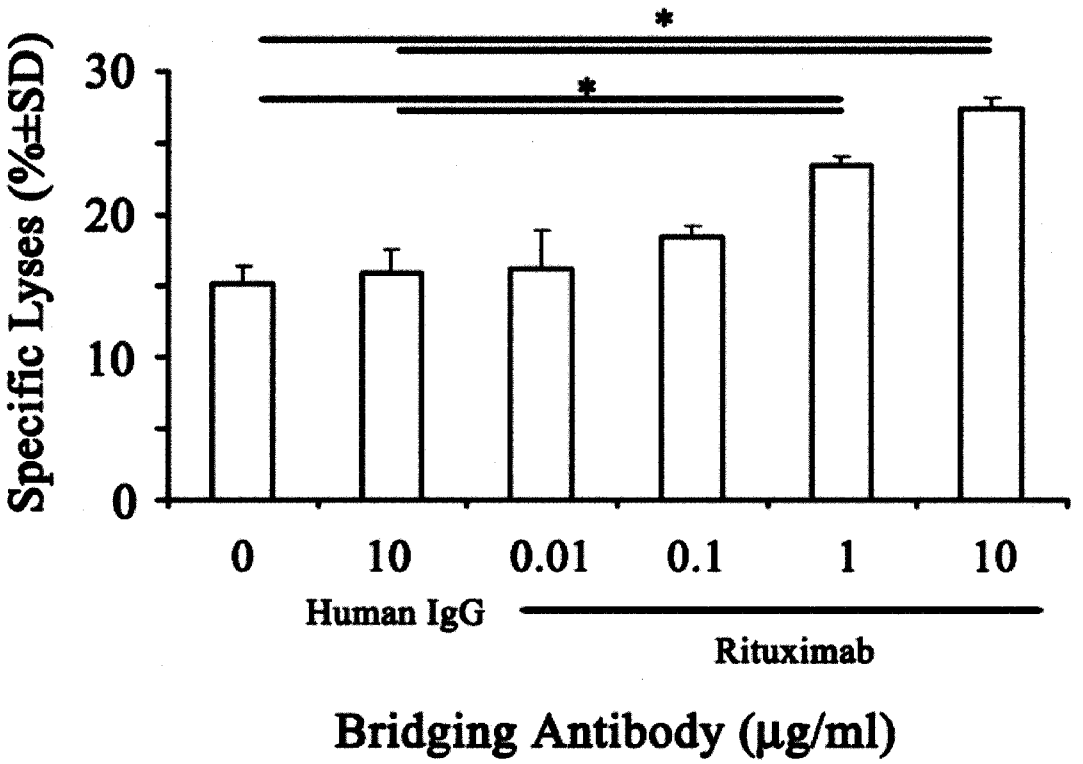


Figure 23. ADCC increases with the increasing rituximab concentration.

Differentially labelled Raji cells were used as targets and the ADCC assays were repeated. The percentage lyses of 10 μg/ml human IgG labelled Raji did not differ from the non-labelled control at E:T ratio studied , nor did the two groups of rituximab at lower concentrations (0.01 and 0.1 μg/ml). However, when rituximab concentration reached 1 μg/ml or above, the ADCC lysis was significantly elevated compared with both controls. * $p<0.05$ by ANOVA. Figure is representative of at least three different experiments.



3.6.3 γδ T cell ADCC is E:T ratio dependent

Target cells labeled with or without rituximab were co-incubated with purified γδ T cells at various E:T ratios as indicated (Fig. 24). γδ T cells demonstrated some spontaneous (NK-like) killer activity towards target cells where they were able to lyse the targets in the absence of rituximab; and this cytotoxicity increased in respond to the increasing E:T ratios (Fig. 24, open bars). However, with the same E:T ratio, the specific lysis of the targets by γδ T cells was significantly augmented by the presence of rituximab. At all 3 E:T ratios lysis in the presence of the antibody was increased by at least 85%. The differences were statistically significant when the E:T ratio was at or above 4:1 (Fig. 24).

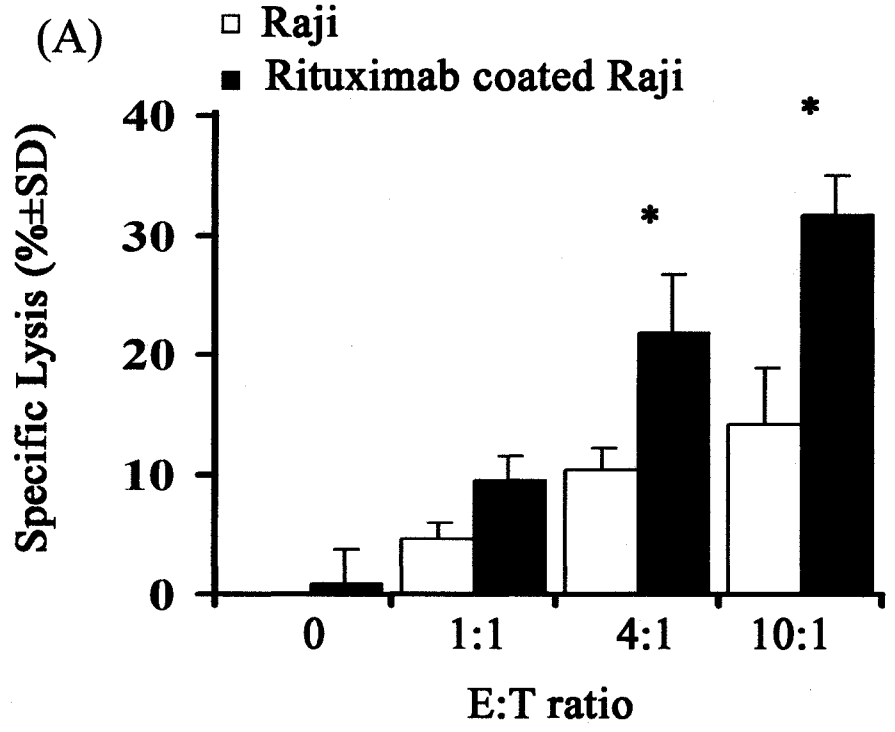
NK cells were used in parallel as a positive control. All the NKs derived and purified (by using a commercial Miltenyi NK cell isolation kit) were CD16 positive. Not surprisingly the NK cells lysed the targets regardless of whether or not there was rituximab. However the presence of rituximab significantly increased the target killing by NKs, in a way analogous to that of γδ T cells, possibly through the same CD16 surface marker.

A similar E:T ratio response was also observed when lower concentrations of rituximab were used, however the overall lysis was also relatively lower (Fig 25).

Collectively, these results were further demonstrations that the γδ T cell ADCC lyses were consequences of specific process that depends on both E:T ratio and antibody concentrations.

Figure 24. γδ T cell ADCC cytotoxicity is E:T ratio dependent.

(A) Target cells labeled with or without rituximab were co-incubated with purified γδ T cells at various E:T ratios as indicated for 18h at 37 °C in a CO_2 incubator. Cytotoxicity was tested as described above. γδ T cells demonstrated some natural killing activity towards target cells where they were able to cytolyse the targets in the absence of rituximab (open bars), however, with the same E:T ratio, the specific lysis of the targets by γδ T cells was augmented by the presence of rituximab and reached statistical significance when the E:T was at or over 4:1. (B) NK cells were used as a positive control. $*p<0.05$ by *t*-test. Figure is representative of at least five different experiments.



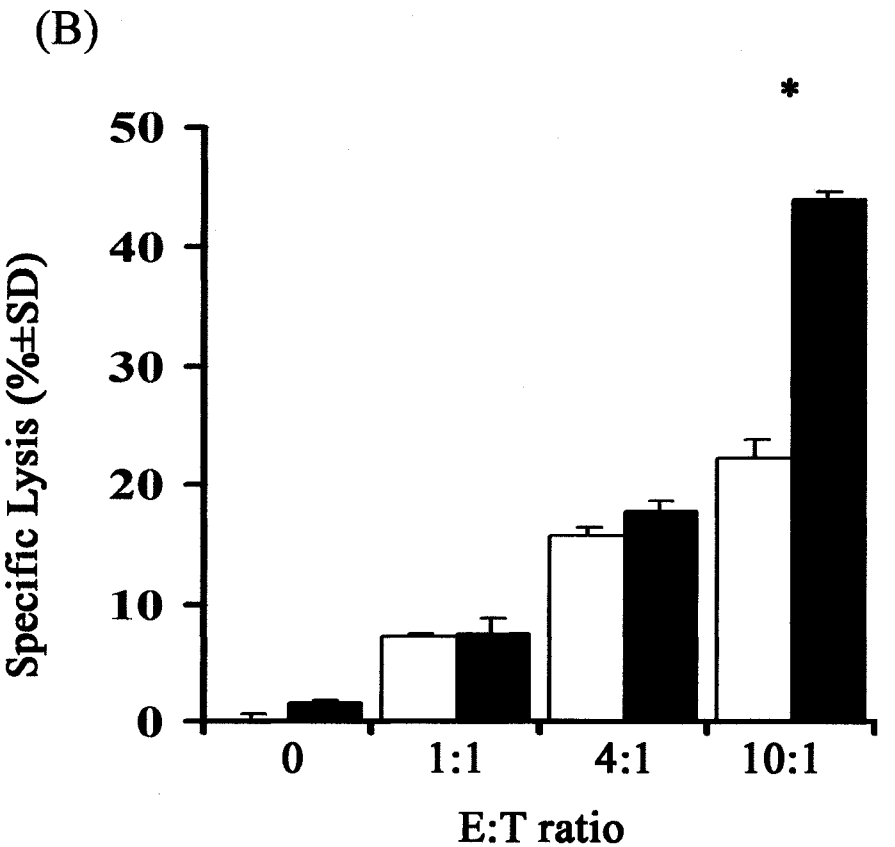
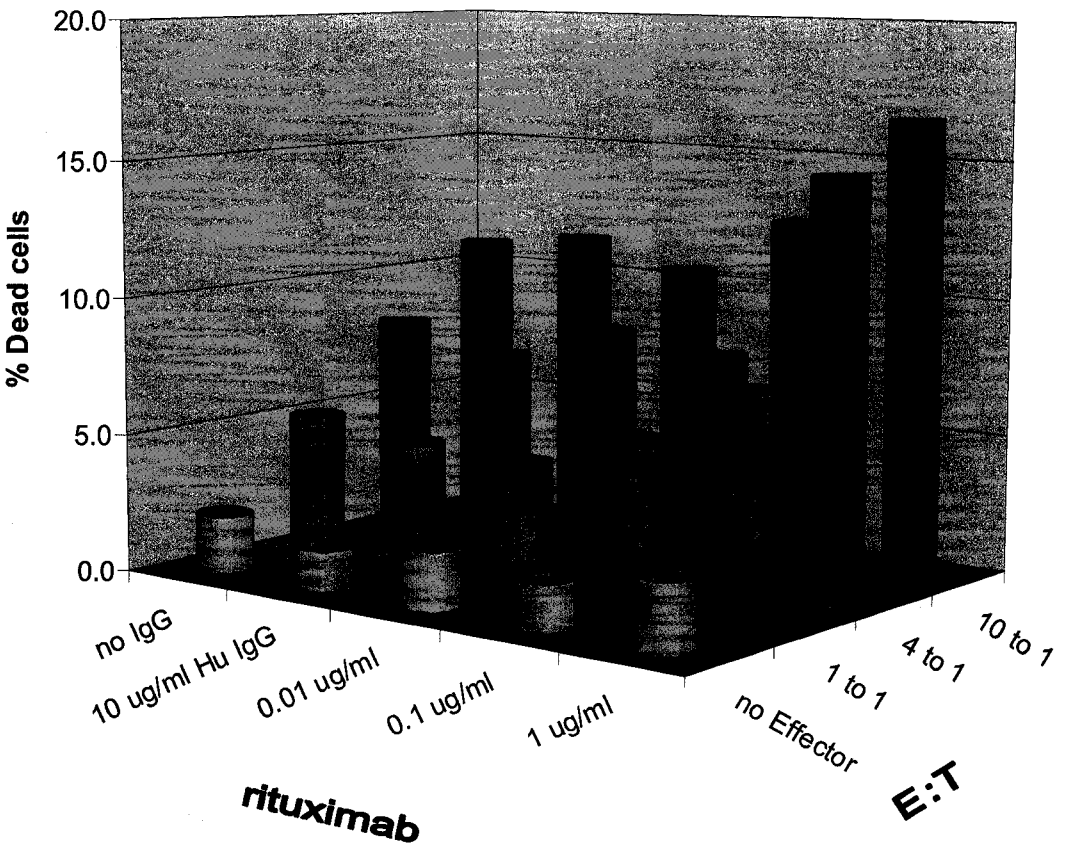


Figure 25. γδ T cells ADCC lysis at sub-optimal rituximab concentration.

A similar E:T ratio response was also observed when lower concentrations of rituximab were used, however the overall lysis was also relatively lower.



3.6.4 ADCC using $F(ab')_2$ fragment of rituximab as bridging antibody

To allow for further corroboration that the observed ADCC lyses complied with the proposed model as illustrated in Figure 1A, the Fc fragment of rituximab was removed by pepsin hydrolysis, in anticipation that using such treated Fc deficit antibody would negate rituximab mediated γδ T cell ADCC.

3.6.4.1 Preparation and quantification of rituximab $F(ab')_2$

$F(ab')_2$ fragments were generated by enzymatic digestion of the Fc portion of rituximab. The concentration of purified $F(ab')_2$ fragments were evaluated with a commercial kit and reconstituted to 10 μg/ml in PBS-1 buffer, the same concentration as that of rituximab optimized in the ADCC assay. However, this apparent identicalness may actually reflect higher molar concentration of $F(ab')_2$ than rituximab because of the reason that the MWs of rituximab and $F(ab')_2$ are different. Coating target cells with these two preparations would result in more $F(ab')_2$ attached to the targets than rituximab did. But this should not be a concern because this would only exaggerate the negative control result and therefore further support the hypothesis of ADCC killing.

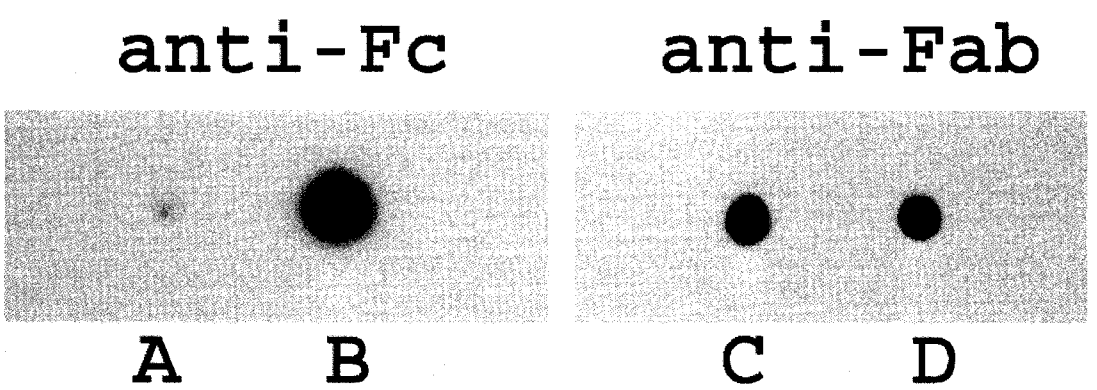
3.6.4.2 Determination of the purity of $F(ab')_2$

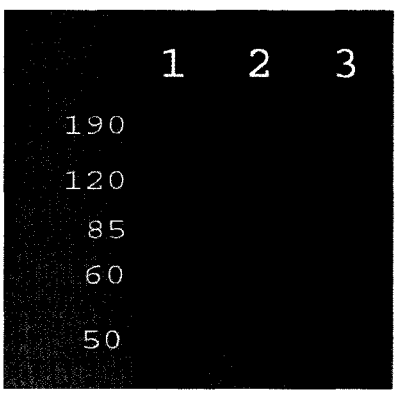
The purity of the $F(ab')_2$ preparation was quantified by dot blot assay coupled with densitometry and further confirmed with Western blot and flow cytometry.

In the first method, the dots of $F(ab')_2$ and rituximab were visualized by chemiluminescence and captured by a cooled CCD camera (Fig. 26 upper panel). When

Figure 26. Immuoblot assays examining the purity of the $F(ab')_2$ fragment of rituximab.

Upper panel: the dots of $F(ab')_2$ (A and C) and rituximab (B and D) were visualized by chemiluminescence and captured by a cooled CCD camera. When detected with anti-IgG Fc specific antibody (left), only rituximab showed positivity but not $F(ab')_2$ fragment; when detected with anti-IgG Fab specific antibody (right), both $F(ab')_2$ and rituximab demonstrated positivity. The pixel densities of the dots were quantified with AlphaEaseFC software and the purity of enzymatically generated $F(ab')_2$ could reach around 95% of total protein (see Table 4). Lower Panel: these results were further confirmed with western blot detected by anti-Fc antibody: lane 1: MW marker; lane 2: rituximab; lane 3: $F(ab')_2$ fragment.





detected with anti-IgG Fc specific antibody, only rituximab showed positivity but not the $F(ab')_2$ fragment; however, when detected with anti-IgG Fab specific antibody, both $F(ab')_2$ and rituximab demonstrated positivity. The pixel densities of the dots were quantified with AlphaEaseFC software and were listed in Table 4. The purity of such enzymatically generated $F(ab')_2$ could reach around 95% of total protein.

Table 4.
Densitometry analysis for dot blot assay.

Dot #	IDV	B/(A+B)%*	Dot #	IDV	D/(C+D)%*
A	3262	2.5	C	68327	50.4
B	126266	97.5	D	67121	49.6

IDV=Integrated Density Value
* Based on IDV
Table is representative result of at least three experiments.

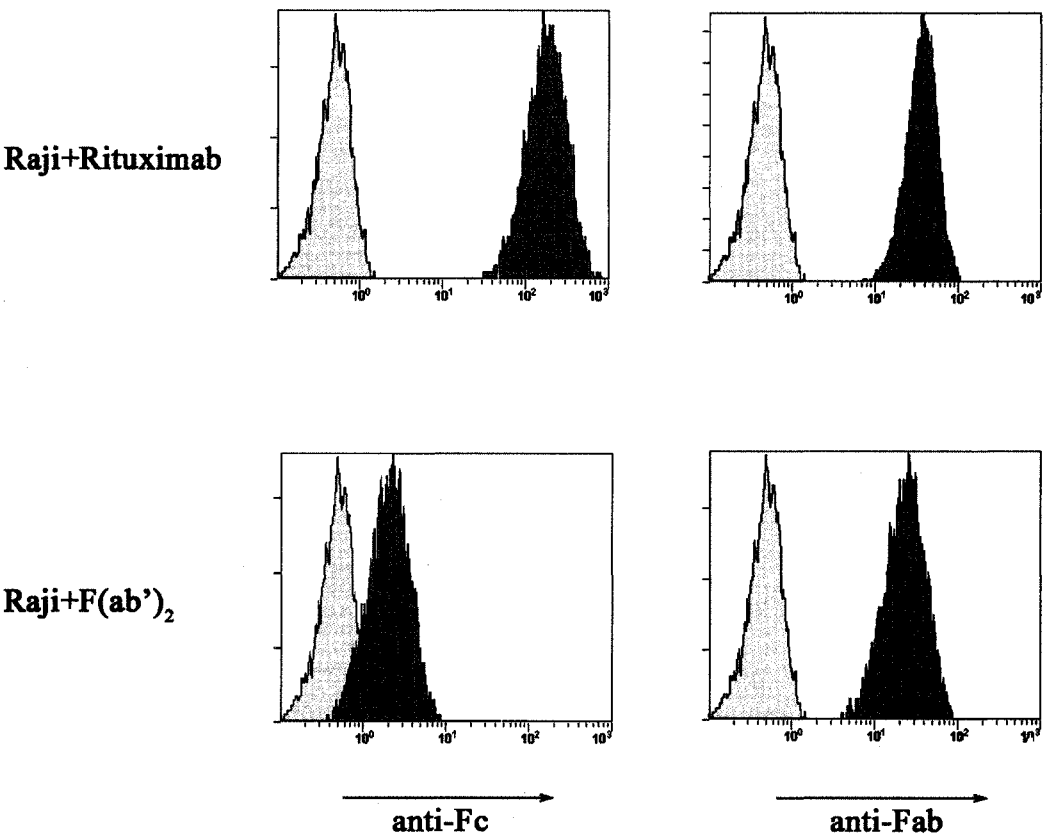
This result was further confirmed by Western blot using an anti-IgG Fc-specific antibody (Fig. 26 lower panel). It can be seen that almost no Fc portion could be detected in the purified $F(ab')_2$ fragment of rituximab (lane 3).

To further confirm the purity and functionality of $F(ab')_2$ fragments, both $F(ab')_2$ fragments and rituximab were used to label Raji cells, followed by secondary PE-conjugated anti-IgG (Fc specific) or anti-IgG (Fab specific) antibodies (Fig. 27). As expected, rituximab labeled Raji cells (upper panel) were positively stained by both antibodies, but $F(ab')_2$ fragments labeled cells (lower panel) illustrated positivity only by anti-IgG (Fab specific) not anti-IgG (Fc specific) antibody.

In conclusion, pepsin digestion effectively removed Fc fragments from rituximab. The purified $F(ab')_2$ fragments were still capable of coating $CD20^+$ Raji cells, but their Fc tails were no longer obviously detectable by Western blot or flow cytometry with an anti-IgG (Fc specific) antibody.

Figure 27. Purity of $F(ab')_2$ as determined by flow cytometry.

Both $F(ab')_2$ fragments and rituximab were used to label Raji cells, followed by secondary PE-conjugated anti-IgG (Fc specific) or anti-IgG (Fab specific) antibodies. It can be seen that rituximab labeled Raji cells were positively stained by both antibodies, but $F(ab')_2$ fragments labeled cells illustrated positivity only by anti-IgG (Fab specific) not anti-IgG (Fc specific) antibody.



3.6.4.3 ADCC was abrogated by removal of Fc fragment from rituximab

$F(ab')_2$ fragments were used in parallel with rituximab to label the target cells, which were then co-cultured with γδ T cells to test ADCC activity. In agreement with the results above, the presence of rituximab significantly increased the cytotoxicity of γδ T cells (column 5, Fig. 28). However, when $F(ab')_2$ fragments were used in place of rituximab, the ADCC lysis was nrarly abolished, resulting in a specific lysis level comparable to that of γδ T cells alone (columns 6 and 4, Fig. 28). Neither rituximab nor $F(ab')_2$ fragments alone showed any significantly increased cytotoxicity towards the targets in these experiments (columns 2 and 3, Fig. 28).

3.6.5 ADCC is driven by $CD16^+$ γδ T cells

FACS sorted $CD16^+$ and $CD16^-$ γδ T cells were reconstituted to yield an artificial CD16 percentage gradient (Table 3 and x-axis of Fig. 29). The ADCC test was repeated using these cells as effectors and rituximab labeled (10 μg/ml) or unlabeled Raji as targets, with an E:T ratio at 10:1.

ADCC type cytotoxicity was totally abrogated when $CD16^+$ cells were removed from the effector pool, resulting in a level of specific lysis similar to that of unlabeled target cells. ADCC lysis increased with the increasing CD16 percentage, reaching its peak level when positively sorted γδ T cells were used as effectors. The specific lyses between ADCC and NK-like cytotoxicity were significantly different when $CD16^+$ cells accounted for more than 40% of the total effector population (Fig. 29).

Figure 28. ADCC was abrogated by removal of Fc fragment from rituximab.

$F(ab')_2$ fragment was used in parallel with rituximab to label the target cells, which were then co-cultured with or without γδ T cells. The presence of rituximab significantly increased the cytotoxicity of γδ T cells (column 5), however, when $F(ab')_2$ was used in place of rituximab, the ADCC lysis was almost eliminated, resulting in a specific lysis level comparable to that of γδ T cells alone (columns 6 and 4). Neither rituximab nor $F(ab')_2$ fragment alone showed any cytotoxicity towards the targets (columns 2 and 3). * $p<0.05$, NS: not significant, tested with ANOVA. Figure is representative of at least six different experiments.

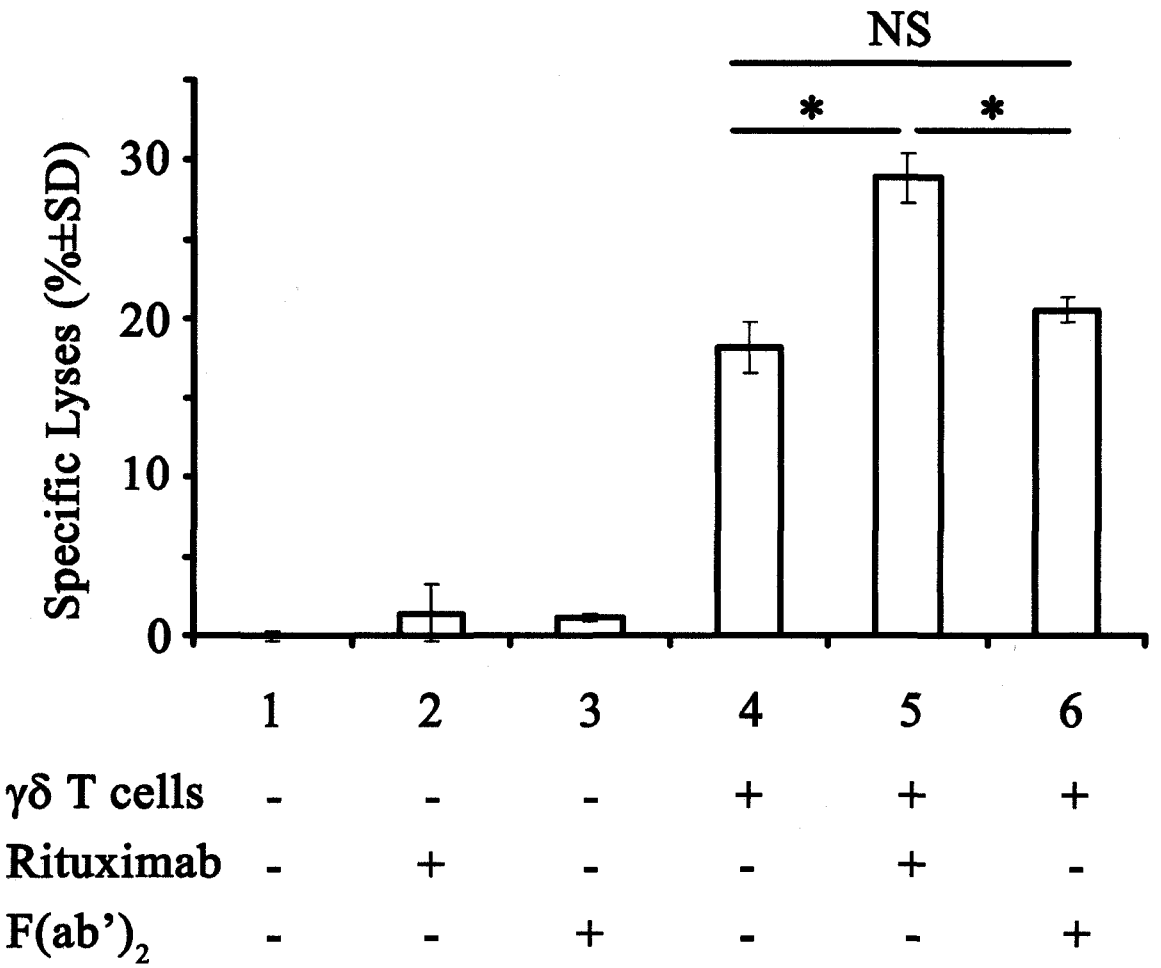
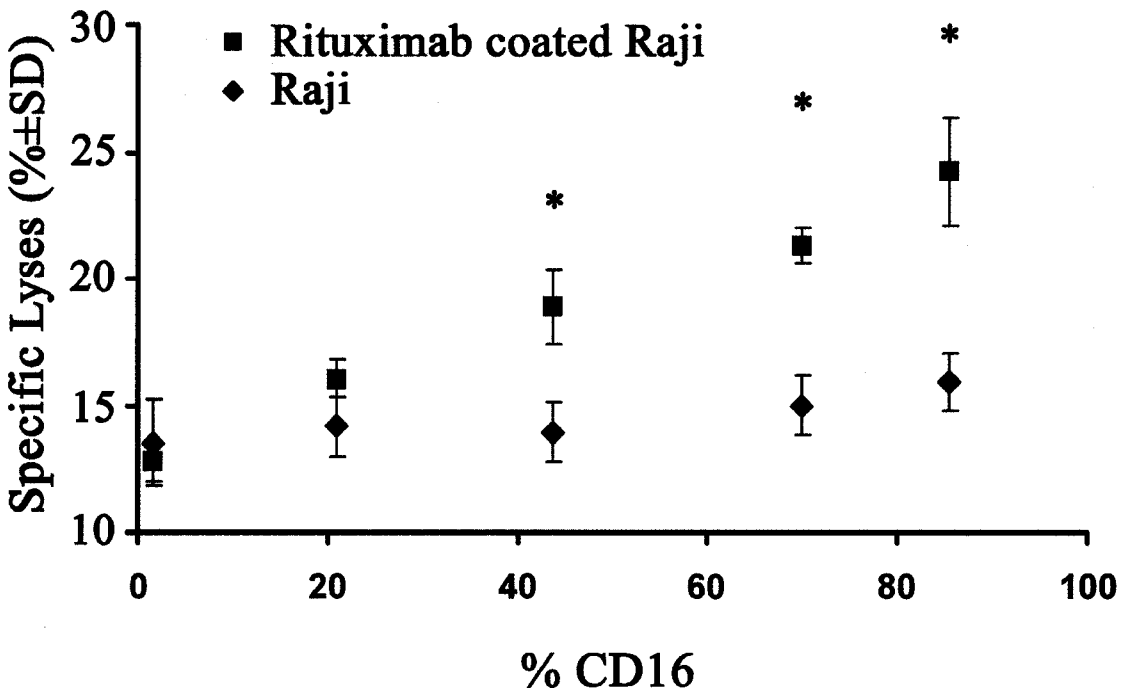


Figure 29. γδ T cells ADCC is driven by CD16.

Purified γδ T cells were sorted by MoFlo to separate $CD16^+$ and $CD16^-$ populations. The CD16 positive and negative cells were then proportionally mixed to yield an artificial CD16 percentage gradient and confirmed by flow cytometry (x-axis). ADCC test was repeated using these cells as effectors and rituximab labelled (■) or unlabelled (◆) Raji cells as targets, at E:T ratio of 10:1. Rituximab-dependent ADCC was totally abrogated when $CD16^+$ cells were removed from the effector pool, resulting in a similar level of specific lyses comparable to that of unlabeled cells. ADCC lyses increased with the increasing number of $CD16^+$ cells. The difference in specific lyses between ADCC (■) and unlabeled cytotoxicity (◆) reached statistical significance when $CD16^+$ cells accounted for more than 40% of the total effector population. *$p<0.05$ by t-test. Figure is representative of three different experiments.



3.6.6 γδ T cells from patients or HC show similar extent of ADCC at a per cell level

%$CD16^+$ γδ T cells were increased in MS patients vs. healthy controls (see Figs. 5 and 6), therefore I compared the rituximab-dependent cytotoxicity of γδ T cells derived from either MS patients or healthy controls, on a "per $CD16^+$ cell" level. Data were obtained from experiments performed at optimal rituximab concentration (10 μg/ml) and E:T ratio (10:1) under the conditions described above. The rituximab-dependent cytotoxicity contributed by per $CD16^+$ γδ T cell was calculated according to the following formula:

$$\%\text{cytotoxicity per } CD16^+\text{-cell} = \frac{\text{ADCC lysis} - \text{NK-like lysis}}{\text{Percentage of } CD16^+ \gamma\delta \text{ T cells}}$$

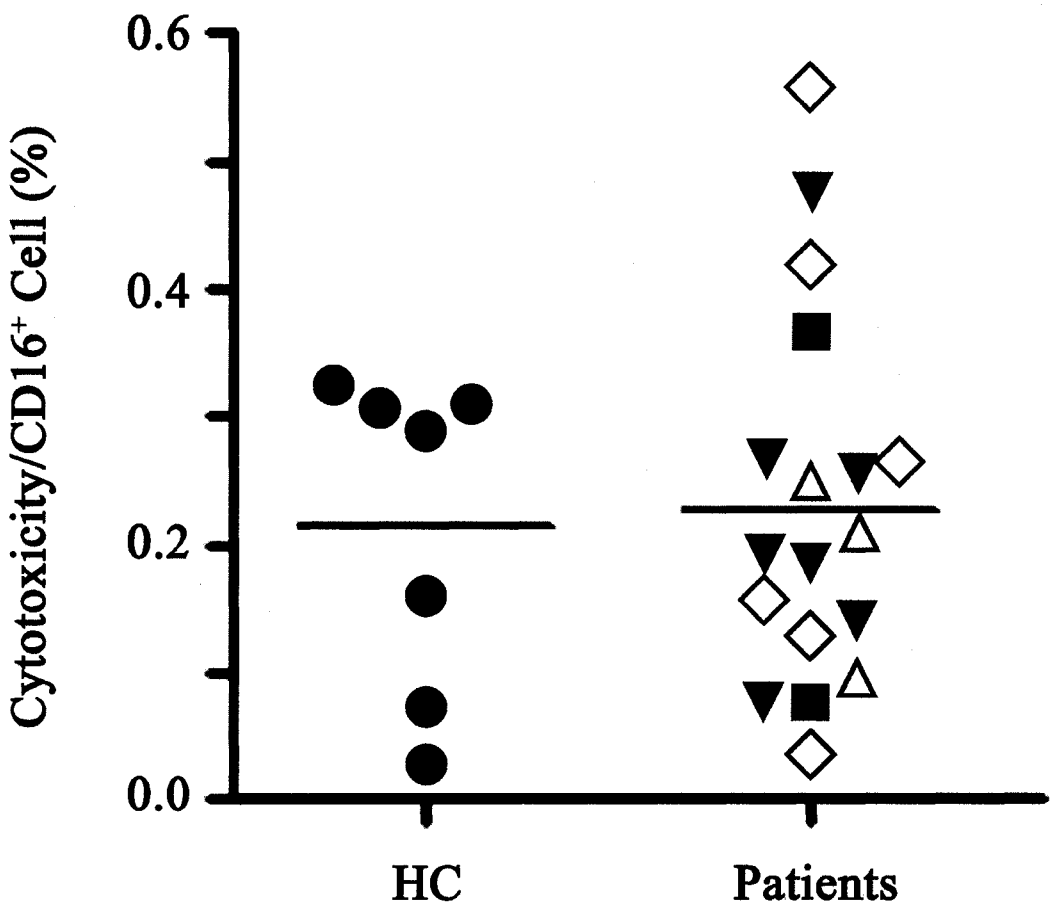
There was no difference between HCs and MS patients regarding their cytotoxicity at per $CD16^+$-cell level, under current experimental scheme, as shown in Fig. 30; neither were there any differences among all subgroups (CIS, RR, SP, and PP-MS) of patients (p=0.787 by ANOVA).

3.7 Cytokine production by γδ T cells upon CD16 engagement

γδ T cells are known to be potent cytokine producers and it has been shown that they release high level of TNF-α when their CD16 molecules are cross-linked *in vitro* (171). However there lacks a study showing the overall profile of their cytokine production, that includes both Th1 and Th2 types, through CD16 stimulation. To offer insight into an additional functional significance of CD16 engagement on γδ T cells, I examined the

Figure 30. γδ T cells from patients or HCs show similar extent of ADCC at a per $CD16^+$-cell level.

Cytotoxicity at per $CD16^+$-cell level was calculated as described in materials and methods. Means (bar) between patients and HCs showed no statistically significant difference as examined by student's *t*-test. Subgroups of the diseases: ◇ Clinical Isolated Syndromes, ■ PPMS, △ SPMS, ▼ RRMS.



production of both Th cytokines following the stimulations of CD16, compared with other typical stimuli such as TCR cross-linking or PMA/Ionomycin.

3.7.1 Intracellular cytokine production by cross-linking of CD16

Purified γδ T cells were cultured under a variety of activating conditions and their cytokine production is measured. Cross-linking of surface receptors such as TCR or CD16 via plate-bound antibodies serves as a good source of stimulation, which leads to proliferation, production of various cytokines, or in some situations apoptotic death (189, 190). Combination of the compounds of PMA/Ionomycin has a strong effect on activating protein kinase C and motivating the calcium influx, and subsequently stimulates the production of cytokines and is therefore used as a standard positive control (191, 192).

γδ T cells that are CD16 positive or negative were distinguished on flow cytometry dot plots by setting two gates according to the MFI in channel FL-5, as shown in Figure 31. The MFI value used to define the boundary of the two gates was obtained by running an isotype matched control antibody. Cells in these two gates were respectively analysed for their intracellular fluorescence positivity of various cytokines that represent either Th1 (IFN-γ and TNF-α) or Th2 (IL-4 and IL-10) subtypes.

Cross-linking of TCR markedly increased the fraction of γδ T cells that produce IFN-γ, in both CD16 positive and negative populations, when compared with medium-alone control (Fig 32). However, it is apparent that the CD16 negative γδ T cells are more responsive to this stimulus than CD16 positive cells in that almost a third to half of the CD16 negative cells produced IFN-γ while about only a quarter of the CD16 positive γδ T cells did. A similar result could be seen when PMA/Ionomycin was used as positive controls, except that

Figure 31. Illustration of intracytoplasmic staining for detection of cytokines from $CD16^+$ and $CD16^-$ γδ T cells.

γδ T cells were cross-linked either by plate bound anti-TCR antibody or anti-CD16 antibody and cultured for 18 hrs. PMA/Ionomycin stimulation was used as the positive control. Cells were stained following the protocol and separated according to their CD16 positivity on flow chart. Both populations were further analyzed for their intracellular production of various cytokines (see Fig. 32 to 35).

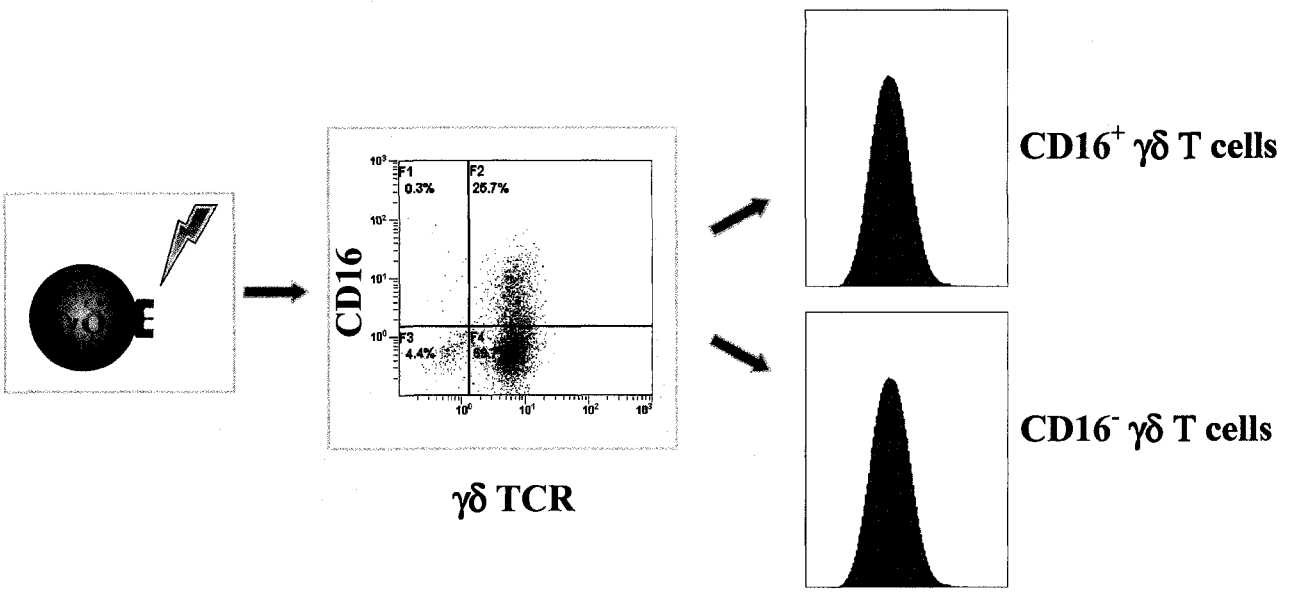


Figure 32. Production of IFN-γ by γδ T cells through cross-linking of TCR or CD16.

Cross-linking of TCR markedly increased the fraction of γδ T cells that produce IFN-γ, in both CD16 positive and negative populations, when compared with medium-alone control. However, it is apparent that the $CD16^-$ γδ T cells are more responsive to this stimulus than $CD16^+$ cells in that more than a third of the $CD16^-$ cells produced IFN-γ while about only a quarter of the $CD16^+$ γδ T cells did. In parallel with the results of TCR cross-linking, cross-linking the CD16 molecules also resulted in moderate increases of IFN-γ producing γδ T cells in the $CD16^+$ population. PMA/Ionomycin treated $CD16^-$ γδ T cells demonstrated strong ability of secreting IFN-γ, while IFN-γ secreting $CD16^+$ cells also increased in number, though to a lesser extent.

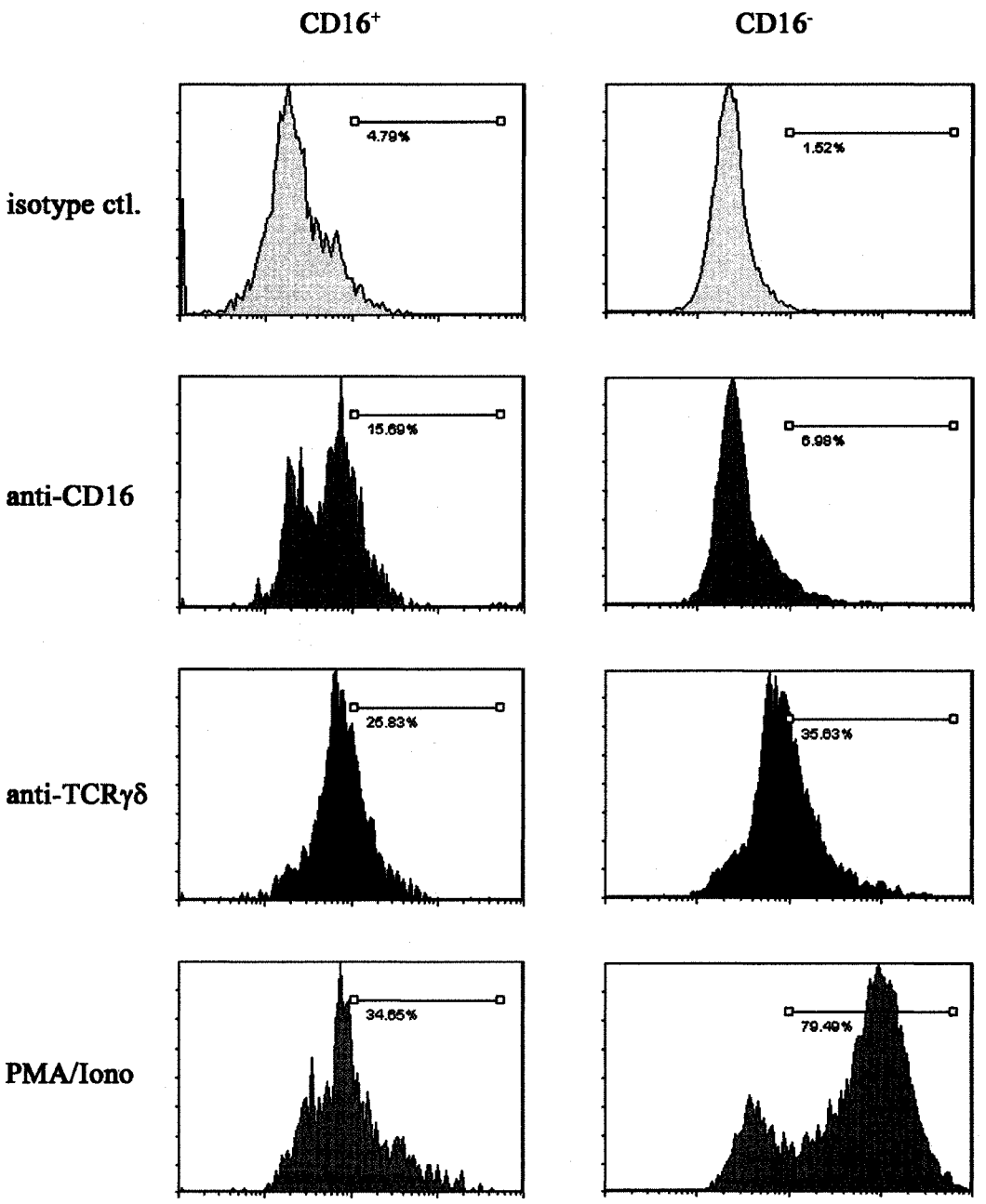
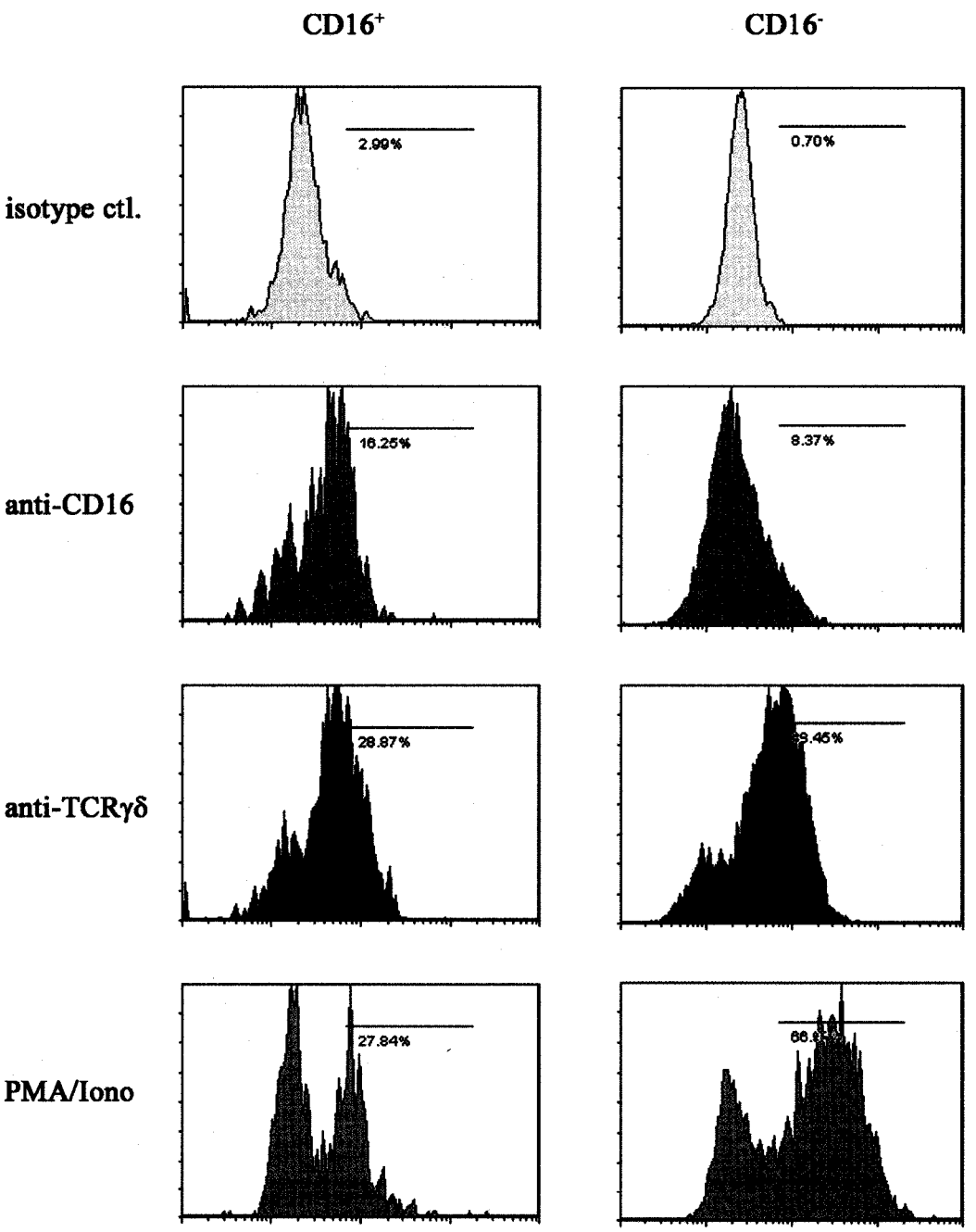


Figure 33. Production of TNF-α by γδ T cells through cross-linking of TCR or CD16.

Cross-linking of TCR markedly increased the fraction of γδ T cells that produce TNF-α, in both CD16 positive and negative populations, when compared with medium-alone control. However, similar to that of the IFN-γ, the $CD16^-$ γδ T cells are more responsive to this stimulus than $CD16^+$ cells. Also, cross-linking the CD16 molecules resulted in a moderate increase of TNF-α producing γδ T cells in the $CD16^+$ population.



the positivity is quite higher. In parallel with the results of TCR cross-linking, cross-linking the CD16 molecules also resulted in a moderate increase of IFN-γ producing γδ T cells in the CD16 positive population. Surprisingly, the CD16 negative γδ T cells also showed positivity in IFN-γ production, though to a much lower extent. This was unexpected because the cross-linking of CD16 molecules would not have any direct impact on these cells that do not express CD16. A possible explanation would be that activated CD16 positive γδ T cells were able to release yet unidentified cytokines that could function on the CD16 negative population in a "paracrine" manner, in which these unidentified cytokines would subsequently act on this population to stimulate the production of IFN-γ.

The production of TNF-α by γδ T cells under these conditions followed that of the INF-γ, and is also in agreement with results reported by others (171). CD16 cross-linking not only caused the increase of TNF-α production by CD16 positive cells, but also moderately by the CD16 negative γδ T cells. However, the CD16 negative cells are also more potent than the CD16 positive cells in TNF-α production under the general stimulations schemes (TCR cross-linking or PMA/Ionomycin) (Fig. 33).

The production of Th2 cytokines are shown in Figs. 34 and 35. Under all the stimulation conditions, almost no IL-4 or IL-10 can be detected in CD16 negative populations. However, cross-linking of TCR or CD16 resulted in increased amounts of CD16 positive γδ T cells that produce IL-4 to the extent similar to that of the PMA/Ionomycin control. IL-10 production was also increased in CD16 positive populations when their CD16 molecules are cross-linked, but only slightly when the TCR receptors are linked.

Figure 34. Production of IL-4 by γδ T cells through cross-linking of TCR or CD16.

Under all the stimulation conditions almost no IL-4 can be detected in $CD16^-$ populations. However, cross-linking of TCR or CD16 resulted in increased number of $CD16^+$ γδ T cells that produce IL-4 to an extent similar to that of PMA/Ionomycin control.

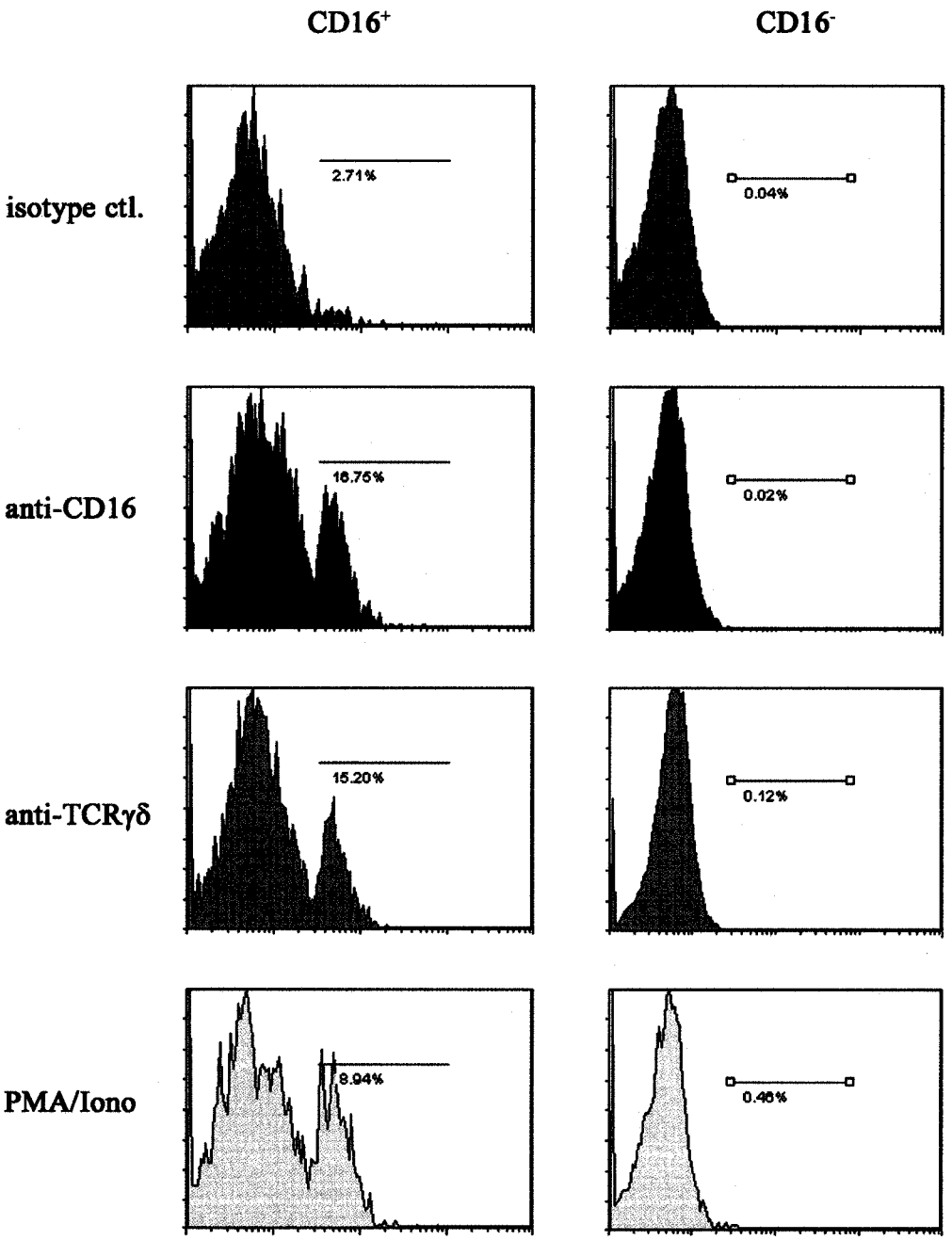
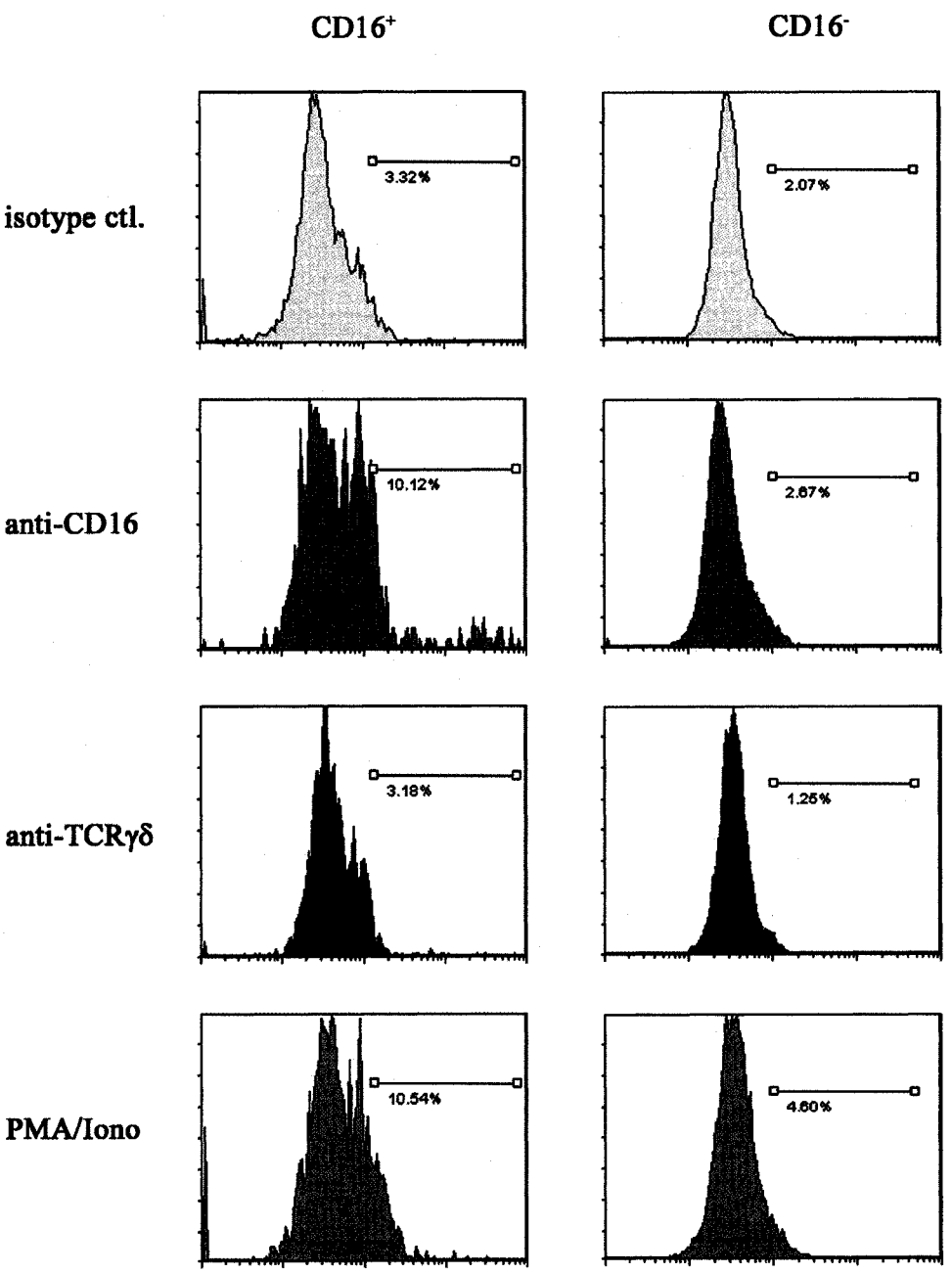


Figure 35. Production of IL-10 by γδ T cells through cross-linking of TCR or CD16.

Under all the stimulation conditions almost no IL-10 can be detected in $CD16^-$ populations. However, cross-linking of CD16 resulted in an increased number of $CD16^+$ γδ T cells that produce IL-10 to an extent similar to that of the positive control. Cross-linking of TCR did not remarkably increase IL-10 production in either population.



Taken together, both CD16 positive and negative γδ T cells are able to produce TNF-α and IFN-γ under various conditions tested, however the CD16 positive population is less potent in doing so than the CD16 negative. On the opposite note, only CD16 positive γδ T cells could be detected producing Th2 cytokines such as IL-4 or IL-10.

3.7.2 Cytokine production of γδ T cells stimulated by antibody coated cells

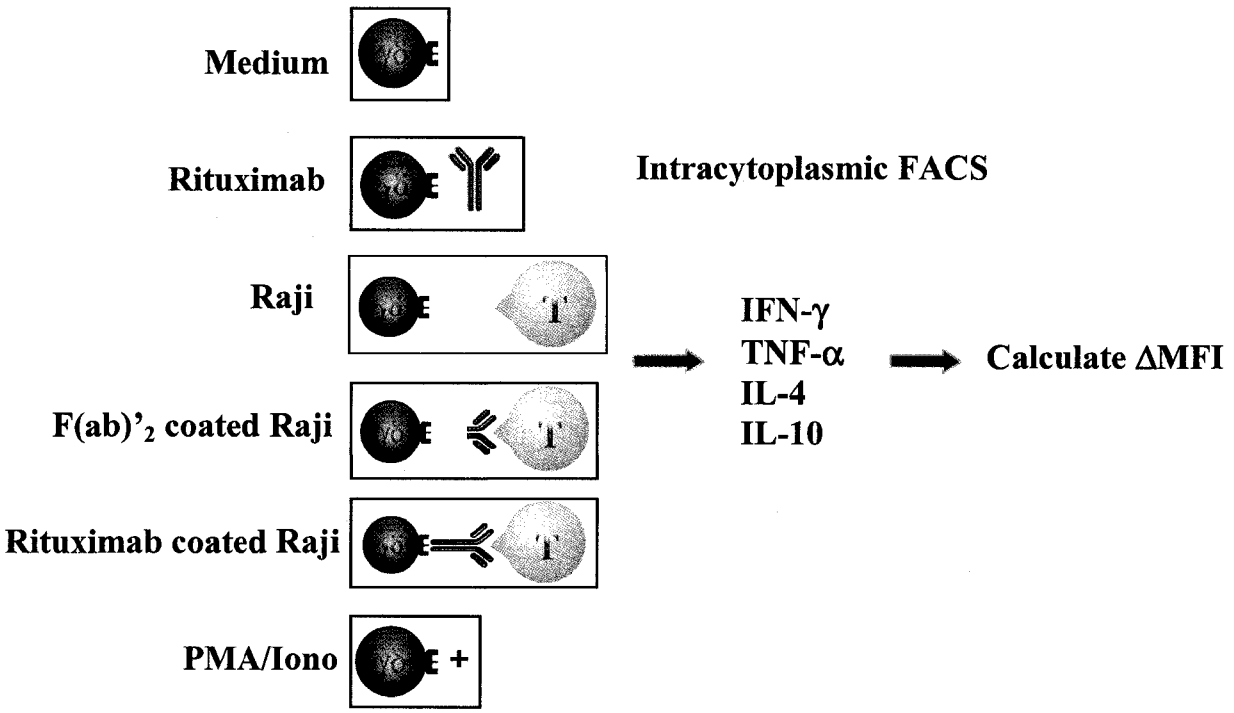
Cross-linking of surface antigens such as CD16 on γδ T cells offers an artificial model to study cytokine production, but the bio-physiological functions of CD16 induced activation of γδ T cells would be better reproduced by engaging this receptor with antibody coated stimulant cells. In the following study, cytokine production of γδ T cells stimulated with rituximab coated Raji cells were examined and quantified, together with various other conditions that include Raji cells alone, rituximab, $F(ab')_2$ fragment, and $F(ab')_2$ coated Raji cells, as shown in Figure 36.

Production of cytokines by γδ T cells under various conditions shown in Fig. 36 was quantified by the change of MFI (ΔMFI), which was defined below:

$$\Delta MFI = \frac{MFI_{sample} - MFI_{control}}{MFI_{control}}$$

In agreement with experiments above, PMA/Ionomycin can efficiently stimulate the production of TNF-α and IFN-γ from both CD16 positive and negative γδ T cells, with CD16 negative population being more potent. However, rituximab antibody, Raji cell alone, or $F(ab')_2$ coated Raji cells did not induce TNF-α or IFN-γ production in either CD16

Figure 36. Illustration of the experimental scheme for cytokine production by γδ T cells through CD16 engagement.



positive or negative γδ T cells. Engagement of CD16 by rituximab coated Raji cells induced production of TNF-α or IFN-γ from CD16 positive γδ T cells ($p<0.05$), but only slightly in CD16 negative γδ T cells (p=ns) (Figs. 37 and 38).

CD16 engagement by rituximab coated Raji cells significantly increased the production of IL-4 (Fig. 39) or IL-10 (Fig. 40) in $CD16^+$ γδ T cells; while no statistical difference was apparent when $CD16^+$ γδ T cells were stimulated with rituximab, Raji alone, or $F(ab')_2$ coated Raji cells. Under all conditions studied, IL-4 or IL-10 was not measurably produced by CD16 negative γδ T cells.

In conclusion, engagement of CD16 on γδ T cells by antibody coated cells induced production of cytokines of both Th1 (TNF-α or IFN-γ) and Th2 (IL-4 or IL-10) subtypes. The increase in Th1 production is moderate and the Th2 producing cells are exclusively CD16 positive γδ T cells.

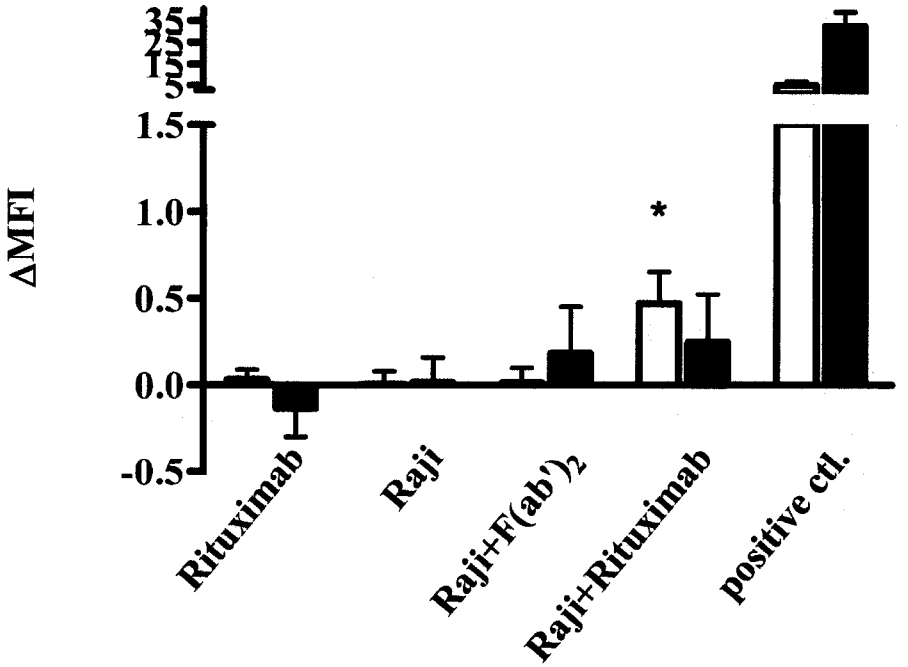
Figure 37. IFN-γ production by γδ T cells through CD16 engagement.

Production of IFN-γ by γδ T cells under various conditions was quantified by the change of MFI (ΔMFI). PMA/Ionomycin can efficiently stimulate the production of IFN-γ from both CD16 positive and negative γδ T cells, with $CD16^-$ population being more potent. However, rituximab antibody, Raji cell alone, or $F(ab')_2$ coated Raji cells did not induce IFN-γ production in either CD16 positive or negative γδ T cells. Engagement of CD16 by rituximab coated Raji cells induced production of IFN-γ from $CD16^+$ γδ T cells, but not, or only slightly, from $CD16^-$ cells. Open bar: $CD16^+$ γδ T cells; closed bar: $CD16^-$ γδ T cells. * $p<0.05$ by ANOVA.

Figure 38. TNF-α production by γδ T cells through CD16 engagement.

Production of TNF-α by γδ T cells under various conditions was quantified by ΔMFI. PMA/Ionomycin can efficiently stimulate the production of TNF-α from both CD16 positive and negative γδ T cells, with $CD16^-$ population being more potent. However, rituximab antibody, Raji cell alone, or $F(ab')_2$ coated Raji cells did not induce TNF-α production in either CD16 positive or negative γδ T cells. Engagement of CD16 by rituximab coated Raji cells induced production of TNF-α from $CD16^+$ γδ T cells, but only slightly from $CD16^-$ cells. Open bar: $CD16^+$ γδ T cells; closed bar: $CD16^-$ γδ T cells.
* $p<0.05$ by ANOVA.

IFN-γ



TNF

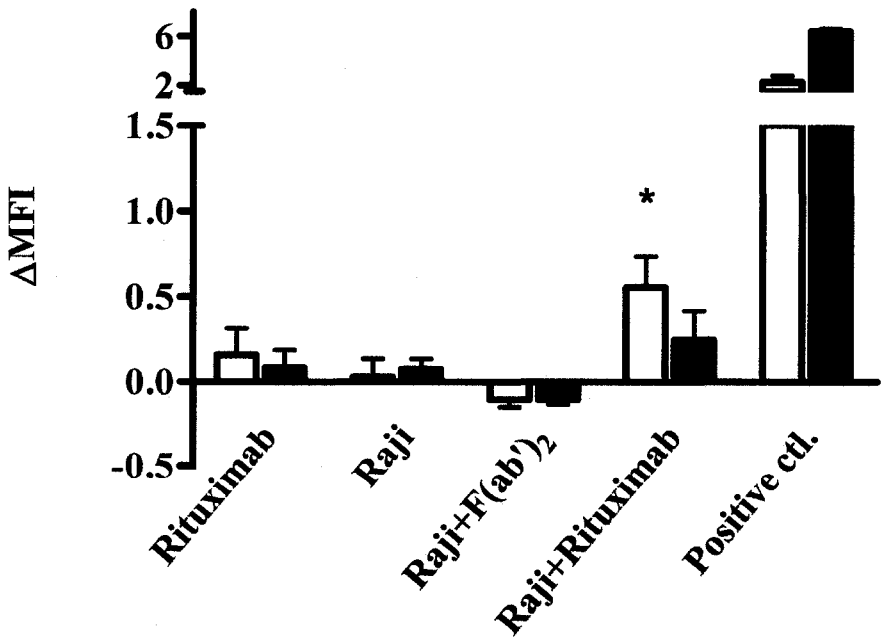


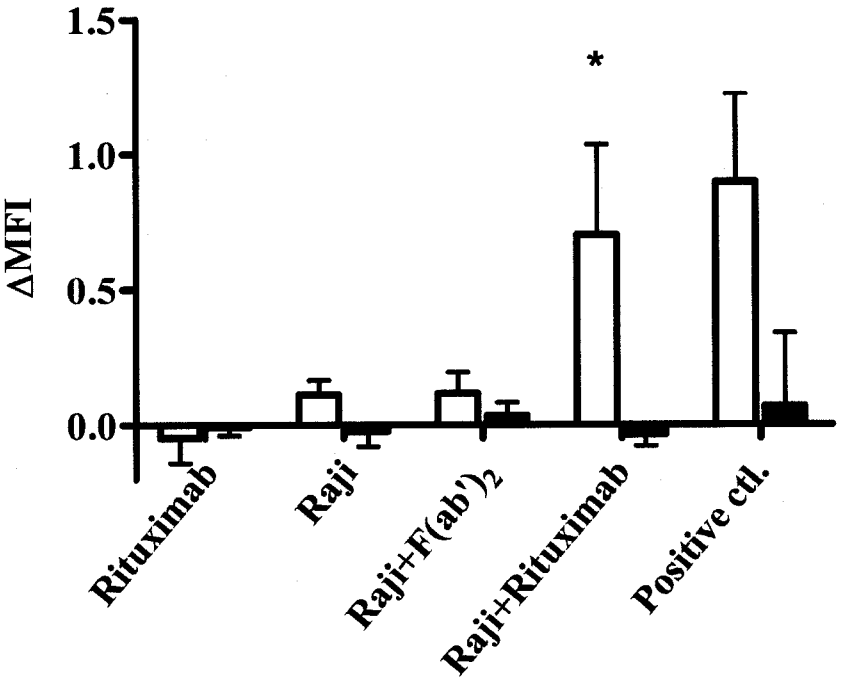
Figure 39. IL-4 production by γδ T cells through CD16 engagement.

CD16 engagement by rituximab coated Raji cells could significantly increase the production of IL-4 in $CD16^+$ γδ T cells; while no statistical difference was noticeable when $CD16^+$ γδ T cells are stimulated with rituximab, Raji alone, or $F(ab')_2$ coated Raji cells. Under all conditions studied, IL-4 was not measurably produced by $CD16^-$ γδ T cells. Open bar: $CD16^+$ γδ T cells; closed bar: $CD16^-$ γδ T cells. * $p<0.05$ by ANOVA.

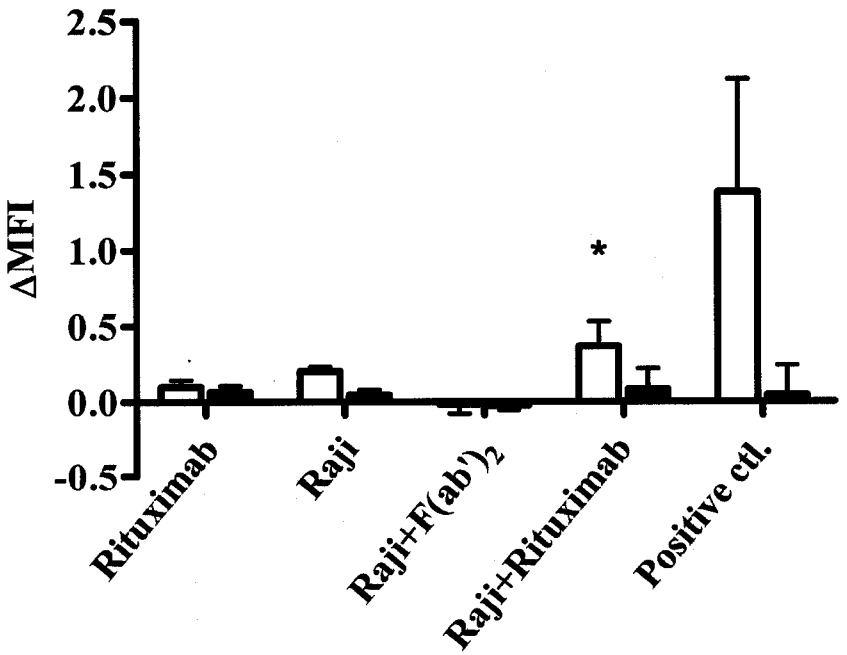
Figure 40. IL-10 production by γδ T cells through CD16 engagement.

CD16 engagement by rituximab coated Raji cells could noticeably increase the production of IL-10 in $CD16^+$ γδ T cells, mirroring the results of that of IL-4. Under all conditions studied, IL-10 was not measurably produced by $CD16^-$ γδ T cells. Open bar: $CD16^+$ γδ T cells; closed bar: $CD16^-$ γδ T cells. * $p<0.05$ by ANOVA.

IL-4



IL-10



4. Discussion

4.1 Overview

My research is based on the hypothesis that a specialised lymphocytic cell group of the innate immune system (γδ T cells) is involved in the immune injury to myelin and axons as part of the immunopathogenesis of MS. I am particularly interested in the role of γδ T cells that express Fcγ receptors. FcγR can have many functions that include both regulations of cell functions, as well as acting as conduits for specialized cytotoxicity. There are many mechanisms that γδ T cells may use to cause injury towards OGC (the cell of the CNS that maintains myelination) *in vitro*. In this work, I examined the potential role of one of the cytotoxic mechanisms used by γδ T cells, that of antibody dependent cell-mediated cytotoxicity (ADCC). Moreover, it was equally important to determine if there were any regulatory functional changes of γδ T cells after their FcγRs were engaged. Such an alteration represented by cytokine production change would help to understand better some of the previously unknown characteristics of γδ T cells in the pathogenesis and development of MS.

The results obtained from this study have revealed that there are many more cells expressing the third subtype of Fcγ receptor (CD16) on γδ T cells derived from MS patients, particularly in those patients with a progressive course. This increase is correlating with patients' disability and length of disease. A possible driving force behind this increase has been identified as some of the proinflammatory cytokines.

By using two in-house developed experimental systems, I was able to demonstrate that γδ T cells, in the presence of proper bridging antibodies, can indeed mediate ADCC-type cytotoxicity *in vitro*, a first of such study by utilising an "all human" components in a situation simulating physiological situations.

Lastly, by using intracytoplasmic staining, I have shown that γδ T cells produce both Th1 and Th2 cytokines. Surprisingly, however, was the discovery that only the $CD16^+$ γδ T cells but not the $CD16^-$ subpopulation were able to produce detectable Th2 cytokines.

Taken together, these results support my hypothesis that CD16 positive γδ T cells are capable of ADCC killing, and these cells modulate immune system by releasing a variety of cytokines. That they are more likely found in patients with progressive and long duration disease indicates these mechanisms may be an important contributor to disease progression which is felt to be due to the loss of myelin and axons.

4.2 Increased presentation of $CD16^+$ γδ T cells in MS

In this study I showed that MS patients have a significant increase in a particular subset of γδ T cells, those bearing the NK-type marker CD16. In addition, the percentage of CD16 expressing γδ T cells (%$CD16^+$) correlated with disease duration and severity suggesting they may be contributing to disease progression.

Particular to γδ T cells, whether derived from patients or healthy controls, the expression of CD16 demonstrated a continuum pattern that no distinct boundary between CD16 negative and positive populations could be identified on a typical flow cytometry plot (which warrants the necessity to include an isotype antibody control in the quantitation process). Also of interest, no dichotomous distribution of CD16 level, (i.e. $CD16^{high}$ vs.

$CD16^{low}$) that is more typically seen on NK cells, was observed on γδ T cells. Indeed, the $CD16^+$ γδ T cells are quite a homogeneous pool.

A higher number of γδ T cells expressing $CD16^+$ has also been observed in other autoimmune diseases such as rheumatoid arthritis (RA) or Sjogren's syndromes, albeit the extent of this elevation in RA is relatively moderate (180, 181). Increased $\%CD16^+$ may reflect their activation status in these diseases as it has been shown that $CD16^+$ γδ T cells concurrently increase the expression of CD25 (178) but decrease the expression of CD27 and CD62L, a phenotype of activation (133). However, it has also been reported that over-stimulation of γδ T cells *in vitro* may actually lead to the down-regulation or shedding of CD16 molecules (180). It is particularly interesting that PHA activated γδ T cells gradually gain the expression of HLA-DR which coincides with the diminishing level of CD16 (181). HLA-DR, the type II MHC molecule, displayed by activated $V\delta2^+$ γδ T cells, has been shown to efficiently process and present antigens and provide co-stimulatory signals, strong enough to match those of the professional APCs such as dendritic cells, for the induction of the proliferation and differentiation of naïve αβ T cells (193). Therefore the timeline of transition from CD16 to HLA-DR may suggest that γδ T cells, by using their Fcγ RIII, capture and internalise immune-complex that they encounter and subsequently feed the HLA-DR groves with the processed antigens, in a way analogous to membrane bound receptors on B cells. This may partially explain the high potency of antigen presentation ability of γδ T cells, because it is known that receptor-mediated uptake and presentation of antigens is 100- to 10,000-fold more effective than endocytosis (194). Certainly, conformational studies are required to prove this argument.

Previous studies had implied that there might be an age related expression of CD16 molecule on NK cells or neutrophils (CD16 expression on neutrophils decreases with aging) (195, 196). However, there was no conclusive data from the literature showing that the percentage of $CD16^+$ γδ T cells correlates with age; actually, no correlation of age and CD16 positivity on γδ T cells was observed in this study (p=0.055 for MS patients and p=0.196 for healthy controls), though there was a trend that older control individuals having higher $\%CD16^+$ compared with younger controls on a population level, but this could be caused by a single outlier in the HC/O subset. Also, the correlation for MS patients group demonstrated a trending to positive. As such, it is highly reasonable to postulate that the trend seen towards an increased representation of $\%CD16^+$ in MS could be the result of disease related factors, one of such may well be a group of inflammatory cytokines.

Increased levels of various pro-inflammatory cytokines were observed in the systemic circulation at different stages of MS (119); in particular, at the SPMS stage heightened levels of IL-2, -12 and -15 were reported (186-188). The observations that these same cytokines significantly increased $\%CD16^+$ suggests that chronic dysregulation and an inflammatory milieu might be the cause for the observed increased $\%CD16^+$ in the SPMS patients.

Although IL-2 and IL-15 are structurally and functionally related cytokines and their receptors share two of the three heterotrimer subunits, IL-2 is produced by activated $CD4^+$ T cells and functions on αβ T lymphocytes; while IL-15 is generated by a large variety of cell types, but preferentially works on γδ T cells (197). In this study the most robust increase in $\%CD16^+$ was in response to IL-15.

The ability of IL-15 to increase CD16 expressing γδ T cells was also noted in other studies (172). Increased %$CD16^+$ may not solely be due to the presence of IL-15 selectively expanding the $CD16^+$ population, since NK cells, both $CD16^-$ and $CD16^+$, proliferate equally in response to IL-15 (134). $CD16^+$ cells might also come from the $CD16^-$ pool in response to antigen-stimulation, such that the increase in $CD16^+$ γδ T cells observed here could actually derive from $CD16^-$ cells in response to unknown antigen stimulation together with the proinflammatory cytokine milieu seen in progressive MS (198). A way to examine this possibility would be to sort the cells into CD16 negative and positive populations first and then analyze their responsiveness to IL-15.

Surprisingly, IL-12, the signatory Th1-inducing cytokine, failed to increase the CD16 expression to the same extent as observed with IL-2 or IL-15 (Fig. 16). This may be explained by the fact that IL-12 has an intrinsic anti-proliferative property that would inhibit the growth of pre-activated cells (199). Indeed, Lin *et al.* has reported an antagonistic effect of IL-12 on IL-15 induced $CD16^+/CD56^+$ NK cell proliferations and ADCC activity (200). Alternatively, it could also be possible that the receptor level for IL-12 on γδ T cells is much lower than that of IL-2/15, or that IL-12 uses different intracellular signalling pathways from those of IL-2/IL-15, which subsequently act differentially on the expression of CD16. Of course, additional work would need to be done to confirm these possibilities.

Studies have shown that immunomodulatory therapies have little effect once patients enter a progressive phase of their illness, reflecting probably more the neurodegenerative disease component (201). However, evidence has also shown that the immune system itself is much more dysregulated in the same progressive stage of MS and that it carries a different, diffusive form of inflammation to the brain (202). This prompted Vaknin-

Dembinsky and Weiner to propose that while abnormalities in adaptive immunity might play an important role in RRMS, innate immunity may be a more important contributor to the disease process in SPMS (202). γδ T cells are a very important and prominent T cell subset involved in innate immune responses and could be postulated to contribute to disease progression by releasing proinflammatory cytokines, or via cytotoxic mechanisms. In agreement with this argument, it has been shown here that %$CD16^+$ γδ T cells was positively correlated with both MS disease severity and progressions (Fig. 9). Because of the progressive nature of MS, it would be unwise to disconnect disease duration and EDSS when considering the impact, or consequence, of CD16 changes in the development of MS. While it can be seen from this study that EDSS correlated with the length of disease, and this intertwined "triangular" relationship among disease duration, EDSS, and %$CD16^+$ is well demonstrated in Fig. 10.

What are the biological significances of this observed elevation of %$CD16^+$ in MS? The answer lies in that CD16 expressing γδ T cells may play a very particular role via ADCC in conjunction possibly with antibodies to myelin elements that are seen in MS patients (85), which is discussed further below.

In summary, the results showing elevated percentage of CD16 expressing γδ T cells correlating with disease severity and progression is a further demonstration that innate immune processes may be particularly involved in mediating the chronicity of MS.

4.3 Development of methods for the quantification of ADCC

The second important focus of this study, was to show, using two different approaches (ELISA and FC_3A), convincingly that $CD16^+$ γδ T cells are capable of mediating ADCC cytolysis *in vitro*.

A confirmational study was lacking that γδ T cells were able to mediate ADCC killing of their targets. Central to this issue was the relative unavailability of a bridging antibody that bears the Fc portion of human origin. To circumvent this problem, an ELISA based system was designed by making use of commercial available polyclonal human anti-Rh_o antibody along with Rh_o^+ human red blood cells. In anticipation of ADCC cytolysis of anti-Rh_o coated red cells by γδ T cells, human hemoglobin released into the supernatant correlated with the amount of dead cells and could be detected by specific ELISA. Indeed, lysis was significantly increased in the presence of the anti-Rh_o compared with the controls (Fig. 18). Unintentionally however, was the finding that one of the mechanisms that γδ T cells use to cytolyse targets was to release pore-forming proteins (perforin) upon CD16 activation, which resulted in the membrane rupture of red blood cells and discharge of hemoglobin and they did this in the presence of anti-Rh_o.

To scale-up one step further by using a nucleated cell line as target, which may differ from red cells in death mechanisms, and still align with the requirements of a "human" antibody in the composition of ADCC killing, I set up another system by taking advantage of the humanized anti-CD20 antibody and developed a flow cytometry based cellular cytolysis assay, namely FC_3A, to measure ADCC induced by γδ T lymphocytes. However the application was not restricted to this purpose but could be readily adapted to study

ADCC-mediated or even non-ADCC cytotoxicity of other immune cells, so long as the effector and target differ in at least one surface marker that could be fluorescently labeled to distinguish the two populations.

The cornerstone underlying the successful application of FC_3A was the ability to discriminate effector and target populations, which made further characterization of target cells' fate feasible. The distinction was normally achieved by labeling either target cells or effector cells with membrane binding fluorochromes before mixing them together. However, this may result in artificial staining of the other population during co-culturing because of spontaneous dye leakage (albeit at a low level) (203) or inter-cellular membrane transfer through immunological synapses (204). In this study, a newer approach was applied to use fluorochrome conjugated target-specific mAb to label the target cells after co-culturing (203). This procedure allowed to clearly separate the effector and target populations cytofluorometrically and to avoid any unintentional contamination.

This approach was especially favourable when prolonged co-culturing time was desired. In this study, co-culturing times of 4 hr and 18 hr were compared (Fig. 21) and the 18 hr protocol was chosen because it was found that this longer culturing period yielded better signal-to-background ratio on the results. Another reason that the dye leakage could be a particular concern in this study was because it had been shown that γδ T cells had a great tendency to snatch membrane patches off Burkitt's lymphoma cells (Daudi or Raji) through immunological synapse, a biological phenomenon termed as "trogocytosis" (204), and therefore inadvertently acquired the fluorescent dye and blurred the border between E:T populations on a typical flow cytometric plot.

To ensure an accurate quantification of ADCC lysis, it was also critical to make certain that the surface marker used to distinguish target cell populations (i.e. CD19) did not manifest a significant change effected from the impact of the apoptotic death of these cells, at least during the period of co-culturing. This concern was assured by inducing apoptosis of target cells with X-ray irradiation and tracking their CD19 changes with the passing of time. It could be seen from Fig. 41 that the CD19 level did not noticeably diminish even after 24 hrs since the induction of apoptosis.

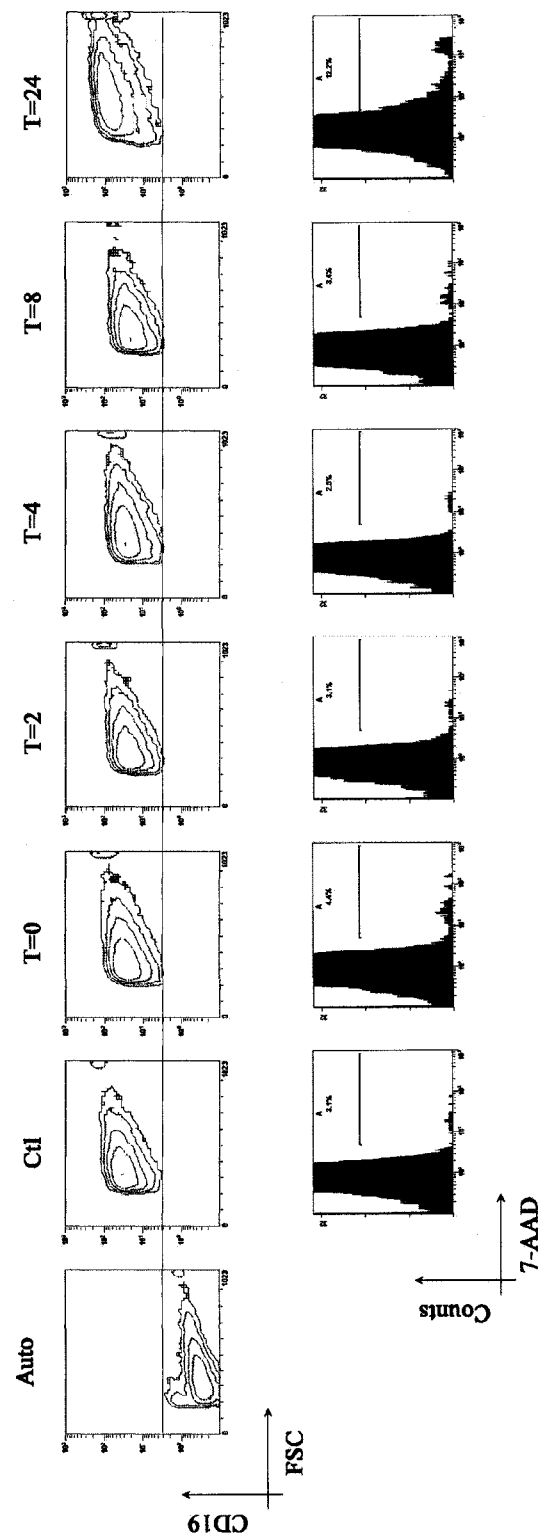
4.4 CD16$^+$ γδ T cells mediate ADCC

A limited number of previous studies attempted to show that γδ T cells could lyse antibody-coated target cells based on the hypothesis that the CD16 expressed on γδ T cells is fully functional. However a careful literature search has demonstrated that this is the first report of its kind to confirm this hypothesis on a cellular level by using an "all human" system simulating physiological situations (Fig. 1A), and by dissecting the functional contributions of each of the key individual players in the cytolysis process. In this study, by utilizing a humanized anti-CD20 mAb (rituximab), it has been shown that CD16 positive γδ T cells are able to mediate ADCC type of cytotoxicity *in vitro*, and the specific lysis correlates with antibody concentration, E:T ratio, presence of Fc portion of the antibody, and the presence of CD16 on the surface of the γδ T cells.

Recently, a Japanese-Australian international group also reported a similar result by using the trio of lymphoma + rituximab + γδ T cells (205). Though their research perspective is oncology oriented, the similarity of the results could lend support to the conclusions of this project that CD16$^+$ γδ T cells are capable of ADCC.

Figure 41. CD19 does not diminish with the apoptotic death of target cells.

Apoptotic death of Raji cells was induced by X-ray irradiation at 30 Gy dosage. Cells were re-plated and cultured in a CO_2 incubator and tested for their CD19 expression over time. T: hours after irradiation. Cell death was confirmed by positive staining of 7-AAD, nevertheless, CD19 expression was not diminished even though 7-AAD$^+$ cells increased over time.



In this study, two dose-dependent curves were shown illustrating the rise of specific lysis in response to the increase of the antibody concentration or the γδ T cell numbers, two critical components in the ADCC system (Figs. 23 - 25). Also serving as an indirect proof of the CD16 involvement in this process, the elimination of Fc portion from rituximab totally abolished the ADCC lysis (Fig 28).

Because of the inability to find an antibody capable of blocking CD16 on γδ T cells, which was a simple but compelling direct proof of γδ T cells' ADCC capability, I applied a strategy whereby $CD16^+$ cells were either nearly completely removed or titered back to examine their contribution to ADCC. The ADCC lysis could be abrogated by removing the $CD16^+$ population from the γδ T cells effector pool, and augmented by increasing the numbers of CD16 expressing γδ T cells (Fig. 29). The choice of using clone LNK16 (FITC-CD16) mAb to sort the cells was two-fold: first, to avoid any interference with the subsequent FC_3A assay, which would utilize at least two fluorochromes (PE and 7-AAD, in this case) that were detected in FL-2 and FL-4 respectively; and secondly to circumvent any potential interactions between the IgG1 antibody and CD16 molecules on γδ T cells, which may lead to self destruction of these cells, because it is known that human CD16 has very low or no affinity towards murine isotype γ1 IgG (177).

Interestingly though, when γδ T cells derived from either MS patients or healthy donors were compared with regard to their ADCC cytotoxicity at per $CD16^+$-cell level under the current experiemental scheme, no statistically significant difference was observed (Fig. 30). This result suggested that the highly differentiated subset of $CD16^+$ γδ T cells held intrinsic cytotoxic potential against targets, irrespective of disease state. However, the cells were derived from peripheral blood and the relevance to MS and other disease states might

well lie in their skewed presence under proinflammatory situations where $CD16^+$ γδ T cells had been found to be increased in several autoimmune disorders including MS, rheumatoid arthritis, and Sjogren's syndrome (180, 181, 206). In line with this hypothesis, I and others had shown that the percentage of $CD16^+$ cells in the γδ T cell pool could be elevated in response to Th1 cytokines including IL-2 and IL-15 (Fig. 16, and Ref (172, 206)).

Raji and Daudi cells, targets used in this project, express FcγRII, another low affinity Fcγ Receptor (207). However the observation that high concentrations of human IgG (10 μg/ml) only marginally labeled these cells demonstrated that the binding of antibodies to this FcγR on the target cells was insignificant, and its interference with ADCC was negligible (Fig. 23).

The human-mouse chimeric monoclonal antibody rituximab is the first mAb to be approved by the US Food and Drug Administration for treatment of malignancy, specifically $CD20^+$ B cell non-Hodgkin's lymphoma. The variable region of this antibody is cloned from a mouse anti-human CD20 antibody and the constant regions of both κ light chain and γ1 heavy chain are copied from human sequences (98). The humanized Fc portion of this antibody not only significantly reduces its antigenicity as a foreign protein, more importantly it helps rituximab to interact with and activate the machinery of the host immune system, which subsequently participates in the elimination of the $CD20^+$ B cells (208). Although most of the B cells are supposedly eliminated through complement mediated lysis, our current work suggests that $CD16^+$ γδ T cells could assist in this elimination via ADCC (99). Given that these cells are increased in progressive MS (206) and rituximab is currently involved in clinical trials for this form of MS in particular, these observations could also explain any perceived benefit of this treatment.

4.5 Immuno-modulation of γδ T cells via CD16 activation

Although it has been shown that by default the majority of γδ T cells are biased towards producing Th1 cytokines such as IL-2 and IFN-γ (209), they are capable producers of cytokines of both Th types (210). Which lineage they are committed to is largely influenced by the cytokine milieu in which they are initially primed by the antigenic stimulation (211). A Th1 inducing cytokine such as IL-12 may activate the STAT4/T-bet intracellular signaling pathway, which leads to the production of Th1 cytokines and inhibition of Th2, whereas in the opposite scenario, the STAT6/GATA-3 pathway would be motivated and the relevant genes activated to produce Th2 cytokines and reciprocally depress Th1 cytokine production (212).

Results of this study showed that both CD16 positive and negative γδ T cells were able to produce IFN-γ upon TCR activation, but the CD16 expressing γδ T cells were much less potent in this mode (Fig. 32). This finding may fall in line with the observation of others that $CD16^+$ γδ T cells were less proficient in IFN-γ production, but rather are a stronger performer in the process of cytotoxicity (133).

As a co-activating receptor secondary to TCR, CD16 engagement on γδ T cells also leaded to the production of IFN-γ, though to a lesser degree. Unexpectedly, though, the CD16 negative population also produced detectable amount IFN-γ after the antibody-coated stimulant cells were added to γδ T cells cultures, though failed to reach statistical significance. A possible explanation could be that by means of "paracrine" cytokine actions, the $CD16^+$ γδ T cells released some kinds of cytokines, possibly including IFN-γ, that acted on the $CD16^-$ population, which in turn synthesized fairly low level of IFN-γ. A

conformational study needs to address this possibility. This could be achieved by first separating these two populations before the stimulation is applied, and their cytokine secretion examined in tandem.

IFN-γ is strongly related with MS, which is believed to be a disease of inflammation in nature. Demyelination of the CNS induced by IFN-γ may involve [a] classical activation of macrophages/microglia which produce nitric oxide and are essential in the effective phase of CNS injury (213); or [b] by eliciting direct cytotoxicity towards OGCs (214). It may also help worsen the disease by inhibition the process of re-myelination (200). There also exists argument, however, that IFN-γ is immune-regulatory at the height of MS or EAE because it has been shown that IFN-γ exerts suppressive effects on activated $CD4^+$ encepholitogenic cells (215).

By strict definition, TNF-α belongs to neither Th1 nor Th2 cytokine subset (216). However, it has close proximity to IFN-γ in biological functionalities and the two were found up-regulated in parallel in MS (200). Figs. 33 and 38 demonstrate that the production of TNF-α by γδ T cells under various stimulation conditions, irrespective of CD16 positivity, follow those of the IFN-γ. These results may serve as a further confirmation that γδ T cells, especially those that are CD16 deficient, contribute to the inflammatory response in the development of MS.

TNF-α is one of the earliest cytokines to be released by the host in response to an immune attack (217). It recruits and stimulates innate immune cells such as macrophages to the site of inflammation and can also in its own power induce apoptosis of target cells (218). Evidence exists that TNF-α has great pathogenic potential in MS by various mechanisms including up-regulation of adhesion molecules, stimulation of secretion of inflammatory

mediators, direct injury to myelin, or interference with impulse propagation (219). However, clinical trials attempting to neutralize TNF-α in MS patients by administration of either anti-TNF-α antibody or its soluble receptors have failed to ameliorate disease (115, 220), reflecting the "redundancy" feature of cytokines and as well the complexity of MS.

Another novel observation in this study, rather unexpectedly, was that $CD16^+$, but not $CD16^-$, γδ T cells produce detectable amount of Th2 type cytokines such as IL-4 and IL-10 upon TCR or CD16 stimulation (Figs 34 and 35). This phenomenon may strengthen the argument that γδ T cells are capable of regulating immune responses, additional to the point that they were of "traditional" killer cells. Th2 cytokines were known to be inflammation suppressors whereby they antagonize inflammatory action of the Th1 subset, though their principal effector functions include stimulating eosinophils, the clearance of helminths or Ig-E induced allergic reactions (221). Consistent with this conception, it has been shown that the remission stage of MS is predominantly associated with Th2 cytokines (222, 223).

IL-4, the centrepiece of Th2 cytokine, can be detected in some of the $CD16^+$ γδ T cells upon CD16 engagement (Fig. 39). Being dubbed as an anti-inflammatory cytokine, IL-4 is important in cell-mediated inflammation resolution (224). It achieves this by repressing the effects of IFN-γ on the activation of macrophages (225), or alternatively, it may induce an impaired activation status of macrophages, which has increased MHC II expression but reduced NO and proinflammatory cytokine secretion (226). IL-4 is also an important hormone for B cell and plasma cell growth, thus that it may claim a proper role in autoimmune process by stimulating the production of auto-antibodies (227).

Data suggest that IL-10 is synthesized upon CD16 engagement on γδ T cells (Fig. 40). One similar function to IL-4 is that IL-10 can contain inflammation by inhibiting the

activation of macrophages, which would subsequently reduce cytokine production and co-stimulator expression (228). More importantly though is that IL-10 assumes a critical role in the class switch of immunoglobulin G. It has been shown that γ1 and γ3 switch by B cells are highly dependent on IL-10, together with a secondary co-stimulation through CD40 molecules (229). Interestingly, CD16 embraces higher affinity to IgG1 and IgG3 than other types of IgGs with different γ heavy chains (230). Therefore it is tempting to suggest that some of the $CD16^+$ γδ T cells, which constantly sense their micro-environment for existence of antibody-antigen complex, would produce IL-10 upon binding with these complexes, which in turn direct the B cells to secrete antibodies with heavy chain types that favour the binding to CD16, a positive feedback loop.

It does not tell us however, from the results of this study whether the Th1 producing $CD16^+$ γδ T cells are also the source of Th2 cytokines (i.e. a single $CD16^+$ γδ T cell produces both Th1 and Th2 cytokines). However it would be reasonable to postulate that there are actually two separate lineages due to the "mutual exclusion" characteristic of these two subsets of cytokines. Indeed, some animal studies have shown that γδ T cells with different usages of Vγ genes may be committed to develop into different Th subsets (231, 232). Therefore it is possible that γδ T cells express other surface markers, other than CD16, to allow for the identification of potentially distinct Th lineages. This may be confirmed by an immunocytochemistry assay to determine the activation status of either one of the two transcription factors T-bet or GATA-3.

4.6 Implications in MS

Although the aetiopathogenesis of MS is largely unknown, it is well established that the damage to the CNS is the result of inflictions from a dysregulated and confused self immune system. γδ T cells are an important innate immune player that has been implicated in the development of MS by ways of MHC-non-restricted cytotoxicity or cytokine production. The results of this study showing that the number of $CD16^+$ γδ T is increased in MS patients especially in the progressive phase of the disease, suggests that they are particularly involved in the chronicity of MS, and indicates that inflammation induced by innate immunity is prominent in the later stage of MS (202).

Involvement of B cells and autoantibodies to myelin-forming cells in the CNS have been suggested to play a significant role in MS (233). Which antibodies are involved in this process are still not unambiguously identified, but this work shows that it is possible that γδ T cells, known to be increased in MS lesions and maintain activated status in the blood of MS patients (145) could possibly be implicated in the process by binding them and causing damage of CNS via ADCC, a new discovery which affixes a novel killing mechanism to the γδ T cells in the pathogenesis of MS. Moreover, what makes this new insight even further appealing is the observations that, through the same CD16 engagement, this special sub-type of γδ T cells releases cytokines that could possibly either temper the inflammation mediated by cellular immunity or sustain the responses carried by the humoral immunity. The findings of this work tie together the innate and adaptive immune responses through an interaction that involves the stimulation of antibody production, possibly against CNS molecules such as myelin, via CD16 expressing γδ T cells that could use these antibodies to enact a guided attack on CNS targets via the mechanism of ADCC. This study offers further

novel insights into the possible roles of γδ T cells in MS that can involve both potentially pathogenic and immunoregulatory functions (Fig. 42).

Meanwhile, this study also offers a clue into the understanding of one potential therapeutic mechanism of rituximab in the treatment of B cell mediated diseases such as MS, whereby the $CD16^+$ γδ T cells, working in concert with other immune actions, clear the disease causing B cells from the circulation of MS patients.

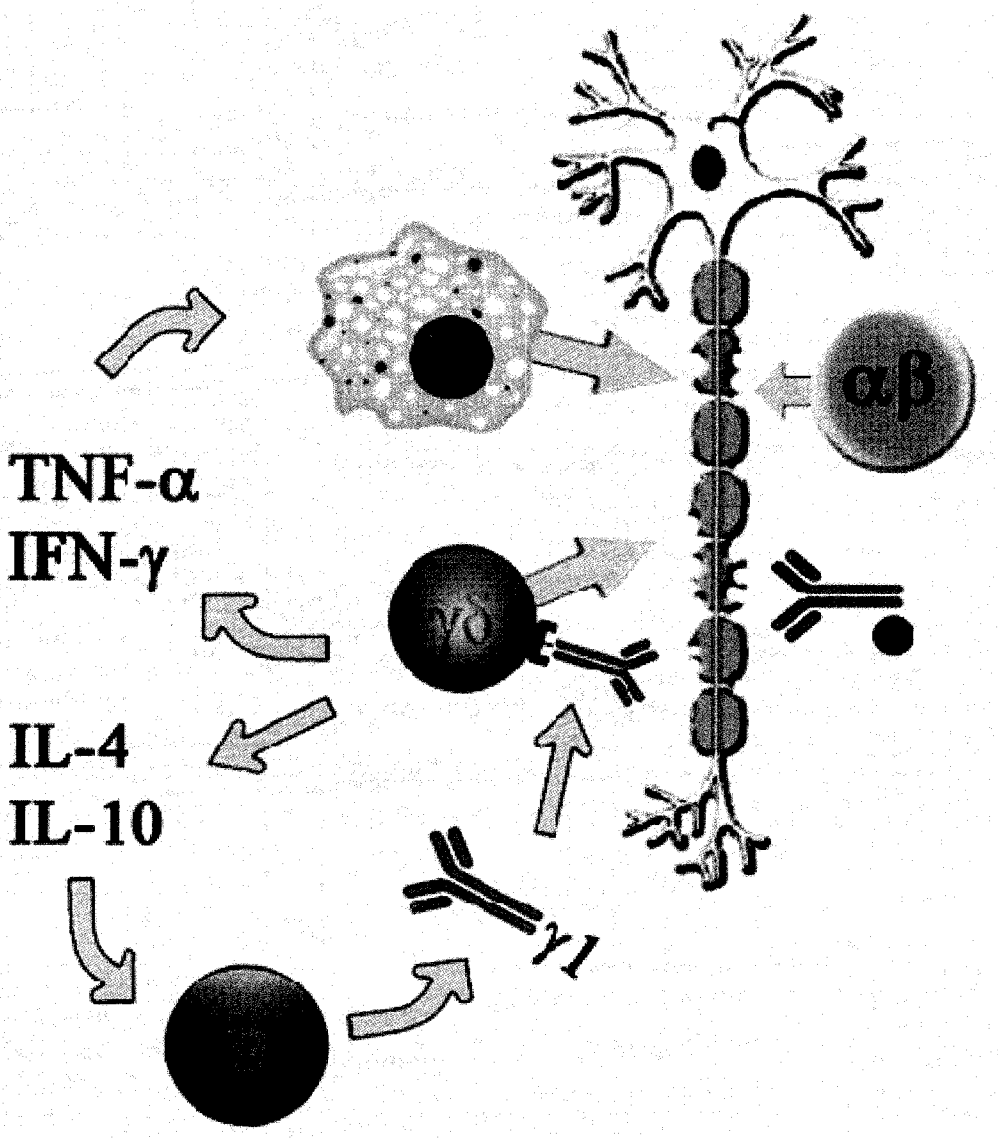
4.7 Future work

At the time of preparation for this manuscript, collaboration is established with Dr. B. Hemmer's group in München, Germany to study anti-MOG autoantibody directed γδ T cells mediated OGC lysis. Using an avant-garde technological design, where they transfect an oligodendroglioma cell line with a full length human MOG gene, the German group has revealed that a "native" form of serum anti-MOG IgG is much concentrated in MS patients than in HCs (90), which disputes previous studies that anti-MOG antibody shows no differences in patients or healthy subjects. This discrepancy could possibly be due to the failure of previous study in identifying "native" immunoglobulin molecules in the circulation (234).

With the human MOG-gene transfected oligodendroglioma as targets and MS patients sera that are high or low in anti-MOG IgG titre as bridging antibody, it would be exciting to look at the ADCC cytotoxicity of γδ T cells in this paradigm. This is currently under investigation in the lab.

Figure 42. Illustration of CD16 mediated γδ T cell function in MS.

Both adaptive immunity (αβ T cells and autoantibody mediated complement lysis) and innate immunity (macrophage, microglia and γδ T cells) contribute to the damage of myelin sheath in MS patients. Results from this work showed that a special subpopulation of γδ T cells that express CD16 are increased in MS patients. This molecule can mediate cell damage by γδ T cells through binding to antibody that specific to the target cells. Upon CD16 engagement, γδ T cells produce Th1 cytokines (TNF-α, IFN-γ) that function on macrophage or microglia cells to enhance their cytotoxicity; they also produce Th2 cytokines (IL-4 and IL-10) that promote the proliferation of B cells and help them produce IgG with γ1 isotype that favours CD16 binding.



One of the other future studies might involve examining if antibodies directed against human CNS components could facilitate $CD16^+$ γδ T cell-mediated cytolysis of the neuronal cells via ADCC. A perfect example of a pathogenic antibody might be anti-neurofascin found in periphery of MS patients (91). $CD16^+$ γδ T cells could affix these antibodies and use them to directly attack axons, a mechanism that could also underlie the pathogenesis of disease progression.

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ACADEMIC BACKGROUND AND TRAINING:

DEGREE	SPECIALTY	ORGANIZATION	DATE
Doctorate (PhD)	Neuroimmunology	University of Ottawa	04/2009
Master's	Biochemistry	China Pharmaceutical University	07/1998
Bachelor's, Honours	Biochemistry	China Pharmaceutical University	07/1995

MEMBERSHIP:

Member, International Society of Neuroimmunology

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DISTINCTIONS AND CREDENTIALS:

AWARDS AND SCHOLARSHIPS:

2006-09	Studentship, Multiple Sclerosis Society of Canada
2006	Travel grant, International Society of Neuroimmunology
2006	Travel grant, Faculty of Graduate and Postdoctoral Studies, U of Ottawa
2006	Ontario Graduate Scholarship*, Ministry of Education, Ontario
2004-06	Post Graduate Scholarship, Natural Science and Engineering Research Council of Canada
2004-09	National Excellence Scholarship, University of Ottawa
2004	Ontario Graduate Scholarship for Science and Technology*, Ministry of Education, Ontario
2003-05	Strategic Areas of Development Award, University of Ottawa
2003-04	Admission Award, University of Ottawa
1995	Jiang'an Scholarship, China Pharmaceutical University
1992-95	University Scholarships, China Pharmaceutical University

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CREDENTIAL:

1997	MVP, intercollegiate volleyball tournament, China Pharmaceutical University
1997	Excellent Student Union Leader, China Pharmaceutical University
1997	Third Place, National University Student Science-Technology Competition, Ministry of Education, China,
1992-94	Excellent Student, China Pharmaceutical University

WORK EXPERIENCE:

POSITION	ORGANIZATION	DATE
Lab Chief	Beijing Anapure Bioscientific Ltd.	1998-2002
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PROJECTS AND RESPONSIBILITIES:

PHD THESIS:

- Discovered that a special subgroup of γδ T cells that express CD16, the low affinity Fcγ receptor, has an increased presence in MS patients;
- Examined $CD16^+$ γδ T cells mediated ADCC killing with an in-house developed flow cytometry based assay;
- Observed that $CD16^+$ γδ T cells produce various cytokines upon CD16 engagement, suggesting that these cells assume an immunoregulatory role in MS.

LAB CHIEF:

- Established a series of Standard Operation Procedures (SOPs) for the analysis of raw materials and final products according to the current Good Manufacturing Practice (cGMP);
- Conducted quality controls for exporting products including animal sera and serum proteins;
- Developed an efficient processing method for the purification of human C-reactive Protein;
- Conducted isolation and enrichment of $CD34^+$ stem cells from human umbilical cord blood.

MSC THESIS:

- Optimized the enzymatic reaction of genetically engineered SHMT (serine hydroxylmethyl-transferase) bacterium;
- Designed a pilot scale bioreactor for the production of serine from glycine;
- Participated in the synthetic preparation of the co-enzyme THFA (tetrahydrofilic acid).

HONOUR'S PROJECT:

- Purified the 2.5S Nerve Growth Factor from mouse submaxillary glands and studied its biochemical properties.

TEACHING ASSISTANT:

- Directed undergraduate students' biochemistry lab; marked their scores according to their performances.

SPECIALTIES:

My research interests are in the following areas:

Neuroimmunology, Immunology, Cellular Immunology, Molecular Immunology, Autoimmunity, Pharmaceutics, Vaccinology, Biochemistry

EXPERTISE:

Cell culturing and primary cell culture preparation, Multi-parameter flow cytometry, ELISA, Immuno-blot, Protein isolation and purification, Immunocytochemistry, Level III bio-safety facility

PEER-REVIEWED PUBLICATIONS:

1. **Chen Z** and Freedman MS. (2008) $CD16^+$ γδ T cells mediate antibody dependent cellular cytotoxicity: potential mechanism in the pathogenesis of multiple sclerosis. *Clin Immunol.* 128:219-227

2. Darlington P, Podjaski C, Horn K, Costantino S, Blain M, Saikali P, **Chen Z**, Baker KA, Newcombe J, Freedman M, Wiseman PW, Bar-Or A, Kennedy TE, Antel JP. (2008) Innate immune-mediated neuronal injury consequent to loss of astrocytes. *J Neuropathol Exp Neurol.* 67:590-9

3. **Chen Z** and Freedman MS. (2008) Correlation of specialized $CD16^+$ γδ T cells with disease course and severity in multiple sclerosis. *J Neuroimmunol.* 194: 147-52

4. Saikali P, Antel J, Newcombe J, **Chen Z**, Freedman M, Blain M, Hall J, and Arbour N. (2007) Contribution of NKG2D ligands in selective cytotoxicity directed on oligodendrocytes in multiple sclerosis. *J Neurosci.* 27:1220-8

5. Sun J, Wu W, and **Chen Z**. (2000) Synthesis of coenzyme tetrahydrofilic acid (THFA) and determination of its relative activity. *J Pharma Biotech.* 7: 38

6. **Chen Z**, Wu W, and Nie K. (1999) Immobilization – a strategy for stability improvement for genetically engineered bacteria. *J Pharma Biotech.* 6: 122

7. **Chen Z**, Wu W, and Xiang B. (1998) The activity assay of recombined serine hydroxymethyltransferase (SHMT) and the optimization of the enzymatic reaction. *J Pharma Biotech.* 5: 75

SYMPOSIUM AND CONFERENCE PRESENTATIONS:

1. **Chen Z** and Freedman M. (2008) γδ T Cells Mediate Antibody Dependent Cell Cytotoxicity and Potentially Regulate Humoral Immunity. The 9th ISNI Congress, Fort Worth, TX, USA

2. **Chen Z.** (2008) CD16 on γδ T cells derived from MS patients. Research Seminar, University of Ottawa, Canada

3. **Chen Z** and Freedman M. (2007) $CD16^+$ γδ T Cells Increase in Peripheral Blood of Multiple Sclerosis Patients and Mediate Antibody Dependent Cellular Cytotoxicity *In Vitro.* The second Canadian endMS Conference, Banff, AB, Canada

4. **Chen Z.** (2007) CD16 expression is elevated on peripheral blood γδ T cells derived from multiple sclerosis patients: positive correlation with disease progression. Student symposium, University of Ottawa, Canada

5. **Chen Z** and Freedman M. (2006) γδ T Cells Derived from Multiple Sclerosis Patients Mediate Antibody-Dependent Cell Cytotoxicity via Fcγ Receptor III. The 8th ISNI Congress. Nagoya, Japan

6. **Chen Z** and Freedman M. (2006) γδ T Cells Demonstrated potential of Mediating Antibody-Dependent Cell Cytotoxicity via Fcγ Receptor III. Research Day, Ottawa Health Research Institute, Canada

7. **Chen Z** and Freedman M. (2005) γδ T Cells from Multiple Sclerosis Patients Mediate Antibody-Dependent Cell Cytotoxicity via CD16. Research poster, University of Ottawa, Canada

8. **Chen Z.** (2004) The Role of Fcγ Receptors in the Regulation of γδ T Cells in Multiple Sclerosis. Research Seminar, University of Ottawa, Canada

9. **Chen Z**, Cai Y, and Wu W. (1997) Construction of SHMT Genetically Recombinant Bacterium and Its Use in the Production of L-Serine. The Fifth "Challenge Cup" National University Student Science and Technology Competition. Nanjing, China

10. **Chen Z**, and Gao X. (1995) Purification of 2.5S Nerve Growth Factor from Mice Submaxillary Gland and Its Property Study. Presentation, China Pharmaceutical University