THE ROLE OF CHRONICALLY STIMULATED AND SENESCENT T CELLS IN AUTOIMMUNITY

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DEDICATION

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"New opinions are always suspected, and usually opposed, without any other reason but because they are already common."

John Locke (1632-1704)

THE ROLE OF CHRONICALLY STIMULATED AND SENESCENT T CELLS IN AUTOIMMUNITY

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THE ROLE OF CHRONICALLY STIMULATED AND SENESCENT T CELLS IN AUTOIMMUNITY

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Myelin-reactive T cells have been hypothesized to play a role in the pathogenesis of MS. If this is the case, these T cells would be expected to be repeatedly stimulated over the course of this disease because of the episodic breakdown of myelin membranes. Continuous simulation of myelin-reactive T cells may also change the phenotype of these T cells. For example it may drive autoreactive T cells into senescence. Senescent T cells would be unable to divide but may still retain effector functions such as the ability to lyse target cells.

The existence of senescent autoreactive T cells in MS patients could explain why therapies that limit the proliferation of T cells have had little effect on the course of MS, particularly later in the course of the disease. Human T cells adopt a CD28-CD57+ phenotype in chronic viral infections and this has been hypothesized to result from chronic stimulation, however this phenotype may also be due to direct viral effects on T cells. Here I make use of human MS patients before and after chronic *in vivo* administration of the antigen glatiramer acetate to test this hypothesis. Before the initiation of glatiramer acetate treatment, glatiramer acetate -specific CD8 T cells were either CD28-CD57- or CD28+CD57-. This response changed to a predominantly CD28-CD57+ response after one year of continuous stimulation. This phenotype was only observed after chronic stimulation and not in a recall response to mumps. These cells were shown to contain perforin, indicating they likely play a cytotoxic role *in vivo*. Furthermore CD28-CD57+ CD8 T cells displayed a reduced proliferative capacity indicating they may be senescent or pre-senecent. When myelin-reactive T cell responses were examined a CD28-CD57+ CD8 T cell response could be detected in MS patients but not in healthy controls. These T cells contained mRNA consitant with a cytotoxic role and the abilty to home to the cerebrospinal fluid of MS patients. This observation may explain why therapies that limit the proliferation of T cells have had little effect on the course of MS, particularly later in the course of the disease.

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CFSE	5 -(and -6) -Carboxyfluorescein diacetate Succinimidyl Ester
CMV	Cytomegalovirus
CPM	Counts Per Minute
CTL	Cytotoxic T Lymphocyte
EAE	Experimental Autoimmune Encephalomyelitis
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Florescence Activated Cell Sorting
GA	Glatiramer Acetate (Copaxone®, COP-1)
HC	Healthy Control
HIV	Human Immunodeficiency Virus
IFN	Interferon
LDA	Limiting Dilution Assay
MBP	Myelin Basic Protein
MOG	Myelin Oligodendrocyte Glycoprotein
MS	Multiple Sclerosis
MST	Multiple Sclerosis Treated (GA) patient
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PLP	Proteolipid Protein
PMA	Phorbol 12-Myristate 13-Acetate (12-O-Tetradecanoylphorbol-13-acetate)

PP	Primary Progressive
RA	Rheumatoid Arthritis
RR	Relapsing Remitting
SEM	Standard Error of the Mean
SP	Secondary Progressive
TCL	T Cell Line
TNF	Tumor Necrosis Factor
TMEV	Theiler’s Murine Encephalomylitis Virus
TREC	T cell Receptor Excision Circles

CHAPTER ONE INTRODUCTION AND REVIEW OF THE LITERATURE

I. Description of multiple sclerosis

A. History of multiple sclerosis

Although various aspects of multiple sclerosis (MS) had been recognized and treated by other doctors, Jean-Martin Charcot was the first to offer a unified clinical concept of a disease in 1868, and to use the term *sclérose en plaque* which was subsequently changed to multiple sclerosis. His description of the disease was characterized by lesions in the spinal cord. In addition, using stains he was able to show that myelin tissue and not axons were damaged in MS patients. In the nineteen twenties, it was debated whether MS was an inflammatory, or degenerative disease or both. In these early years many experiments were conducted using the Koch postulates to link MS to an infectious agent (Murray 2000).

B. Types and incidence of MS

MS has been recognized to have an incidence of about 1 out of 1,000 persons in populations found in temperate climates, and an incidence of about 1 out of 100,000 persons in populations in equatorial regions (Kurtzke 1993). Whether this is due to genetic or environmental differences is unclear. A further complication is that MS manifests itself in several forms. In its Relapsing Remitting (RR) form, MS patients are stricken with episodes of paralysis usually affecting the extremities first. Although usually affecting coordination and causing paralyses, cognitive dysfunction can occur. These relapses are followed, in one

to two months, by remissions. Remission is not always complete however, and patients will slowly accumulate permanent disability. The rate at which disability accumulates may vary considerably. Whether changes in the relapse rate affect changes in accumulated disability has not been ascertained. RR MS can change to a form without relapses and remissions, to one defined by slowly accumulating disability. This form is termed Secondary Progressive (SP). Some patients present with a slowly accumulating disease from the outset, and this type is termed Primary Progressive (PP) MS. However the situation is further complicated because there is heterogeneity in even these classifications (Sotgiu, Pugliatti et al. 2004), and defining more subgroupings may be necessary to gain a better understanding of this disease.

C. Genetics and Environment: Recent Findings

Important progress in has been made over the years in understanding how susceptibility is conferred. These data not only impart a better understanding of this disease, but also impart important clues which help define the range of possible mechanisms that are responsible for initiating and maintaining MS. Epidemiological studies have shown the relative risk of developing MS is increased in northern latitudes. This individual susceptibility changes if an individual moves from a high risk zone to a low risk zone before the age of 15 (Kurtzke 1993), but after this age the relative risk does not change. This data indicates an environmental factor, such as a virus or other infectious agent, may lead to MS when individuals are exposed before the age of 15. While MS does display familial aggregation, the incidence of MS was found to be no higher in the adoptees of families with

histories of MS, than in the general public. This indicates a shared microenvironment plays little if any role in susceptibility to MS (Ebers, Sadovnick et al. 1995). So while environmental factors may play a role in population wide susceptibility, they do not seem to play a role on an individual level. This effect was confirmed with half siblings (Sadovnick, Dyment et al. 1996). This second study also demonstrated that the rate of MS was the same if the father or mother had a history of MS. This indicates maternal genomic imprinting does not influence susceptibility to MS, although this is controversial. Furthermore, when the incidence of MS was examined in twins, dizygotic twins had a concordance rate of 5.4 % which is similar to the concordance rate of siblings and much lower than a concordance rate of 25.3% observed in monozygotic twins (Willer, Dyment et al. 2003). This observation demonstrates that both genetic and environmental factors play a role in the development of MS.

a) Viral and Degenerative disease hypothesis

Although the case for MS being an autoimmune disease is strong, other hypotheses for the cause of this disease have been put forward. One idea put forward is that MS may be the result of a degenerative prossess (Barnett and Prineas 2004). Although this remains a possibility, data to support this theory is rather sparse. Another possibility is that MS is the result of damage from a virus that infects brain tissue. It was with this idea that IFN-γ was administered to MS patients (Panitch, Hirsch et al. 1987). Patients receiving this treatment appeared to have more relapses and the trial was immediately halted. Since IFN-β also has

antiviral properties, it was administered to MS patients after IFN-γ. This therapy lessened relapse rates (Jacobs, Cookfair et al. 1996) and importantly, was ultimately shown to reduce disability accumulation as well (Rudick, Goodkin et al. 1997). While this could be taken as evidence that MS is caused by a virus, IFN-β also has imunomodulatory and immunosupressive properties as well (Genc, Dona et al. 1997; Furlan, Bergami et al. 2000). While this therapy reduces disease accumulation to some degree, more effective therapies are needed. Recently, treatment with anti-VLA-4 (Natalizumab, Tysabri), which blocks lymphocyte extravasation, has been approved by the FDA for the treatment of MS (Chaudhuri 2006; Polman, O'Connor et al. 2006). However, anti-VLA-4 has safety issues (Van Assche, Van Ranst et al. 2005). More effective and safe therapies will likely only come about with a greater understanding of the disease process that occurs in MS patients.

(1) Animal models

There are several animal diseases that incorporate viral agents, which phenotypically resemble MS, and have been used as models. One example is Theiler's Murine Encephalomyelitis Virus (TMEV), which initiates an anti-viral response which then changes into an anti-myelin response. This change may occur by epitope spreading or molecular mimicry (Oleszak, Chang et al. 2004). Thus, this model incorporates an autoimmune component. Indeed animal models of MS can be understood as a continuum, from viral mediated damage, to both viral and autoimmune components, to purely autoimmune mediated diseases such as Experimental Autoimmune Encephalomyelitis (EAE).

(a) Experimental Autoimmune Encephalomyelitis

EAE is induced by subcutaneous injection of myelin proteins in an emulsion of complete Freund's adjuvant and Phosphate Buffered Saline (PBS). Pertussis toxin is also administered and acts as additional adjuvant. EAE can be induced with many antigens although Myelin Basic Protein (MBP), Proteolipid Protein (PLP), and more recently Myelin Oligodendrocyte Glycoprotein (MOG), are among the most commonly used. In addition to active immunization, this disease can be transferred to naïve mice by purified, activated myelin-specific Th1 $CD4^+$ T cells without an adjuvant such as pertussis toxin (Mokhtarian, McFarlin et al. 1984). So, autoreactive Th1 $CD4^+$ T cells are necessary and sufficient to cause this disease. Although early experiments did not reveal a role for CD8 T cells in pathogenesis several more recent reports support a role for CD8 T cells being involved in pathogenesis (Huseby, Liggitt et al. 2001; Sun, Whitaker et al. 2001). Some strains of mice are more susceptible than others to EAE. This indicates genetics does play a role in determining susceptibility to this disease. It should be pointed out that through genetic manipulation of mice, spontaneous forms of EAE have been generated. Mice that have a transgenic TCR specific for MBP Ac1-11 develop spontaneous EAE, although the penetrance is low, which makes conducting experiments on this population of mice difficult. The incidence of EAE can be increased by breading these mice onto a rag -/- background (Lafaille, Nagashima et al. 1994).

b) Autoimmune hypothesis

MS has been hypothesized to result from an autoimmune process and although the initiation most likely differs from the disease course, EAE and MS share similarities. In addition, susceptibility to MS is linked to HLA DR15. Specific MHC molecules may induce susceptibility to MS in individuals by binding and presenting specific peptides to T cells. Alternatively, MHC molecules play a role in selecting the TCR repertoire in the thymus (Messaoudi, Patino et al. 2002). MHC molecules may provide susceptibility to autoimmunity by skewing the TCR repertoire selected in the thymus. Although, it has not been ruled out that a locus close to this MHC gene is responsible for disease susceptibility, this is strong evidence that MS has an immune component. It should be pointed out that most known genes in this area function immunologically. Furthermore mononuclear infiltrates are found in MS lesions and breakdown of the blood brain barrier is considered to be an early event. As stated before when IFN-γ was addminitered to MS patients the relapse rate appered to increase and the trial was halted (Panitch, Hirsch et al. 1987). Lastly blocking lymphocyte entry into the CNS results in decreased disease accumulation (Polman, O'Connor et al. 2006).

D. Autoimmunity

Autoimmunity initially was coined to refer to the process whereby the system of cells and organs that normally protect higher vertebrates from infection instead “attacks” self, which leads to disease. This process may be a fairly common phenomenon that only rarely

leads to disease. Nevertheless, autoimmunity generally is thought to result when an adaptive immune response is mounted against a self-epitope. How tolerance is maintained strikes at the heart of how the adaptive immune system functions. Generally accepted is the central tolerance model. Here lymphocytes go through a process of education during their maturation process so that mature lymphocytes are only specific for non-self antigens. All antigenic determinants for self are thought to have been eliminated during maturation. This elegant theory is supported by much data, and so has a dominant position in the minds of current immunologists. However work in transplant immunology has lead to the danger theory of immunity proposed by (Matzinger 2002). Here an immune response is only initiated when a danger signal is detected by the immune system. While the central tolerance model will not likely be discarded, some modification will continue to be necessary.

a) Possible events that initiate MS as autoimmune disease

There are many possible ways the immune system could break tolerance to self antigens. The animal models discussed above each present a possible scenario for the initiation of an autoimmune response. The evidence most strongly supports a role for thymus derived lymphocytes in the initiation and maintenance of MS. However B cells may play a role as well as oligoclonal banding is associated with MS and B cells can be found in MS lesions.

(1) Molecular mimicr

The possibility exists that a virus or other pathogen will contain epitopes that are also found in self myelin proteins. If a T cell response is generated against these epitopes the possibility exists that T cells bearing this TCR specificity will also target myelin antigens as well. Furthermore T cell receptors are capable of binding more than one peptide, and the viral and myelin epitope need not share sequence identity for molecular mimicry to occur (Wucherpfennig and L. 1995).

(2) Alteration of homeostasis

While it has been known that low affinity TCR MHC interactions are necessary for T cell survival in the periphery, a surprising finding was made that in conditions of lymphopenia, recognition of self peptides are capable of driving homeostatic T cell proliferation (Ernst, Lee et al. 1999). These self activated T cells also expressed CD44, a marker typically expressed on activated T cells in mice (Ernst, Lee et al. 1999; Cho, Rao et al. 2000). CD8 T cells expanded in this fashion display other markers typical of memory T cells such as CD122 and notably are capable of killing (Goldrath, Bogatzki et al. 2000). However this phenotype was shown to be transient as it diminished after 45 days after the transfer of cells and the initiation of homeostatic expansion. How long activated cells actually remain is controversial. This expansion may favor specific TCR specificities over others and lead to a skewing and restriction of the TCR repertoire (La Gruta, Driel et al. 2000). This indicates that a small number of receptors may be responsible for stimulating

TCR's. In addition, this expansion and activation of T cells specific for self can induce autoimmunity (King, Ilic et al. 2004). This observation may explain why lymphopenia has long been correlated with both systemic and organ specific autoimmunity (Nishizuka, Tanaka et al. 1973; Sakaguchi and Sakaguchi 1990; Smith, Lou et al. 1992; Penhale, Farmer et al. 1973). Viral infections such as measles may lead to lymphopenia (Okada, Kobune et al. 2000; Tumpey, Lu et al. 2000) and MS has been associated with viral infections such as measles (Hernan, Zhang et al. 2001).

As stated above, epidemiological data implicates an environmental agent such as a virus or other pathogen that may lead to MS when individuals are infected before the age of 15 (Kurtzke 1993). Interestingly few individuals develop MS by age 15 or 16 (Kurtzke 1993). In fact, the median age of developing MS is around 30 years (Kurtzke 1993). This presents the possibility that the environmental insult that leads to MS occurs years before the emergence of the disease. This leaves open the possibility that the autoimmune process has been ongoing for several years before patients are diagnosed with definite MS. Furthermore, chronic infections may lead to release of self antigens, which is followed by an immune response.

(3) RA may result from expansion of T cells

There is data indicating that this process can lead to organ specific autoimmunity in humans. Rheumatoid Arthritis (RA) is postulated to result from auto reactive T cells (Panayi, Corrigall et al. 2001). It has been demonstrated that Rheumatoid Arthritis (RA)

patients contain clonally expanded populations of CD4 and CD8 T cells (Goronzy, Bartz-Bazzanella et al. 1994; Fitzgerald, Ricalton et al. 1995; Schmidt, Goronzy et al. 1996). These T cells are stable over 2 years (Wagner, Koetz et al. 1998). There is also a loss of diversity in the TCR repertoire of RA patients which may result from the peripheral expansion of T cells (Wagner, Koetz et al. 1998). CD4 T cells from RA patients are more frequently in the S-G2/M dividing phase of the cell cycle indicating CD4 T cells may undergo increased proliferation in RA patients (Wagner, Koetz et al. 1998). Telomeres steadily decline in RA patients, in healthy controls they decline after age of 40 (Koetz, Bryl et al. 2000). Furthermore, the CD4 T cells of RA patients harbor shorter telomeres (Wagner, Koetz et al. 1998). Few T cell Receptor Excision Circles (TREC) are found in RA patients (Koetz, Bryl et al. 2000; Ponchel, Morgan et al. 2002). This may result from an increased propensity of naïve T cells to proliferate in RA patients (Hazenberg, Borghans et al. 2003), or from lymphopenia. In total, this data supports the hypothesis that RA results from the activation of self-reactive T cells by a homeostatic mechanism. In support of this concept, thymic output decreases with age, and susceptibility to RA increases with age.

II. Attempts to identity and phenotype autoreactive T cells

Although the mechanism that initiates MS has not been uncovered, advances have been made in identifying autoreactive lymphocytes in MS patients. A major advance in immunology occurred with the recognition that T cell growth factor (IL-2) could be used to culture T cells (Morgan DA 1976). After this advance, the culture of antigen-specific T cells

became possible and many properties and functions of T cells were discovered. Since susceptibility to MS is linked to MHC and lymphocytes have been found in MS lesions, attempts were made to isolate autoreactive T cells from MS patients.

A. Long term culture

a) Frequency of MBP reactive T cells in MS patients and healthy Controls

While myelin-reactive T cells can be isolated from MS patients specific for many antigens found in EAE, such as MBP, PLP and MOG, it was also discovered they can be isolated from healthy controls as well (Burns J 1983; Jingwu, Medaer et al. 1992; Joshi, Usuku et al. 1993). Although two of the subjects in one of these studies may have been exposed to myelin proteins in the laboratory, they make the case that, although it may be necessary, the existence of T cells specific for myelin epitopes is not sufficient to cause MS. When T cells were subsequently cultured up to two months, they found almost all responding T cells were $CD4^+$ and not $CD8^+$. As discussed below, this study has subsequently been confirmed by numerous investigations. Identical twins who are discordant for MS appear to have similar numbers of auto-reactive T cells (Martin R 1993). In this study only 3 discordant twin sets were examined, and true limiting dilution assays were not performed, and so a suppressor cell population could mask a higher frequency. In another study to try to limit the effect of genetic variance, the frequency of MBP reactive T cells was examined in

siblings that were discordant for familial MS. Again no difference was detected (Joshi, Usuku et al. 1993). In another study after 4 weeks of culture with MBP, the existence of cytotoxic class II restricted $CD4^+$ T cells were demonstrated (Martin, Jaraquemada et al. 1990). Again the same T cells could be isolated from healthy controls. This data indicates myelin-reactive T cells exist in healthy controls.

Progress has been made however, demonstrating that myelin-reactive T cells have been previously activated in MS patients, while myelin-reactive T cells from healthy controls are naive. Initially, this was done by selecting clones with a mutant copy of the house keeping gene, hprt (Allegretta, Nicklas et al. 1990) It was assumed that this natural population of T cells would be enriched for cells that had been actively dividing *in vivo*. This population from MS patients was then shown to contain T cells that were reactive with myelin while the population from healthy controls did not. Furthermore, the T cells from patients with progressive MS were shown to contain more hprt mutations in general, indicating T cell turnover was higher in these patients.

Subsequently it was that shown a higher frequency of T cells could be detected in MS patients when Peripheral Blood Mononuclear Cells (PBMC) were initially cultured with IL-2, and then cultured with MBP or PLP, as opposed to culture with only MBP or PLP for the same amount of time (1.3×10^{-6} verses 0.3×10^{-6}) (Zhang, Markovic-Plese et al. 1994). This increased frequency and difference between MS patients and healthy controls was not found when tetanus toxoid was used as an antigen. The frequencies were determined using 17 day cultures with limiting dilutions, and calculated using the Poisson distribution. This compares

with a frequency of 1x 10^{-6} in the peripheral blood of unimmunized rats, and a 10x 10^{-6} in the peripheral blood of rats at the onset of EAE (Vandenbark AA 1992). Subsequently, T cell lines were generated from positive wells generated from IL-2 or MBP stimulation. These cells were then analyzed by flow cytometry. When this was done 92 out of 93 were $CD4^+$, and one was $CD8^+$. This indicated that myelin-specific $CD4^+$ T cells exist in MS patients in an altered activation state. While this data could be interpreted as indicating these cells were anergic, it was subsequently shown using a similar approach, autoreactive T cells can be detected at significantly higher frequencies when CD95 (Fas) stimulation is blocked. This indicates these cells may be more susceptible to apoptosis (Zang, Kozovska et al. 1999).

Our laboratory has shown myelin reactive T cells from MS patients have altered costimulation requirements, when compared to myelin reactive T cells from healthy controls (Lovett-Racke, Trotter et al. 1998). Specifically, myelin-reactive T cells in MS patients are less dependent on costimulation through CD28 in their proliferative response to MBP, indicating they have been previously activated *in vivo,* and are of a memory or activated phenotype. These experiments are confirmed by experiments with APC with and without CD80 (B7-1) and CD86 (B7-2), which are the ligands for CD28 (Scholz, Patton et al. 1998). Taken together these data indicate that while myelin-reactive $CD4^+$ T cells exist in MS patients and healthy controls, in MS patients they are in an altered state. Since memory T cells are less dependent on co-stimulation (Croft, Bradley et al. 1994), this indicates they may be of a more memory phenotype.

B. Molecular Techniques

In contrast to these experiments, when mRNA was examined for TCR transcripts specific for MBP, frequencies of 3.2 x 10^{-3} and 1.3 x10^{-3} were reported (Bieganowska, Ausubel et al. 1997). This value was arrived at by two similar methods. In both, a previously generated T cell clone that recognized MBP 84-99 with a Vα3.1 and Vβ17.1 chain was selected, and the CDR3 region of the TCR was sequenced (Wucherpfennig, Zhang et al. 1994). In one method the frequency of this particular CDR3 region was determined in cDNA from PBMC by amplifying this sequence by PCR, transfecting it into bacteria via a pCRII vector, growing the bacteria in 96 well plates and probing blots made from the plates with primers specific for Vα3.1 and CDR3 sequence that was previously determined to be specific for MBP 85-99. The occurrence of this sequence in the peripheral blood was then calculated based on the frequency of Vα3.1 and positive wells. In the second method Vβ17.1^{+} and Vβ17.1^{-} T cells were sorted by FACS, and the frequency determined as above. In these experiments, only subjects that had a high frequency were selected for further examination. When they examined healthy controls that had a similar frequency of MBP reactive T cells by LDA, they did not observe this high frequency. However, small numbers of patients were examined, and it is hard to determine if the MS patients and controls were similar. Nevertheless, this paper indicates that at least some MS patients harbor a high frequency of MBP reactive T cells. Further evidence supporting this interpretation is the fact that studies employing ELISPOT have demonstrated frequencies of around 1 x 10^{-4} myelin-reactive T cells in the PBMC of both MS patients and healthy controls (Pelfrey, Rudick et al.

2000; Hellings, Baree et al. 2001; Van der Aa, Hellings et al. 2003). Why these cells do not proliferate *in vitro* is not clear, and what role they play in MS, such as whether they are anergic or suppressor T cells, was not examined. In viral situations such as HIV infection, there is evidence of higher frequencies of T cells than that which is observed by limiting dilution assays (Gotch FM 1990; Moss, Rowland-Jones et al. 1995; Butz EA 1998). Among other explanations, it is possible that these antigen specific T cells may be unable to proliferate because they undergo apoptosis or are terminally differentiated.

III. Overview of chronic stimulation of T cells and why it may be important in MS

The existence of cells that respond by ELISPOT, but not by proliferation may be why therapies based on immunosuppression have limited effectiveness. While myelin-reactive $CD4^+$ T cells are readily cloned and studied from MS patients, determining the nature of the high frequency, of non-proliferating cells may facilitate the development of more effective therapies for MS. While it may seem inconsistent that MS patients contain memory myelin-reactive T cells that do not proliferate, this may be due to the fact that chronic stimulation of autoreactive T cells reduces the replicative capacity of these cells in MS patients. If the frequency of autoreactive T cells is indeed very high, there are several possibilities why these cells do not proliferate enough to be detected in an LDA. One is that these cells undergo apoptosis when stimulated. As mentioned above, when CD95 stimulation was blocked, the frequency of responding cells increased from 6.9×10^{-7} to11.3×10^{-7} (Zang, Kozovska et al. 1999). These cells may be anergic, and thus division of these cells is not observed, or these

T cells may be terminally differentiated or senescent. Both cell types would have diminished proliferative capacity. While senescence implies a greater replication history, functionally these two populations would be difficult to differentiate. In support of this possibility, MS is a chronic disease and in most cases lasts for many decades, and MBP specific clones can be detected in MS patients up to 13 months apart (Wucherpfennig, Zhang et al. 1994). Understanding the exact nature of the T cell response against myelin proteins will be very important for understanding this disease. Indeed, the MBP specific T cells examined previously may have been peripheral to the autoimmune process in MS. For this reason, I was interested in understanding the changes that occur in terminally differentiated or senescence T cells in general.

A. Senescence

Regardless of the signals that drive proliferation, T cells, like all somatic cells with the possible exception of some stem cells, have a finite replicative ability (Pawelec, Sansom et al. 1996) (Hayflick and Moorhead 1961) (Grubeck-Loebenstein, Lechner et al. 1994). After a certain number of divisions, somatic cells will lose the ability to divide. At this point, they will reach a developmental stage called senescence and lose some functions such as an ability to divide, even in the presence of IL-2 (Perillo, Naeim et al. 1993). This is characterized by cells entering G0 and remaining there with an inability to enter the S phase of cell division (Perillo, Naeim et al. 1993; Jaruga, Skierski et al. 2000). Perhaps not surprisingly, these cells show reduced expression of the cell cycle regulator p21(CIP1) which

facilitates entry into S phase (Herbig, Jobling et al. 2004). They may also lose the ability to upregulate hsp70 (Effros, Zhu et al. 1994), which may indicate a general loss of function. However, senescent T cells also gain some functions and thus are not anergic. They may release TNF-α and IL-6 (Effros 2003), and importantly may still possess cytotoxic abilities (Perillo, Naeim et al. 1993). From this one can conclude that senescence is not a general break down, but merely a change in cell function, similar to terminal differentiation. There are several theories as to why cells become senescent. It may be a mechanism to block cells from becoming transformed and so provides protection against cancer (Sager 1991; Campisi 2000). It is probably the case that the ability of cells to continue replicating, or longevity, is subjected to evolutionary pressures and evolves over time like other traits such as an organism's height or cell size. Whether or not T cells persist in this state is a matter of controversy. While numerous articles show evidence that senescent T cells are less susceptible to apoptosis and have increased Bcl2 (Campisi 2001) (Spaulding, Guo et al. 1999)., there are also numerous studies demonstrating they are more susceptible to apoptosis. Both CD4 and CD8 T cells from aged individuals have increased CD95 expression and display increased apoptosis (Phelouzat, Laforge et al. 1997; Aggarwal and Gupta 1998; Potestio, Caruso et al. 1998; Potestio, Pawelec et al. 1999). Aged cultures of CD8 T cells show decreased susceptibility to apoptosis *in vitro* (Spaulding, Guo et al. 1999). CD4 T cells have been reported to undergo increased apoptosis (Hyland, Barnett et al. 2001). There are many similarities between T cells stimulated to divide until exhaustion *in vitro*, and *in vivo* isolated senescent T cells. One difference may be susceptibility to apoptosis. (Pawelec,

Adibzadeh et al. 2000). This may result from the outgrowth of resistant T cells *in vivo*. If senescent cells do not persist, this could lead to clonal exhaustion. It is clear that at least in some situations clonal exhaustion can occur (Welsh and McNally 1999). Chronic stimulation may result in a responding cell population that still has effector function and an impaired ability to proliferate. If this is the case in MS, then studies involving the long-term culture of T cells may be expanding T cells that are incidental to the disease process. To determine if this is the case, I wished to use the highly sensitive flow cytometric technique, which may be employed to assay cells without an inherit bias towards T cells that proliferate well. To accomplish this goal, it was necessary to examine molecules that would only be expressed on chronically stimulated or senescent T cells with priority given to molecules expressed on the cell surface.

B. Markers studied

Most studies of chronic stimulation of the human immune system have been conducted in situations of chronic viral infection. Understanding the changes that occur during this type of immune response (Appay and Rowland-Jones 2004) has been confounded by several factors. One is the difficulty in knowing with certainty when a chronic immune response is initiated. Because of this, there is always ambiguity knowing which stage of an immune response is being studied (Zhang, Shankar et al. 2003). Nevertheless, in an effort to identify T cells that have been chronically stimulated, expression of various cell surface molecules were investigated to determine if their regulation correlates with perceived chronic

stimulation (Seder and Ahmed 2003; Appay and Rowland-Jones 2004). This line of investigation suffers a second problem because viral agents can modulate the immune response to evade clearance (Tortorella, Gewurz et al. 2000). Furthermore, persistent viral infections may alter an antiviral T cell response itself (Lenkei and Andersson 1995; Kalams and Walker 1998; Zajac, Blattman et al. 1998; Tortorella, Gewurz et al. 2000), making identification of chronically stimulated T cells in viral infections problematic. A study examining expression of CD28, CD27, and CD45RA has shown that T cells responding to persistent infection adopt different phenotypes depending on the type of infection or virus to which they are responding (Appay, Dunbar et al. 2002). Conversion of the CD45RA isoform to CD45RO is commonly used to distinguish naïve from memory cells, however long lived quiescent memory cells can re-express CD45RA (Weekes, Wills et al. 1999). Because of this, CD45RO can be a marker of activation as opposed to memory, and it is not an ideal marker to study chronic immune responses. Other studies suggest chronically stimulated T cells express cell surface markers commonly associated with NK cells (Tarazona, DelaRosa et al. 2000; McMahon and Raulet 2001). The frequency of T cells that downregulate CD28 and express the NK cell marker CD57 (HNK-1 Leu-7) increases with age (McNerlan, Rea et al. 1998), such that $CD28^{-}CD57^{+}$ T cell are postulated to be chronically stimulated cells. It has been shown that when stimulated to divide *in vitro*, T cells lose CD28 expression (Effros, Boucher et al. 1994). CD28 is a costimulatory molecule involved in activating naïve T cells and memory T cells are less dependent on CD28 costimulation. CD57 is defined by the binding ability of the mouse IgM antibody HNK-1 (Abo and Balch 1981). The HNK-1

epitope is present on at least 2 N- linked oligosaccharides, sulfoglucuronosyl paragloboside (SGPG) and Sulfoglucuronosyllactosaminyl paragloboside (SGLPG) (Kanda, Yamawaki et al. 1995). While this epitope was first described on NK cells (Abo and Balch 1981), it has been well described on the surface of oligodendrocytes, Schwann cells, and brain microvascular endothelial cells and is reported to function as a neuronal adhesion molecule (Shy, Gabel et al. 1986; Mikol, Gulcher et al. 1990; Filbin and Tennekoon 1991; Field, Wing et al. 1992; Filbin and Tennekoon 1993; Needham and Schnaar 1993). On these cells, it is believed to function as an extracellular adhesion molecule. In this regard, it is found that P0 (a protein expressed on the surface of Schwann cells) binds in a homophilic manner only when two interacting P0 molecules are glycosolated with the HNK-1 epitope, although it is not known how this interaction takes place (Filbin and Tennekoon 1991). In addition, it was found that molecules bearing the HNK-1 epitope bind to L-selectin and P selectin (Needham and Schnaar 1993; Kanda, Yamawaki et al. 1995). These studies indicate that functionally, CD57 is involved in cell adhesion. Although when expressed on T cells, it may also be involved in binding IL-6 (Cebo, Durier et al. 2002). Another study suggests $CD57^+$ T cells have fewer TREC (Brenchley, Karandikar et al. 2003) and indicates these cells may have divided on average at least four times more than $CD57^-CD45RO^+$ T cells. $CD28^-CD57^+$ T cells have been shown to possess less replicative ability and shorter telomeres than $CD28^+CD57^-$ T cells (Effros, Allsopp et al. 1996). This is taken as evidence that $CD28^-CD57^+$ T cells have undergone more rounds of replication than $CD28^+CD57^-$ T cells. However, these observations may be due to lack of costimulation for two reasons. First,

CD28 provides costimulation to T cells and enhances their proliferative responses. Second, most somatic cells do not express telomerase and their telomeres shorten with every division. T cells are able to induce telomerase when activated, and this induction has been shown to be dependent on CD28 costimulation (Buchkovich and Greider 1996; Hathcock, Weng et al. 1998; Valenzuela and Effros 2002). Since $CD28^{-}CD57^{+}$ T cells cannot receive CD28 costimulation, they may be unable to induce telomerase. The shortened telomeres may be due to lack of telomere extension, and not more cell division. Lastly, based on studies of CD8 T cells in the absence of a viral infection, it has been proposed that currently activated effector T cells and not chronically stimulated T cells will be $CD28^{-}$ and express NK cell markers (Speiser, Pittet et al. 1999). Alternatively, T cell lines can be generated *in vitro* that suppress $CD4^{+}$ T cells and this suppression was shown to reside in the $CD8^{hi}CD28^{-}$ fraction (Liu, Tugulea et al. 1998). In this report, all stimulation was either allogeneic or xenogeneic in nature and both displayed the suppressive phenotype. This was done by allogeneically, or xenogeneically stimulating PBMC twice in a two week culture period. These suppressor cells did not kill and although they did not show the data, they reported cytotoxic potential resided only in the $CD28^{+}$ fraction and the $CD28^{-}$ T cells suppressed by releasing cytokines. They showed a decrease in CD80 and CD86 expression on APC in the presence of $CD8CD28^{-}$ TCL. In addition $CD8^{+}CD28^{-}$ T cells may inhibit the activation of CD4 cells by the inducing the inhibitory receptors ILT 3 and ILT4 on APC (Chang, Ciubotariu et al. 2002).

a) GA treatment

Glatiramer Acetate (GA, Cop-1, Copaxone®) is a random copolymer of glutamic acid, lysine, alanine, and tyrosine in a constant molar ratio of 6.0 : 1.9 : 4.7 : 1.0, similar to MBP. It has an average molecular weight of 23,000kda. GA was originally synthesized to investigate how MBP initiates EAE (Teitelbaum, Meshorer et al. 1971; Einstein, Chao et al. 1972; Teitelbaum, Webb et al. 1972). GA was shown to inhibit EAE and was determined not be generally immunosuppressive (Teitelbaum, Meshorer et al. 1971; Teitelbaum, Webb et al. 1972). GA was also shown to inhibit graft survival in graft vs. host disease (Aharoni, Teitelbaum et al. 2001). In addition to inhibiting EAE, it was also shown to have the ability to treat established disease, and this is important as established disease is considered to resemble MS more closely. After further investigations, it was found to be effective in treating relapsing remitting MS (Johnson, Brooks et al. 1995; Johnson, Brooks et al. 1998). In the human setting, it has been demonstrated that $CD4^+$ T cells specific for GA are induced to proliferate in its presence (Duda, Krieger et al. 2000; Crawford, Yan et al. 2004). More recently $CD8^+$ T cells are also induced to proliferate in its presence. Since patients are injected daily with GA, this presents a unique model system to study chronic stimulation of CD4 and CD8 T cells in MS patients.

Initially a series of experiments were set up that examined two hypotheses. One is that repeated stimulation of T cells results in a $CD28^-CD57^+$ phenotype. The second is that CD8 GA-reactive T cells are chronically stimulated when MS patients undergo GA treatment. These two hypotheses could lead to circular arguments however. This was

avoided by independently verifying the first hypothesis, and the fact that some evidence to support the second has already been established. To test the first hypothesis, I decided to use MS patients treated with daily injections of GA. This system is advantageous because I have samples from patents before they initiate therapy and every 4 months thereafter. This allowed us to precisely monitor the time patients have responded to GA. This is important as responses may change overtime. For example initially, the response may be a robust memory response, this may change to a less potent response after chronic treatment, and finally to clonal exhaustion and a minimal response. Thus, studies on T cells in humans that are chronically infected with viruses may generate conflicting results because of the uncontrolled variable of time. This effect could always be lessened by studying a larger number of patients, but it cannot be formally removed. Studies on animals are informative, but as discussed above, the only way to know for sure they recapitulate the human condition is to examine humans. Another advantage is that since GA is composed of multiple specificities, the response it generates is robust. There are however, a few draw backs to this approach. One is GA itself, which is a random polymer. Because of this, GA may have many effects that are difficult to eliminate. For example, it may bind to and affect T cells directly, or it may directly affect another cell type that indirectly effects a change on T cells. A second concern is that this study will only be conducted on MS patients as healthy controls will not be given this drug. This may limit the generality of the findings. There is little reason to suppose that MS patients will have altered responses to GA as opposed to healthy

controls and in any case, knowing the effect on the MS population is informative for the purposes of this study.

Objectives

Myelin-reactive T cells have been hypothesized to play a role in the pathogenesis of MS. If this is the case, these T cells would be expected to be repeatedly stimulated over the course of this disease because of the episodic breakdown of myelin membranes. The major objective of this thesis was to examine how this repeated stimulation would affect the phenotype of suspected auto reactive T cells in MS. In addition to revealing the possible nature of these autoreactive T cells, this approach would provide additional evidence for the role of these auto reactive T cells in the pathogenesis of MS. Another objective was to use this system to examine if T cells in MS patients have undergone significantly more homeostatic expansion than healthy controls, as this has been shown to result in autoimmunity.

Examining the effects of chronic stimulation was also conducted on MS patients injected daily with the antigen GA. In addition to providing information on the effects of repeated stimulation, it provided insight into possible mechanisms of action of GA as a therapeutic agent.

CHAPTER TWO MATERIALS AND METHODS

I. Overview of methodology

In this thesis, I have primarily used a flow cytometric approach to identify and phenotype cells of interest. This is a powerful technique, and enables the analysis of individual cells on a rapid basis. In this technique, fluorochromes are covalently attached to antibodies, which in turn specifically bind to cellular molecules of interest. Usually this binding role is performed by antibodies. The fluorochormes will emit electromagnetic radiation at separate frequencies when excited by a laser. Some overlap of spectra may be eliminated electronically, and is acceptable.

In this thesis, I have primarily examined cell responses in two ways, CD25 (IL-2Rα) up regulation and 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution. On CD8 T cells CD25 is expressed upon activation and is part of the IL-2 receptor. The same is the case for CD4 cells. However on CD4 T cells it is also expressed on a population of regulatory cells. This assay can detect activated cells regardless of whether they have divided. CFSE is a dye which is taken up by cells and bound covalently to intracellular proteins. Once bound it is remains inside the cell membrane. As cells divide they dilute the amount of dye they contain by one half with each division. Thus, dividing cells can be identified as well as the number of divisions. Cells that divide only one or two

times over the course of a week can be identified with this assay. Furthermore, it should be able to detect cell types overlooked by an LDA.

II. Reagents

Human media: 500ml RPMI 1640 (Cellgro, Herndon, VA, USA) supplemented with 5% human AB serum (Nabi, Miami, FL, USA), 5ml HEPES (Cellgro), 5ml Pen-Strep (Cellgro), and 5ml L-Glutamine (Cellgro).

Phosphate-Buffered saline (PBS): 0.144g/l KH2PO4, 9.0 g/l NaCl, 0.8 g/l Na_2HPO_4 (anhydrous), (Cellgro).

80% freezing media: 80ml Human Serum AB, and 20ml RPMI 1640.

20% DMSO Freezing media: 40ml RPMI 1640, 10ml DMSO, 400 μl HEPES buffer, and 400 μl L-glutamine.

PBS-Tween: 250μl tween 20 (Fisher Biotech, Subiaco, WA, USA) was mixed into 500ml of PBS.

PBS-Tween-BSA: 0.5g BSA (Sigma) was mixed with 50mls of PBS-Tween and used fresh.

ELISPOT Blocking solution: 0.5g BSA was mixed into 50ml PBS and used fresh.

AEC Solution: 100mg 3-amino-9-Ethyl carbozole (Sigma), was mixed into 10ml DMF (N, N, Dimethylformamide), in a glass vial in a fume hood.

0.1M Acetate Buffer: 148ml of 0.2M acetic acid (11.5 ml glacial acetic acid per lL of DI water) to 352 ml of 0.2M sodium acetate (27.2g in 1L DI water). The pH was adjusted to 5.0.

ELISPOT Visualization Solution: 800µl of AEC solution was mixed into 24ml 0.1M Acetate Buffer. This solution was passed through a 0.45µm filter to remove particulates, 12µl H_2O_2 was added and solution was used immediately.

FACS Juice: 1 100ml bottle of FACS Lysing Solution (Becton Dickson), was placed in a dark brown Nalgene bottle, and as it was stirred with a stir bar exactly 250µl tween 20 (PCR grade) was added.

DNase I: 50 U/ml DNase I solution (Sigma) was dissolved in 4.2mM $MgCl_2$, 10.15M$NaCl_2$ and the pH was adjusted to 5.0.

Antibodies: The following mouse anti-human fluorochrome conjugated antibodies were purchased from Pharmignen, anti-BrdU-PE, IgM anti-CD57-FITC, anti-CD8α–APC, anti-CD3ε-PE-Cy5 (Cy-Chrome), anti-CD25-PE-Cy5 (Cy-Chrome), anti-CD28-PE-Cy5 (Cy-Chrome), anti-CD28-PE, anti-CD4-APC. IgM anti-CD57-PE was purchased from Southern Biotech, Birmingham, AL, USA. The following conjugated isotype control mouse antibodies were used IgM-FITC, $IgG_1\kappa$–PE, $IgG_1\kappa$PE-Cy-5 (Cy-Chrome), $IgG_1\kappa$–APC (PharMingen), and IgM-PE (Southern Biotech). And anti-human mouse IgG_{2b} anti-PerforinFITC, or, mouse IgG_{2b}-FITC or, mouse IgG_{1K} IFN-γ FITC, or mouse IgG_1-FITC (Pharmingen) was used. Anti-human IFN-γ (Endogen, Rockford, IL, USA), rat anti-human/mouse IL-5(PharMingen, San Diego, CA, USA), mouse $IgG_{2a}\kappa$ anti-human CD3 (PharMingen), anti-IFN-γ (Endogen) labeled secondary antibody, 5 μg/ml anti-IL-5 (PharMingen) biotin were used in ELISPOT.

MACS column buffer: PBS supplemented with 0.5% BSA, and 2 mM EDTA. This solution was degassed by applying a vacuum before use.

MACS antibody mouse anti-human CD8 antibody: (isotype: mouse IgG2a; clone: BW135/80)

2-Amino-6-purinethiol (2-Amino-6-mercaptopurine; 6-Thioguanine): 6-Thioguanine is a purine analog that is converted into a toxic compound by the HPRT gene product in dividing cells. Cells with a mutation in the HPRT gene will be able to divide in the presence of 6-

thioguanine while those without will not. In this way 6-thioguanine can be used to select cells with a mutation in the HPRT gene. This provides an approximation of the overall mutation rate in a cell type. 6- thioguanine (Sigma) was suspended in culture medium to a final concentration of 1.5×10^{-3} M.

Glatiramer Acetate (GA, Copaxone®, TEVA Neuroscience LLC, Kansas City, MO, USA): is a random polymer of the four amino acids: L-glutamic acid, L-alanine, L-tyrosine, and L-lysine, with a molar fraction of 0.141, 0.427, 0.095, and 0.338 respectively. It has an average molecular weight from 4.7×10^3 to 1.1×10^4 Daltons. It is administered as an acetate salt. Unused GA was donated from patients. 2ml of PBS was added to each vial of GA containing 20mg. This was kept at 4°C and used within 3 months.

Antigens: 25 μg/ml gamma irradiated Cytomegalovirus (CMV) (Microbix Biosystems Inc., Toronto, Ontario, Canada), or 25 μg/ml gamma irradiated mumps (Microbix Biosystems Inc.), or 1 μg/ml mouse $IgG_{2a}\kappa$ anti-human CD3 (PharMingen) were used. Myelin peptides were synthesized by C S Bio Company Inc, Menlo Park, CA, USA. The panel of peptides consisted of 25 μg/ml proteolipid protein peptides (31-60, 91-120, 111-140, 131-160, 177-206), 25 μg/ml myelin oligodendrocyte glycoprotein peptides (63-87, 81-100, 91-110), and 25 μg/ml myelin basic protein peptides (83-106, 111-129, 143-171), or in some early experiment 25 μg/ml bovine myelin basic protein (Sigma, St. Louis, MO, USA), was used instead of myelin basic protein peptides.

Primers: (IFN-γ forward TTTAATGCAGGTCATTCAGATGTA, reverse CACTTGGATGAGTTCATGTATTGC: Granzyme B forward TGCAGGAAGATCGAAAGTGCG, reverse GAGGCATGCCATTGTTTCGTC: β-actin forward CCTGGACTTCGAGCAAGAGA, reverse ACTTGCGCTCAGGAGGAGCA: CCR4 forward TTGGACTATGCCATCCAGGC, reverse AATTCCCTCTGGAGAAACCC: CXCR3 forward CACTGCCCTTCTCATTTGGAAACT, reverse GCAAATATAGAGGTCTTGGGGAC: TNF-α forward CGAGTCTGGGCAGGTCTACTTT, reverse AAGCTGTAGGCCCCAGTGAGTT)

III. Patient samples

All GA treated MS patients were self-injected subcutaneously once daily with 20mg Glatiramer Acetate (GA, Copaxone®) (TEVA Neuroscience LLC, Kansas City, MO, USA). Healthy volunteers and MS patients were recruited at the University of Texas Southwestern Medical Center and consented to be leukopheresed every four or six months as approved by the University of Texas Southwestern Institutional Review Board (Table 1-Table 4). PBMC were isolated from leukopheresed blood by the following method.

On the day of collection, pheresis product was aseptically mixed with PBS 1 to 1 in 50ml conical tubes. 30ml of this solution was layered over 15ml of room temperature Ficoll Paque (Amersham Biosciences, Uppsala, Sweden).

Table 1 Patient samples: Healthy Controls (HC).

Patient code	Patient number	Age y	Gender
H333	HC1	51	F
H219	HC2	46	M
H504	HC3	34	F
H183	HC4	31	F
H437	HC5	22	M
H948	HC6	28	M
H123	HC7	43	F
H836	HC8	40	F
H727	HC9	41	M
H255	HC10	37	F

Table 2 Patient samples: Untreated MS patients (MS).

Patient code	Patient number	Age y	Type of MS*	Gender	Patient code	Patient number	Age y	Type of MS*	Gender
M466	MS1	31	PP	F	M584	MS14	39	RR	F
M140	MS2	44	RR	F	M792	MS15	45	PP	F
M295	MS3	38	PP	M	M971	MS16	45	RR	F
M311	MS4	51	RR	F	M731	MS17	55	PP	M
M210	MS5	46	RR	F	M934	MS18	32	RR	M
M616	MS6	37	RR	F	M522	MS19	32	RR	F
M833	MS7	52	PP	F	M933	MS20	23	RR	F
M705	MS8	59	PP	M	M396	MS21	32	RR	M
M819	MS9	55	PP	M	M469	MS22	32	RR	F
M222	MS10	50	PP	F	M545	MS23	54	SP	M
M596	MS11	38	PP	F	M808	MS24	49	PP	M
M304	MS12	47	PP	M	M763	MS25	49	PP	M
M626	MS13	50	RR	F	M250	MS26	44	RR	F

*RR= Relapsing Remitting, PP= Primary Progressive, SP = Secondary Progressive.

Table 3 Patient Samples: MS patients Treated with GA (MST).

Patient code	Patient number*	Age y	Type of MS**	Gender
M705	MST8	59	PP	M
M819	MST9	55	PP	M
M222	MST10	50	PP	F
M596	MST11	38	PP	F
M304	MST12	47	PP	M
M626	MST13	50	RR	F
M584	MST14	39	RR	F
M792	MST15	45	PP	F
M971	MST16	45	RR	F
M731	MST17	55	PP	M

*MST = Multiple Sclerosis patient Treated with GA for at least 12 months.

**RR= Relapsing Remitting, PP= Primary Progressive.

Table 4 Patient samples used in Figure 24-Figure 26.

Patient code	Patient no.*	Age y	Type of MS**	Gender	Patient code	Patient no.*	Age y	Type of MS**	Gender
H333	HC1	51	NA	F	M295	MS3	38	PP	M
H219	HC2	46	NA	M	M210	MS5	46	RR	F
H504	HC3	34	NA	F	M616	MS6	37	RR	F
H183	HC4	31	NA	F	M833	MS7	52	PP	F
H437	HC5	22	NA	M	M705	MS8	59	PP	M
H948	HC6	28	NA	M	M222	MS10	50	PP	F
H123	HC7	43	NA	F	M596	MS11	38	PP	F
H836	HC8	40	NA	F	M934	MS18	32	RR	M
H727	HC9	41	NA	M	M522	MS19	32	RR	F
M466	MS1	31	PP	F	M933	MS20	23	RR	F
M140	MS2	44	RR	F					

*HC= Healthy control, MS= MS patient untreated, numbers are not always sequential because some patients were not performed to control for age. **NA (Not Applicable), RR= Relapsing Remitting, PP= Primary Progressive.

This was centrifuged for 30 minutes at 1900rpms in an 8510R centrifuge (Eppendorf, Westbury, New York) with an A-4-62 rotor (eppendorf) with the brake off. The buffy coat was subsequently transferred to a clean 50ml conical tube via a sterile transfer pipette. The buffy coat (15ml) was then mixed 1:2 with 30ml of PBS. This solution was gently well mixed, and centrifuged at 1850 rpms in an 8510R centrifuge for 10 minutes at room temperature. The supernatant was decanted and each pellet was re-suspended in 10ml PBS. This solution was then further diluted with PBS and centrifuged at 4°C for 10 minutes at 750 rpms. The pellet was then suspended in 80% human serum freezing media. The quantity of cells was then determined using a hemocytometer and a Leica ATC 2000 microscope (Leica, 35578 Wetzlar, Germany). Fresh samples were dispensed from this stock, and frozen samples were prepared as follows. The cells solutions were mixed 1 : 1 with 20 % DMSO freezing media, and either placed in an isopropanol freezing container at -70°C for 3 hours, or slowly chilled at approximately -1°C per minute until reaching –90°C. All samples were stored in liquid N_2 until use. Samples were then placed in a 37°C water bath until thawed, and mixed with chilled PBS at a 1:10 dilution and centrifuged at 1,000rpm for 10 minutes. Subsequently, the pellets were resuspended in PBS and centrifuged again at 1,000rpms for 10 minutes. The cells were resuspended, and the quantity of cells determined using a hemocytometer and a microscope (Leica).

IV. Cell Culture and Related Assays

A. ELISPOT

96 well ImmunoSpot M200 plates were pre-coated with mouse anti-human IFN-γ diluted to 4 μg/ml in PBS (Endogen, Rockford, IL, USA) or rat anti-human/mouse IL-5 antibody diluted to1 μg/ml in PBS (PharMingen, San Diego, CA, USA) and incubated overnight at 4°C in a humidified box. The next day the antibody solution was aspirated off and the wells washed 3 times with 200μl PBS each. Subsequently 200μl 1% BSA in PBS was added per well and incubated at room temperature for 1 hour to block non-specific binding. The plate was washed 3 times with 200μl PBS per well. 100μl of human media containing or not containing 100 μg/ml GA, or 2 μg/ml mouse $IgG_{2a}\kappa$ anti-human CD3, (PharMingen) was added. Next 5 $x10^6$ PBMC in 100 μl were added per well. This gave a final concentration of 50μg ml GA and 1μg/ml anti-CD3. The plates were incubated for 36 hours at 37°C in 5% CO_2. Subsequently the plates were washed 3 times with 200μl PBS per well and once with DI water to eliminate background. 100μl of 2 μg/ml anti-IFN-γ (Endogen) or 5 μg/ml anti-IL-5 (PharMingen) biotin labeled secondary antibody diluted in PBS-tween-BSA was added to each well. The plates were then and incubated overnight in a humidified box at 4°C. Plates were washed 4 times with 200μl PBS-tween per well. 100μl Streptavidin-HRP (Dako, DK-Glostrup, Denmark) diluted 2,000 to 1 in PBS-Tween-BSA was added and incubated at room temperature for 2 hours. The plate was washed with 200μl per well PBS-tween 3 times and subsequently washed 3 times with 200μl PBS per well.

200µl visualization solution was added per well and development was watched by eye and allowed to incubate from 10 to 60 minutes as appropriate. Plates were washed with DI water to stop reaction and air-dried in laminar flow hood overnight. SPOTS were counted on an Alpha Imager (Alpha Innotech Corporation, San Leandro, CA, USA).

B. ^{3}H Proliferation assays

PBMC were plated in 96-well round bottom tissue culture plates at a concentration of 5×10^5 cells per well in 200 µl of human media. Quadruplicate cultures were set up with the indicated doses of antigens. Cells were pulsed with 0.25 µCi per well of ^{3}H-thymidine for the last 18 hours of culture. On day 5 of culture, cells were harvested onto glass filters (Wallac, Turko, Finland) and analyzed for incorporation of radioactivity using a Wallac Betaplate liquid scintillation counter (Wallac, Gaithersburg, MD). Results are expressed in Counts Per Minute (CPM) with Standard Error of the Mean (SEM).

C. Limiting dilution assay

PBMC were plated in round bottom plates at the following dilutions 1×10^5, 5×10^4, 2.5×10^4, and 1.25×10^4. PBMC, irradiated with 30 Gy were added to produce a total of 1×10^5 cells per well. 192 replicates were set up for each dilution, half with no antigen and half with 50 µg/ml GA. These cultures were incubated at 37°C in 5 % CO_2. Each well was pulsed with 0.25 µCi [methyl-^{3}H]-thymidine (Amersham) on day 5 and harvested onto glass

filters (Wallac) using an automated harvester (Tomtec) on day 6. Filters were counted using a 1205 Betaplate (Wallac, Turko Finland). Proliferation 2 standard deviations above the mean was considered a positive response. The precursor frequency was determined using the Poisson distribution and chi square minimization.

D. Cell Culture

Cells were re-suspended in human media at 2 $x10^6$ cells per ml. Subsequently the cells were pipetted into sterile capped 12 x 75mm FACS tubes. Subsequently appropriate antigens were added and the cells incubated for the indicated amount of time at 37°C in 5% CO_2. To provide the appropriate amount of surface area, the tubes were angled in the incubator at approximately 30°. For experiments with large numbers of cells, cells were plated in 24 well plates and cultured for the indicated amount of time at 37°C in 5% CO_2. For intracellular cytokine staining, 10μg / ml Brefeldin A was added for the last 12 hours of culture.

V. Flow cytometry

A. CFSE staining

PBMC were diluted to 1 $x10^6$ cells per ml in PBS, and 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR, USA) was added to give a final concentration of 0.25 μM. The cells were subsequently incubated for 7 minutes

in a 37°C water bath in the dark. Next the cells were diluted with PBS and 1 % serum to give a 2 % serum concentration in PBS. The cells were centrifuged at 1,000rpm for 10 minutes. Then the cells were washed with 2 % serum in PBS again and centrifuged for 10 minutes at 1,000rpm. Cells were then resuspended in human media and cultured. After culture CFSE loaded cells were analyzed immediately on a FACS Calibur (Becton Dickinson, Franklin Lakes, New Jersey, USA).

B. Surface Staining

Cells were resuspended in 12 x 12mm tubes in 4 ml of 1 % BSA in PBS and centrifuged for 10 minutes at 1,000 rpm. Next the cells were again resuspended in 4 ml of 1 % BSA in PBS and centrifuged for 10 minutes at 1,000 rpm. The supernatant was decanted which left a volume of 200µl per tube. Appropriate fluorochrome conjugated antibodies and isotype control antibodies were added and incubated for 30 minutes on ice in the dark. Subsequently the cells were washed 2 times with 4 ml of 1 % BSA in PBS as before. Data was acquired either immediately on a FACS Calibur, or cells were fixed with a 1 % paraformaldehyde solution and data was acquired on a FACS Calibur within 3 days.

C. Intracellular flow cytometry

For cytokine staining, 10 ng/ml PMA (Sigma), and 1µg/ml ionomycin (Sigma) was added for the last 12 hours of culture. Cells were surface stained as above. Next cells were

washed 2 times with 1 % BSA in PBS. Next the cells were permeabilized by adding 750µl of FACS Juice per tube. Tubes were incubated for 10 minutes in the dark and washed 3 times with 1 % BSA in PBS. All centrifugations performed after permeabilization were conducted at 1800rpm. The cells were then incubated with intracellular antibodies on ice for 45 minutes in the dark. The cells were washed 2 times with PBS and analyzed as before on a FACS Calibur (Becton Dickinson).

D. 5-bromo-2-deoxyuridine (BrdU), Proliferation Assay

BrdU is an analog of thymidine and is incorporated into dividing cells. Subsequently the cells may be permabilzed and the presence of BrdU detected by anti-BrdU antibodies attached to a reporter molecule, in this case, PE. Protocol was adapted from (Tough and Sprent 2001).

2 x10^6 PBMC per ml were cultured for 7 days as at 5% CO_2 at 37°C as before. 150 µM 5-bromo-2-deoxyuridine (BrdU) was added to culture for the last 3 days of culture. Subsequently PBMC were surface stained as above. Next the cells were washed with 1 ml PBS per tube and resuspended in 0.5ml ice cold 0.15M NaCl. 1.2 ml of ice cold 95% ethanol was added as the cells were agitated and incubated for 40 min on ice. Cells were resuspended in 2 ml PBS and centrifuged for 10 min at 1500rpm. The cells were resuspended in 1 ml DNase I solution and incubated for 10 min at room temperature. The cells were washed with 2 ml PBS and 10µl anti-BrdU was added. The cells were incubated for 30 min on ice, washed and analyzed on a FACS Calibur (Becton Dickinson).

E. Florescence Activated Cell Sorting (FACS)

Cultured or thawed cells were passed though a nylon mesh filter, and labeled with surface antibody as above. Subsequently, cells were washed and sorted into $CD8^{hi}CD28^{-}CD57^{+}$, or $CD8^{hi}CD28^{+}CD57^{-}$ populations on a BD FACStar or FACSVantage SE sorter. Sorted cells were typically 95 % pure. Cells sorted for subsequent culture were collected in human media. Cells sorted for subsequent molecular experiments were sorted into RNAlater (Qiagen Inc, Qiagen Strasse 1, 40724 Hilden, Germany).

VI. MACS sort

A. Positive Selection

Thawed PBMC were passed through a nylon-mesh filter, to remove clumps, washed with MACS column buffer, and 20µl of MACS CD8 microbeads (Miltenyi Biotec, Auburn, CA) per 10^7 cells were added. This was incubated for 15 minutes at 6°C. MACS column buffer was added to wash the cells and the cells were centrifuged at 1200rpm for 10 minutes. The cells were then resuspended in 500ul and applied to a LS MACS Columns (Miltenyi Biotec) in a magnetic field. Cells without bound magnetic beads will pass through the column while those with beads will remain. To remove the unbound cells the column was washed several times with buffer. Subsequently the column was removed from the magnetic

field and cells with antibody bound were eluted with several washings. Cells were typically 95 % pure.

B. Negative Selection

The flow through was collected from the positive selection above and washed with MACS column buffer, resuspended in 500µl MACS column buffer, and passed over an LD MACS Column (Miltenyi Biotec). This column was washed several times to remove all cells. The cells were passed over an LD column as these are optimized for negative selection, and other wise a low purity would result.

VII. RNA isolation

RNA was extracted from FACS sorted cells using micro RNeasy kit (Qiagen) according to the manufactures instructions. Briefly cells were initially lysed by adding 500 µl RLT Buffer and gently vortexed. The solution was homogenized by drawing sample into an 18 gauge needle attached to a syringe 10 times. When working with 3,000 cells or less carrier tRNA was added. 500µl 70% ethanol in molecular biology grade RNase free water was added and mixed. This sample was passed through a column silica-gel-based membrane. The column was washed with 350µl RW1. DNase I was applied to the column to digest any bound DNA leaving only RNA. The column was again washed with 350µl RW1, and with 500µl Buffer RPE, and then with 80% ethanol in molecular biology grade

RNase free water. RNA was then eluted from the column with 14µl molecular biology grade RNase free water.

VIII. RNA amplification

RNA was amplified once using a RiboAmp® RNA Amplification Kits (Arcturus Bioscience, Inc., Mountain View, CA, USA) 1.0µl of mRNA specific primer was and added, and the sample incubated for 5 minutes at 65°C. Next the samples were chilled to 4°C for one minute. To this was added the complete first stand synthesis mix reagents to transcribe RNA into cDNA. The sample was incubated for 45 minutes at 42°C. The sample was chilled to 4°for one minute. 2.0µl nuclease was added and incubated for 20 minutes at 37°C, then at 95°C for five minutes. Next the second cDNA strand was synthesized. First 1.0µl second strand primer was added and the samples were incubated at 95°C for two minutes and then chilled at 4°C for two minutes. Next 30 µl second strand reaction mix was added and the sample incubated in a thermocycler as follows. 25°C 5 minutes, 37°C 10 minutes, 70°C 5 minutes. This double stranded DNA was purified on a column. First a DNA / RNA purification column was acclimated by wetting it with 250µl DNA Binding buffer. After 5 minutes the column was centrifuged at 16,000g for 1 minute and the flow through discarded. Next the double stranded cDNA was diluted with 200µl DNA binding buffer and applied to the column. This was spun at 100 x g for one minute to bind the cDNA to the column and then 30 seconds at 10,000 g to elute the buffer. Next 250µl of DNA wash buffer was added

and the column spun for 1 minute at 16,000 g to elute the buffer. DNA was removed from the column with 16µl DNA Elution buffer and a 1,000 x g centrifugation followed by a 16,000 x g centrifugation for one minute. Purified cDNA was subsequently transcribed into aRNA by adding 24µl IVT Reaction mixture mixed and incubated at 42°C for 3 hours. After this 2µl DNase mix was added to remove cDNA. The amplified aRNA was purified on a column by diluting it with 200µl RNA Binding Buffer, placing it over a column pre-conditioned with 250µl RNA binding buffer, and centrifuging at 100 g for two minutes. This was done to bind aRNA to the column. Subsequently the column was centrifuged at 10,000 x g to remove the flow-through. The column was washed by adding 200µl RNA wash buffer and centrifuging at 10,000 x g for one minute, then washed again by adding 200µl RNA wash buffer and centrifuging at 16,000 x g for one minute, aRNA was removed from the column with 30µl RNA Elution buffer and a 1,000 x g centrifugation for 1 minute was followed by a 16,000 x g centrifugation for one minute. 20µl of this was used to in cDNA transcription.

IX. cDNA transcription

cDNA was transcribed from amplified RNA using a Ready To Go T-primed First-strand kit (Amersham Biosciences) according to the manufactures instructions. 20µl of RNA was diluted to 33µl. This solution was incubated 5 minutes at 65°C, then for 5 min at 37°C. The RNA solution was then applied to a cDNA reaction mix, and incubated for 5 min in a

37°C water bath. Next the sample was mixed by pipetting. The sample was flash centrifuged and incubated for 60 min in a 37°C water bath.

X. Real time PCR

Quantitative real-time PCR assays were performed using 1.25U Taq (Amersham Pharmacia), 1.5 mM $MgCl_2$ 0.2 mM deoxynucleotide triphosphate, 1.2 μM forward and reverse primers in 1 x PCR buffer. The following conditions were used: 95°C for 10 minutes one time (95°C for 45 seconds, 55°C for 1 minute, and 81°C for 1 minute) x 40 cycles, 95°C for 1 minute and 57°C for 25 seconds one time. SYBRGreen I nucleic acid gel stain 10,000 (Molecular Probes) was added at a final dilution of 1 to 50,000. SYBRGreen binds to double stranded DNA but not single stranded DNA and is used to quantify the amount of a PCR product as it is produced. PCR reactions were read by BioRad iCycler thermocycler (BioRad, Hercules, CA, USA). Melting analysis was performed from 55°C to 100°C at 0.5°C per second at the end of the reaction, to determine the specificity of the reaction.

XI. Statistics

Since sample sizes used were generally less than 13, a Gaussian distribution could not be confirmed and was not assumed. Therefore to determine significance between groups a non-parametric Mann-Whitney t test was performed. A one or two tailed test was performed as indicted to determine statistical significance. A paired student t test was also conducted as

appropriate. A p value of less that 0.05 was considered significant. The Poisson distribution and chi square minimization was used to determine the precursor frequency from limiting dilution assays. The precursor frequency was calculated from CFSE dilutions according to

the following formula where U= the number of non-dividing cells, E = the number of events in each division and d= the division number.

$$\text{precursor frequency} = \frac{\sum_{d=2}^{n} \frac{E_d}{2^d}}{\left(\sum_{d=2}^{n} \frac{E_d}{2^d}\right) + U} - \text{precursor frequency of media alone}$$

In GA studies samples with a proliferating fraction of at least one percent above no antigen control and a stimulation index above two were considered positive. In GA studies the same criteria were used for CD25. Namely, the difference in $CD25^+$ expression between sample and no antigen must have been at least one percent, and must have been at least two times above the no antigen control.

XII. Software used

CellQuest (BD Biosciences) was used to collect flow cytometry data. WinMDI 2.8 (The Scripps Research Institute, La Jolla, CA, USA) and CellQuest pro were used to analyze

collected data. GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA, USA) was used to graph data and for statistical analysis. Microsoft Excel (Microsoft C Corporation, Redmond, WA USA) was used to tabulate data. Microsoft Power Point (Microsoft Corporation) and Adobe Photoshop 5.5 (Adobe Systems Incorporated, San Jose, CA) were used to generate figures. Microsoft Word (Microsoft Corporation) was used to write the manuscript and Endnotes 9 (Thomson ResearchSoft, Carlsbad, CA) was used to track references.

CHAPTER THREE RESULTS

I. $CD28^{-}CD57^{+}$ T cells are detectable in MS Patients

Rheumatoid arthritis, another organ specific autoimmune disease, has been hypothesized to result from homeostatic T cell proliferation (Wagner, Koetz et al. 1998). RA patients displayed increased numbers of $CD28^{-}$ and $CD57^{+}$ lymphocytes when the total population of peripheral blood $CD4^{+}$ T cells was examined (Schmidt, Goronzy et al. 1996). I hypothesized homeostatic proliferation generates effector T cells which are myelin-specific and cause MS, and as in RA when this happens higher levels of $CD28^{-}CD57^{+}$ cells would be found. I was first interested in determining if a similar mechanism could be responsible for the initiation of MS. To determine if MS patients possess a greater number of $CD28^{-}CD57^{+}$ T cells, I was first interested in determining if a $CD28^{-}CD57^{+}$ population could be detected in MS patients and if so, if it is increased in size relative to healthy controls. To address this question, frozen PBMC previously collected by leukopheresis were thawed and labeled with CD57 FITC, CD28 PE, CD3 PerCP, and CD4 APC or CD8 APC. These labeled PBMC were subsequently analyzed by flow cytometry. Figure 1A displays the gate (R1) that was used to identify the live lymphocyte population. Shown in Figure 1B is the gate used to identify the $CD3^{+}$ $CD4^{+}$ events (R2, blue). When an isotype matched control antibody bound to APC was used instead of anti-human CD4 mouse antibody bound to APC, only one APC^{+} event was detected, indicating the APC^{+} signal detected in panel 1B is due to increased CD4

expression on the cells in question (Figure 1C). Panel D displays the intensity of CD57 FITC and CD28 PE florescence of all events that were included in both the R1 and R2 gates. While the majority of events fell within the $CD28^{+}CD57^{-}$ quadrant (90.2%), a distinct population fell within the $CD28^{-}CD57^{+}$ quadrant (6.6%). This indicates that $CD4^{+}$ $CD28^{-}$ $CD57^{+}$ T cells can be detected in MS patients. When isotype control IgM FITC antibody was used, this population was not detected (Figure 1E). $CD8^{hi}$ $CD57^{+}CD28^{-}$ T cells were also detected in our MS population. This was expected as most adult humans harbor this population in their CD8 T cell compartment (Phillips and Lanier 1986). When examining CD8 T cells only $CD8^{hi}$ populations were examined to exclude $CD3^{-}$ events, this was not necessary when CD4 T cells were examined. We chose to use frozen PBMC to allow the simultaneous examination of samples from different patients at the same time. To determine what effect, if any, the freezing of PBMC has on this cell type, I analyzed the expression of CD28 and CD57 on CD4 and CD8 T cells before and after freezing (Table 5). As shown no difference was detected in the percentage of CD28 and CD57 expressing cells in CD4 or CD8 T cells populations.

A. MS patients do not harbor a greater percentage of $CD28^{-}CD57^{+}$ T cells

Since I consistently detected $CD28^{-}CD57^{+}$ T cells in MS patients, I wanted to determine if MS patients harbor elevated levels of this cell type. If MS is initiated by lymphopenia induced homeostatic proliferation, MS patients may possess a greater number of $CD28^{-}CD57^{+}$ T cells. To determine if this is the case, I examined previously frozen PBMC from MS patients and healthy controls by flow cytometry.

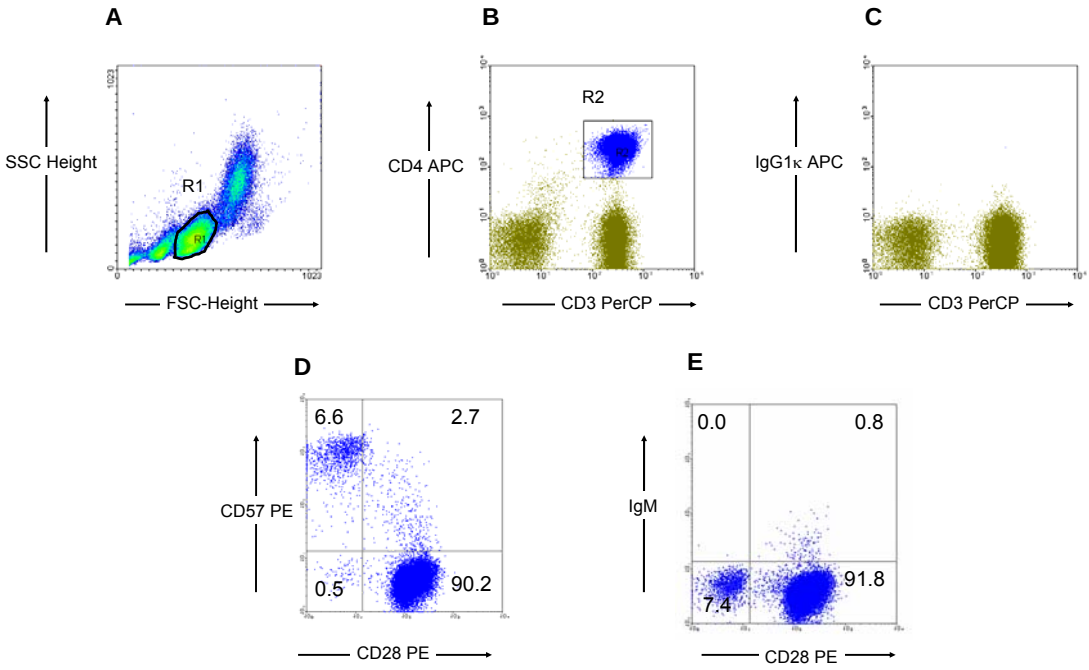

Figure 1 $CD28^{-}CD57^{+}$ T cells can be detected in $CD3^{+}CD4^{+}$ T cells from MS patients.

PBMC from MS patients were labeled with CD57 FITC, CD28PE, CD3PerCP, and CD4 APC or CD8 APC. The PBMC were subsequently examined by flow cytometry. (A) Displays side vs. forward scatter and the live lymphocyte gate (R1). (B) Shows the intensity of PerCP and APC florescence from all events that fall in R1 and the gate used to identify $CD3^{+}$ $CD4^{+}$ T cells (R2), (C) shows the same gate on PBMC labeled with isotype control APC. (D) Displays the expression of CD57 and CD28 on R1 and R2 gated events. Numbers represent percentage of events in each quadrant. E shows the same gate on PBMC labeled with isotype control FITC. One representative MS patient is displayed. As shown in Table 6, this was performed on 9 MS (MS) patients and 7 healthy controls (HC).

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Table 5 Freezing does not significantly alter percent of $CD28^{-}CD57^{+}$ T cells.

Patient code	Patient number		$CD3^{+}$ $CD4^{+}$ CD28- $CD57^{+}$	$CD3^{+}$ $CD4^{+}$ CD28-	$CD3^{+}$ $CD8^{hi}$ CD28- $CD57^{+}$	$CD3^{+}$ $CD8^{hi}$ CD28-
M222	MST10	Fresh	2.34	3.56	41.01	51.65
M222	MST10	Frozen	3.4	4.69	42.59	53.58
M763	MS25	Fresh	0.1	0.13	6.06	9.06
M763	MS25	Frozen	0.22	0.29	6.95	10.24
M250	MS26	Fresh	0.01	0.03	1.45	3.56
M250	MS26	Frozen	0.01	0.05	2.01	4.45

As shown in Table 6, no significant difference was found in the level of $CD28^{-}CD57^{+}$ T cells expressing either the CD4 or CD8 receptor between healthy controls and MS patients. It was however, again confirmed that $CD4^{+}$ $CD28^{-}CD57^{+}$ T cell can be found in MS patients.

B. $CD28^{-}CD57^{+}$ T cells proliferate in seven day assays

One of the objectives of this thesis is to examine how repeated stimulation affects the phenotype of T cells in MS patients. To accomplish this, I sought to identify senescent T cells. I examined the hypothesis that $CD57^{+}$ T cells are senescent. If this is the case they would be unable to replicate even if given a powerful stimulus, such as anti-CD3. To test this hypothesis, I stimulated PBMC with anti-CD3 and examined proliferation by BrdU incorporation. As shown in Figure 2 both CD4 and $CD8^{hi}$ $CD57^{+}$ T cells incorporated BrdU, indicating they were able to divide. This indicated they are not senescent. While $CD57^{+}$ T cells may not be fully senescent, the possibility remained they have lost some of their proliferative capacity but not all and are pre-senescent. To examine this possibility I examined the proliferation of $CD57^{+}$ T cells over time. In this case CFSE dilution was used to detect proliferation, as it allows the determination of the number of cell divisions that have occurred after loading of the dye. CFSE loaded PBMC from MS patients were stimulated with anti-CD3 and the proliferative response examined. CD28 expression was also examined as these cells have been hypothesized to be senescent as well. After 2 days, proliferation was not detected in the $CD8^{hi}CD28^{-}CD57^{+}$ population, but was detected in the other 3 $CD8^{hi}$

Table 6 Overall level of CD28-CD57^{+} T cell is the same in MS and healthy controls.

Patient codes	Patient no.	CD3+ CD4+ CD28- CD57+	CD3+ CD4+ CD28-	CD3+ CD4+ CD28- CD57+	CD3+ CD8hi CD28- CD57+	CD3+ CD8hi CD28-	CD3+ CD8hi CD28- CD57+
H333	HC1	0.03	0.06	0.01	3.37	6.74	2.99
H219	HC2	0.42	0.60	0.00	14.44	19.34	7.76
H504	HC3	0.11	0.22	0.03	10.80	18.50	7.41
H183	HC4	0.21	0.26	0.16	8.53	12.05	6.49
H123	HC7	27.18	46.00	26.03	44.60	71.20	31.70
H836	HC8	0.04	0.60	0.02	16.70	21.90	15.59
H255	HC10	0.29	0.30	0.11	22.50	41.90	13.78
Average		4.04	6.86	3.76	17.28	27.38	12.25
SD		10.20	17.26	9.818	13.50	22.23	9.612
M140	MS2	31.19	32.46	30.25	37.17	46.33	23.45
M626	MS13	6.80	7.40	5.90	26.92	37.08	19.98
M584	MS14	0.24	0.34	0.02	12.36	17.57	11.50
M522	MS19	6.8	7.8	6.04	16.39	27.27	8.38
M396	MS21	0.00	0.08	0.00	5.34	10.22	3.23
M469	MS22	0.77	1.67	0.64	39.92	43.48	39.28
M545	MS23	0.13	0.38	0.03	5.89	9.14	5.56
M808	MS24	0.05	0.14	0.01	35.14	39.82	28.79
M763	MS25	0.16	0.26	0.14	7.70	11.88	7.30
Average		5.12	5.61	4.78	20.76	26.98	16.39
SD		10.18	10.54	9.881	14.14	15.12	12.26

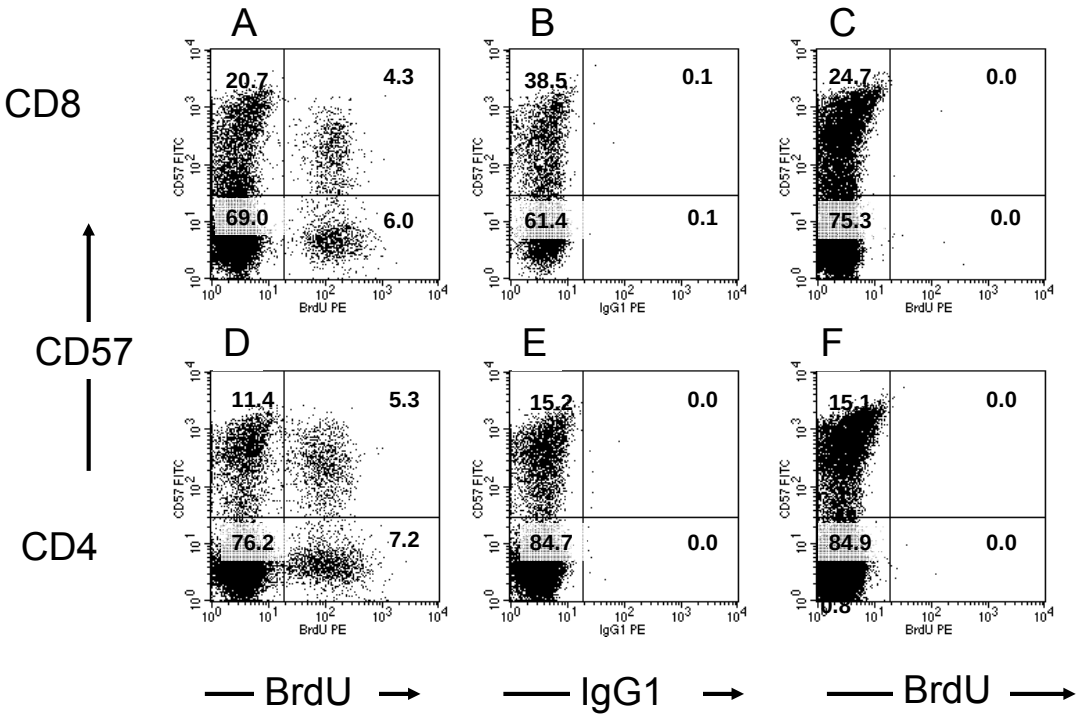

Figure 2 CD57^{+} T cell proliferation can be detected by BrdU incorporation.

PBMC were stimulated for 7 days with (A, B, D, E) anti-CD3 or (C, F) no antigen BrdU was added for the last 3 days of culture. The cells were then analyzed by flow cytometry. (A-C) gated on CD8^{hi} T cells. (D-F) Gated on CD4^{+} T cells. 1 MS patient (MS) and 1 Healthy Control (HC) were examined. The MS patient is shown.

populations made up by CD28 and CD57 expression (Figure 3A). After 3 and 5 days of stimulation, proliferation was detectable in all four $CD8^{hi}$ populations, but still the lowest in the $CD8^{hi}CD28^{-}CD57^{+}$ T cells (Figure 3A). This indicates that $CD8^{hi}$ $CD28^{-}CD57^{+}$ T cells proliferate more slowly than $CD28^{+}$ T cells, and $CD28^{-}CD57^{+}$ T cells may be pre-senescent. Another positive benefit of this result, is that these cells can be detected by proliferation. When $CD4^{+}$ T cells were examined, no proliferation was detected in any population on day 2 (Figure 3B). By day 4, proliferation was robust in the $CD28^{+}CD57^{-}$ population, and by day 7 proliferation was detected in all 4 populations (Figure 3B). The small number of events observed in the $CD4^{+}CD28^{-}CD57^{+}$ compartment on day 4 made interperation of this data difficult. Since the possibility remained that the $CD57^{+}$ T cells observed in the dividing fraction only expressed CD57 after they had undergone division, I wished to confirm that $CD57^{+}$ T cells are indeed able to divide. To accomplish this, $CD4^{+}CD28^{+}CD57^{-}$ and $CD4^{+}CD28^{-}CD57^{+}$ cells were sorted from bulk PBMC loaded with CFSE and stimulated with 1 μg/ml anti-CD3 for 7 days. $CD28^{+}CD57^{-}$ T cells remained $CD28^{+}$ (Figure 4A) and few if any became $CD57^{+}$ (Figure 4B). When $CD4^{+}CD28^{-}CD57^{+}$ T cells were stimulated with 1 μ/ml anti-CD3 for 7 days the majority of cells remained $CD28^{-}$ and $CD57^{+}$ (Figure 4C & D). It appears that some previously $CD57^{+}$ T cells may have down-regulated CD57 during seven days of incubation, although, the possibility of contaminating $CD28^{+}$ T cells cannot be ruled out. I also examined CD8 T cells in the same manner. As before $CD28^{+}CD57^{-}$ and $CD4^{+}CD28^{-}CD57^{+}$ cells were sorted from bulk PBMC, however now $CD8^{hi}$ cells were sorted.

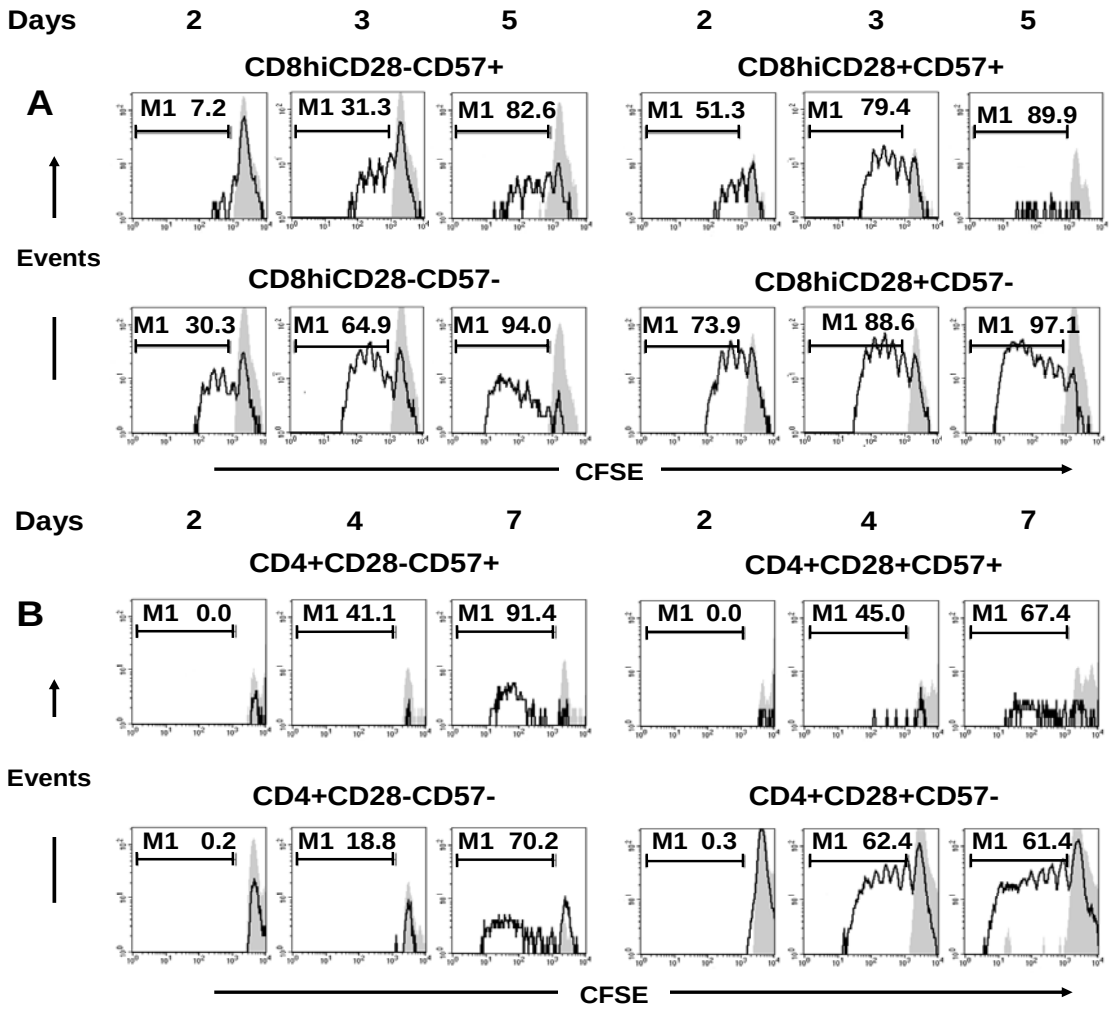

Figure 3 Anti- CD3 induced proliferation can be detected in $CD28^{-}CD57^{+}$ T cells that express either the CD4 or CD8 coreceptor**.**

PBMC were loaded with CFSE stimulated with anti-CD3 (open histograms) or no antigen (closed histograms) for the indicated number of days, and examined by flow cytometry. (A) $CD8^{hi}$ gated T cells and (B) $CD4^{+}$ gated T cells are displayed. The four populations created by CD28 and CD57 expression on are shown for both CD4 and CD8 T cells ($CD28^{-}CD57^{+}$ (upper left); $CD28^{-}CD57^{-}$ (lower left); $CD28^{+}CD57^{+}$ (upper right); $CD28^{+}CD57^{-}$ (lower right). Dividing cells dilute their CFSE content and shift to the left. Numbers denote the percent of $CFSE^{dim}$ events of anti-CD3 stimulated samples. These results are representative of 3 MS patinets, and 1 Healthy Control (HC). An MS patient is shown.

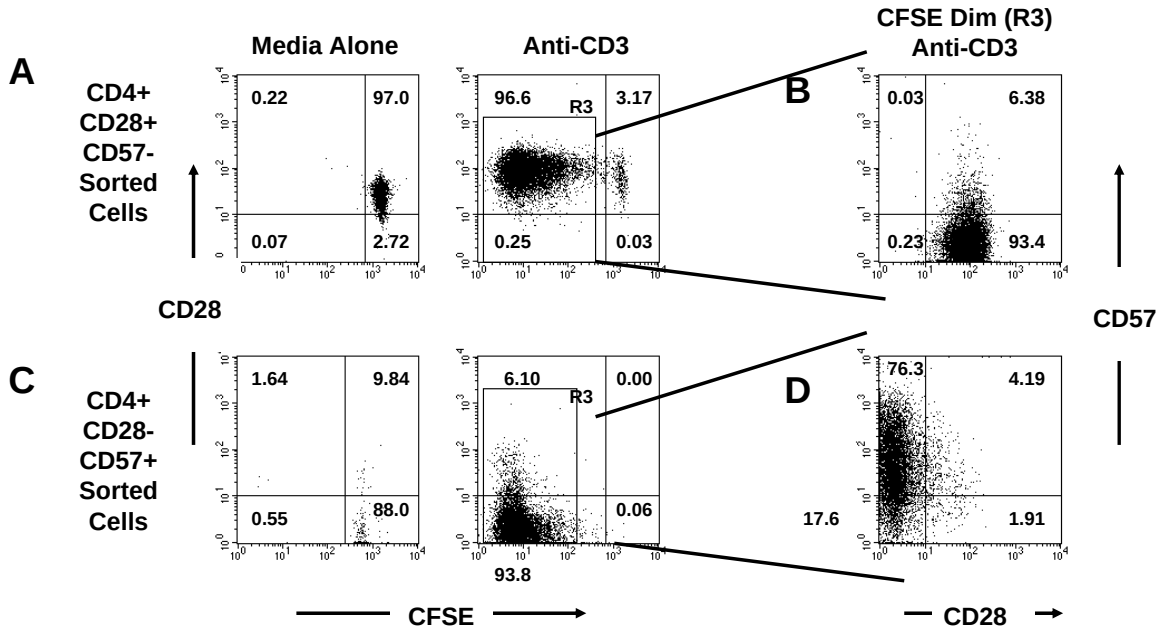

Figure 4 Sorted $CD4^+CD28^-CD57^+$ T cells remain $CD28^-CD57^+$ after stimulation.

$CD4^+$ $CD28^+$ $CD57^-$ and $CD4^+$ $CD28^-$ $CD57^+$ cells were sorted from whole PBMC and loaded with CFSE. Irradiated feeder cells were added to give a final concentration of $2x10^6$ per ml. 1 μg / ml anti-CD3 was added and the cells were incubated for 7 days. Subsequently the cells were analyzed by flow cytometry. Plots are electronically gated for $CD4^+$ lymphocytes. This Experiment was performed once on an MS patient.

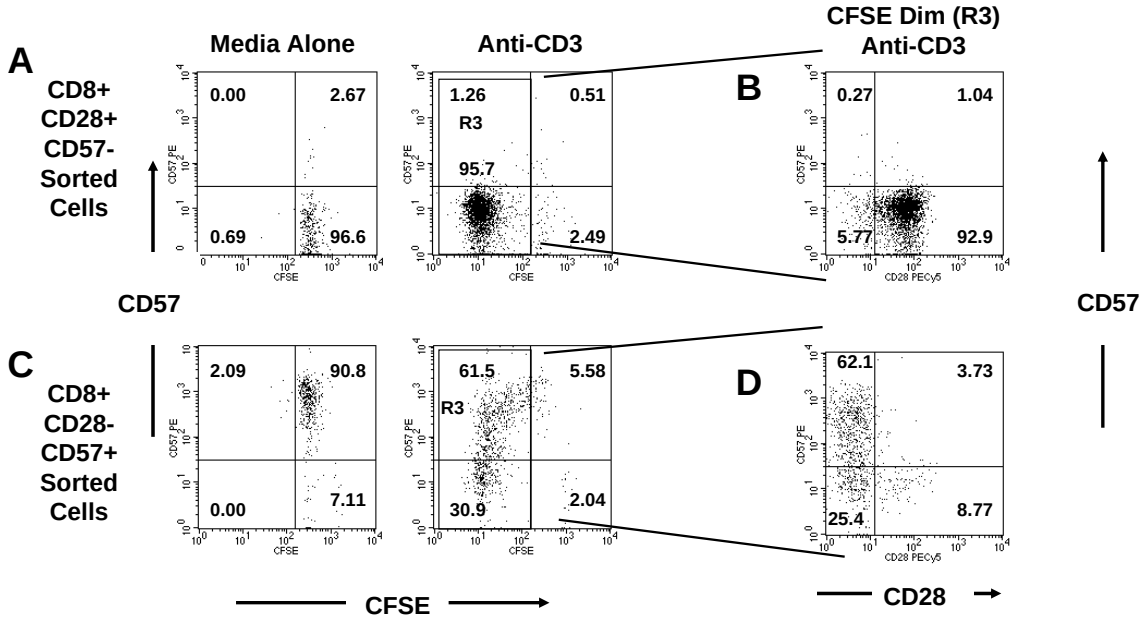

Figure 5 Sorted $CD8^{+}CD28^{-}CD57^{+}$ T cells remain $CD28^{-}CD57^{+}$ after stimulation.

$CD8^{+}$ $CD28^{+}$ $CD57^{-}$ and $CD8^{+}$ $CD28^{-}$ $CD57^{+}$ cells were sorted from whole PBMC and loaded with CFSE. Irradiated feeder cells were added to give a final concentration of 2 $x10^{6}$ per ml. 1 μg / ml anti-CD3 was added and the cells were incubated for 7 days. Subsequently the cells were analyzed by flow cytometry. Plots are electronically gated for $CD8^{+}$ lymphocytes. Experiment was repeated 4 times.

The cells were loaded with CFSE and stimulated with 1 μ/ml anti-CD3 for 7 days. $CD28^+CD57^-$ T cells remained $CD57^-$ (Figure 5A) and few if any became $CD28^-$ (Figure 5B). When $CD8^+CD28^-D57^+$ T cells were stimulated with 1 μg /ml anti-CD3 for 7 days, the majority of cells remained $CD28^-$ and $CD57^+$ (Figure 5C & D). It appears that some previously $CD57^+$ T cells may have down-regulated CD57 during seven days of incubation. Again the possibility that contaminating $CD8^+$, $CD28^-CD57^-$, or $CD28^+CD57^-$ T cells were able to outgrow the $CD28^-CD57^+$ T cells could not be ruled out. These last two experiments confirm that $CD28^-CD57^+$ T cells were able to divide and demonstrate that $CD28^+CD57^-$ T cells do not become $CD28^-CD57^+$ in seven days of culture. Next, I wished to determine if I could detect antigenic responses that were $CD57^+$. To accomplish this, I choose to use PBMC from patients that had been treated with GA.

II. Chronic stimulation with GA leads $CD8^{hi}$ T cells to a $CD28^-CD57^+$ phenotype

A. Background experiments on PBMC response to GA

Initially I was interested in determining if GA treatments led to a global change in T cell responses. To address this question, I examined the proliferative potential, precursor frequency, and cytokine response of PBMC when rechallenged with GA *in vitro*. PBMC from MS patients were analyzed before the initiation of GA therapy, and at 3, 7, and 11 months after initiation of GA therapy. It has been shown that T cell lines generated *in vitro* from MS patients treated with GA display Th2 type cytokines (Duda, Schmied et al. 2000;

Chen, Conway et al. 2002; Dhib-Jalbut, Chen et al. 2003; Schmied, Duda et al. 2003). However, primary PBMCs produce both Th1 and Th2 type cytokines (Farina, Then Bergh et al. 2001). This is because in primary cultures, IFN-γ is produced by cell types such as CD8 T cells which proliferate relatively slowly and are overgrown by CD4 Th2 T type cells in T cell lines. I examined the frequency of IFN-γ and IL-5 producing cells in response to restimulation with 10, 50 or 100 μg/ml GA by ELISPOT. Three months after initiation of treatment, the frequency of both IFN-γ and IL-5 producing cells increased at all concentrations tested (Figure 6). However, after 7 and 11 months, the number of IL-5 producing cells diminished and this effect was most notable at higher concentrations of GA. This is in agreement with a report that PBMC from GA treated patients respond to GA restimulation with Th1 and Th2 cytokines (Farina, Then Bergh et al. 2001). Thus, GA reactive T cells can produce Th1 and Th2 type cytokines. Next, I examined the proliferative potential of these T cells at various concentrations of GA, since it has been shown that PBMC respond to GA stimulation *in vitro* by proliferation (Duda, Krieger et al. 2000; Karandikar, Crawford et al. 2002). PBMC from patients that had been treated for only 3 months proliferated the most robustly at 50 μg / ml (Figure 7). After 7 and 11 months of treatment the proliferative potential was diminished. Using a CFSE based assay, it was also shown that the proliferating fraction of cells from PBMC are $CD3^+$ (Data not shown). I also wanted to examine whether GA treatment induces a change in their responding frequency, since the precursor frequency was also shown to increase by limiting dilution analysis (Schmied, Duda et al. 2003).

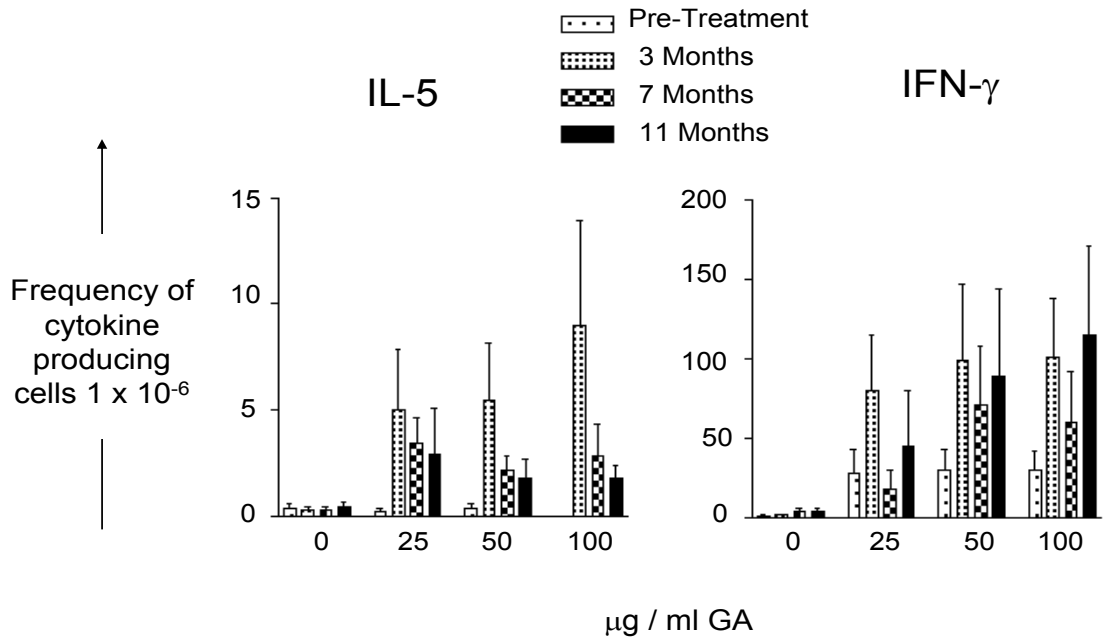

Figure 6 T cells from GA treated patients secrete both Th1 and Th2 cytokines.

5 x10^6 PBMC per well were plated on an ImmunoSpot M200 pre coated with anti-IFN-γ or anti-IL-5 antibody. The plates were incubated for 36 hours with GA and a labeled secondary antibody for IFN-γ or IL-5 was added. Spots were visualized with AEC and counted on an Alpha Imager. Averages from 4 treated MS patients are shown. Error bars represent ±SEM.

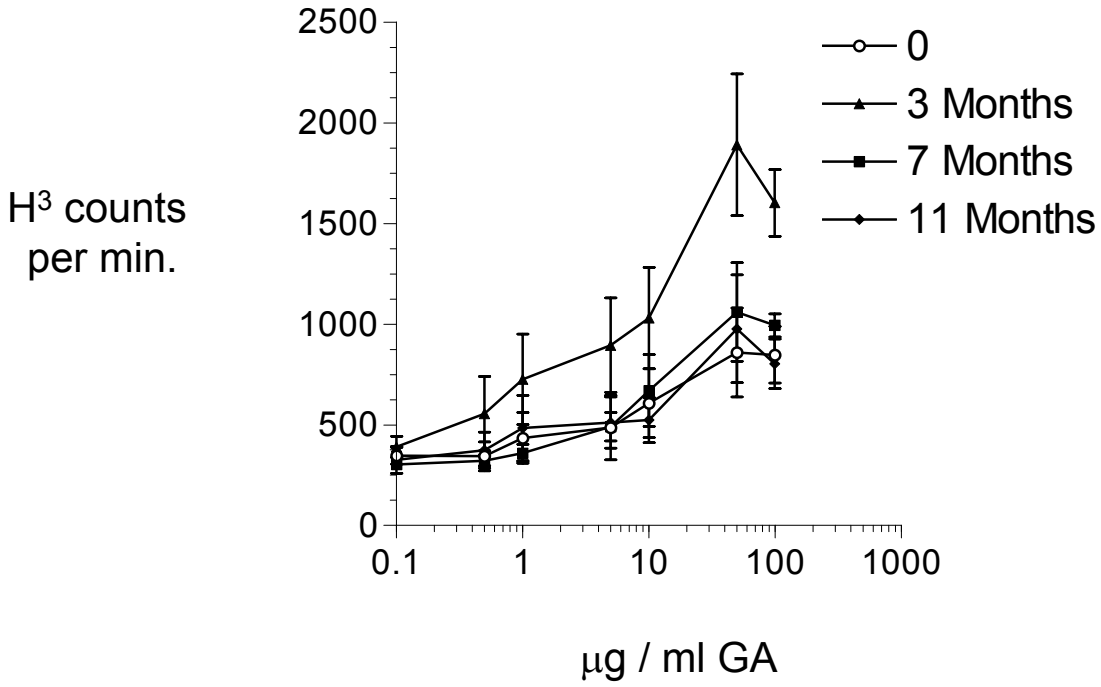

Figure 7 PBMC proliferate when challenged or rechallenged with GA.

Quadruplicate cultures of PBMC from treated or untreated MS patients (0 time point) were rechallenged *in vitro* with the indicated doses of antigens. Cells were pulsed with 0.25 μCi per well of ^{3}H-thymidine for the last 18 hours of culture. On day 5 of culture, cells were harvested onto glass filters (Wallac, Turko, Finland) and analyzed for incorporation of radioactivity using a Wallac Betaplate liquid scintillation counter (Wallac, Gaithersburg, MD). Averages from 4 treated MS patients are shown. Results are expressed in cpm with SEM.

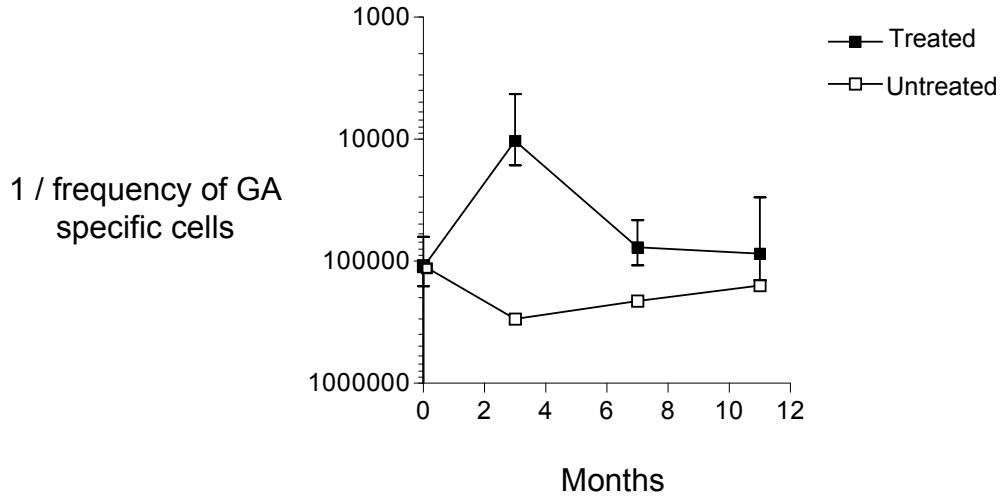

Figure 8 The precursor frequency of GA reactive cells correlates with the proliferation potential.

A limiting dilution of 1 x10^5, 5 x10^4, 2.5 x10^4, and 1.25 x10^4 PBMC were plated in round bottom plates. Irradiated PBMC were added to give 1 x10^5 cells per well. These plates were incubated for 6 days and harvested onto glass filters. Each well was pulsed with 0.25 µCi ^{3}H-thymidine on day 5. 192 replicates were set up for each dilution half with no antigen and half with 50 µg / ml GA. Proliferation 2 standard deviations above the mean was considered positive. Frequency was determined using the Poisson distribution and chi square minimization. The x axis represents the number of months the MS patients were on GA therapy with 0 denoting pre treatment. The precursor frequency of 4 GA treated MS patients and 1 untreated MS patient ±SEM is shown.

After several months of treatment, the proliferative potential and the frequency of GA-reactive T cells had declined (Chen, Conway et al. 2002; Karandikar, Crawford et al. 2002; Schmied, Duda et al. 2003). When I examined the precursor frequency of GA-reactive cells, I observed an initial increase followed by a decrease in GA-reactive T cells (Figure 8). The frequency of GA-reactive T cells in PBMC from an MS patient not undergoing therapy remained unchanged. These results indicate that our patient population responded to GA therapy in a similar manner to previously studied patient populations (Chen, Conway et al. 2002; Karandikar, Crawford et al. 2002; Schmied, Duda et al. 2003).

B. GA treatment leads to a $CD28^{-}CD57^{+}$ phenotype

Since some MS patients are injected daily with GA, I determined if this repeated stimulation resulted in the development of $CD28^{-}CD57^{+}$ GA-reactive T cells. I accomplished this by examining the phenotype of GA-reactive T cells from MS patients that had been treated for 12 months. PBMC loaded with CFSE were incubated with 50 μg/ml GA for seven days, labeled with antibodies and examined by flow cytometry. The responses of a typical MS patient treated with GA (MST) and a typical untreated MS patient (MS) are shown (Figure 9A & B). All events that fall in the $CD4^{+}$ live lymphocyte population are shown (Figure 9A), as is the electronic gate used to identify the proliferating fraction of events. The expression of CD28 and CD57 on these electronically gated events is displayed (Figure 9B). The majority of proliferating $CD4^{+}$ lymphocytes responding to GA from treated

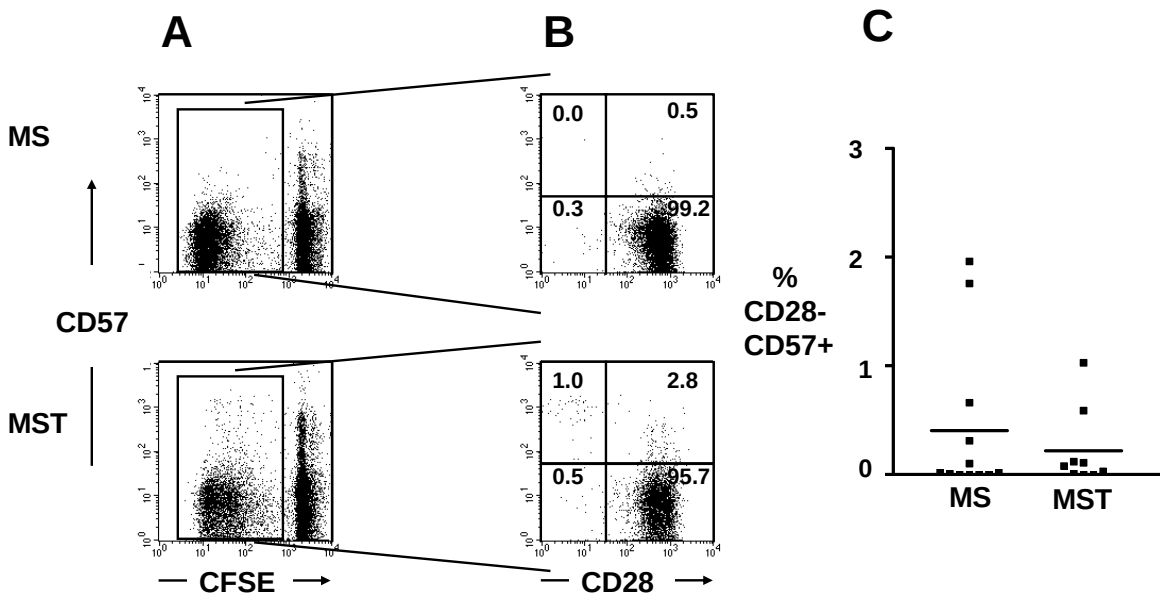

Figure 9 After 12 months of treatment, CD4 GA responses remain $CD28^{+}CD57^{-}$.

PBMC from an untreated MS patients (MS) and MS patients Treated with GA (MST), were incubated for seven days with 50 μg/ml GA. Subsequently the cells were analyzed by flow cytometry. (A) Plots are electronically gated for live $CD4^{+}$ lymphocytes and (B) live $CD4^{+}$ $CFSE^{dim}$ lymphocytes. Numbers represent percent of events in each quadrant. (C) Scattergram showing the percent of $CD28^{-}CD57^{+}$ events in the live $CD4^{+}$ $CFSE^{dim}$ lymphocyte of multiple samples tested. Chauvenet's criterion was applied and one outlier was removed from both conditions.

and untreated MS patients displayed a $CD28^{+}CD57^{-}$ phenotype. The percentage of $CD28^{-}CD57^{+}$ (upper left quadrant) events in the proliferating CD4 population is shown for all samples that responded (according to the criteria in "materials and methods") (Figure 9C). When multiple patient samples were examined, there was no difference in the mean percent of $CD4^{+}CD28^{-}CD57^{+}$ cells between untreated and treated patients (0.2 % ±0.4 vs. 0.4 % ± 0.7). This indicated there is no difference or only a few $CD4^{+}$ cells altered their expression of CD28 and CD57. Alternatively, the $CD4^{+}$ GA-reactive cells may have undergone anergy or apoptosis. To examine if GA treatment has a global effect on the level of $CD4^{+}CD28^{-}CD57^{+}$ T cells I examined the individual responses to anti-CD3 stimulation. As shown in Figure 10, some $CD4^{+}$ $CD28^{-}CD57^{+}$ T cells proliferated in MS patients that seemed to be absent in healthy controls (HC). However this difference was not significant and this was reminiscent of the overall level of $CD28^{-}CD57^{+}$ levels in the CD4 compartment of MS patients as compared to healthy controls discussed earlier (Table 6). When $CD8^{hi}$ T cells from untreated patients were examined similarly, most $CD8^{hi}$ T cells responding to GA treatment were $CD57^{-}$ (Figure 11A). In treated patients, the $CD57^{+}$ population in the dividing fraction was much greater in number. In untreated patients most responding cells were either $CD28^{+}CD57^{-}$ or $CD28^{-}CD57^{-}$, but a small population of $CD28^{-}CD57^{+}$ T cells was detected (Figure 11C-F). In treated patients, the $CD28^{-}CD57^{+}$ population was much greater in number (Figure 11C).

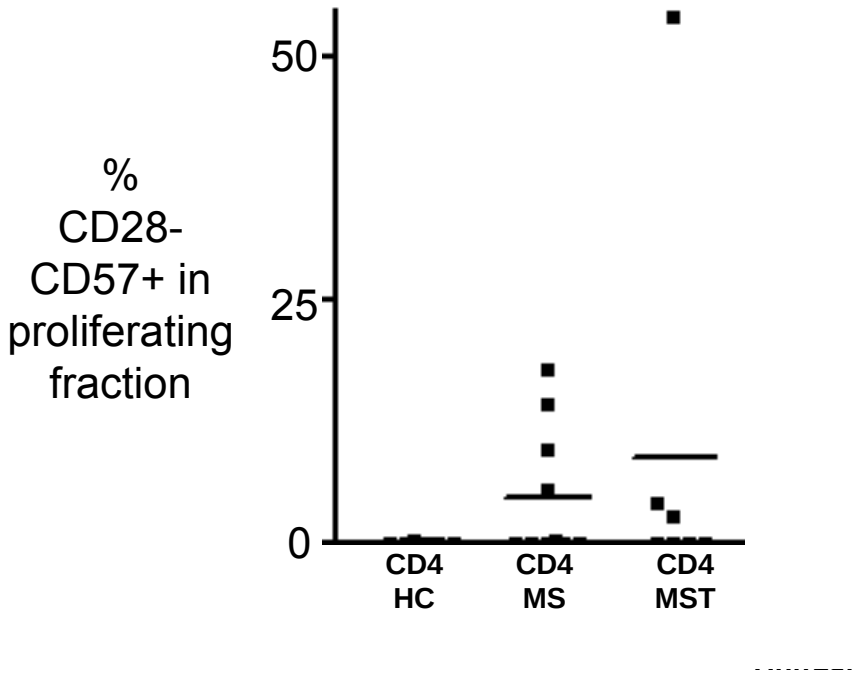

Figure 10 GA treatment does not change global level of $CD4^{+}CD28^{-}CD57^{+}$ T cells.

PBMC from untreated MS patients (MS), MS patients Treated with GA (MST), and Healthy controls (HC) were incubated for seven days with 1 μg/ml anti-CD3. Subsequently the cells were analyzed by flow cytometry. Scattergram shows the percent of $CD28^{-}CD57^{+}$ events in the live $CD4^{+}$ $CFSE^{dim}$ lymphocyte of multiple samples tested.

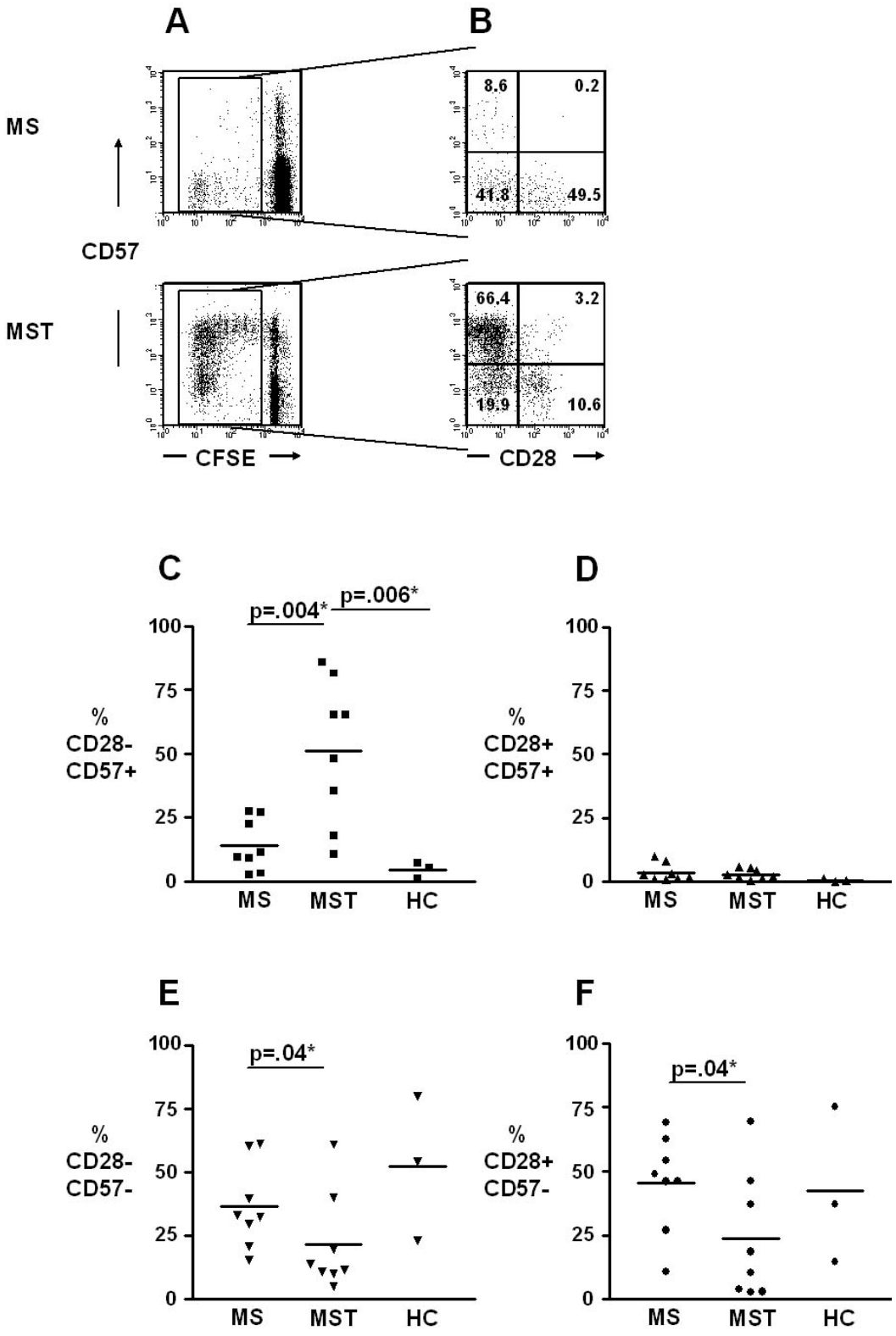
A
B
MS
MST
CD57
8.6
0.2
41.8
49.5
66.4
3.2
19.9
10.6
CFSE
CD28
C
D
p=.004*
p=.006*
% CD28- CD57+
% CD28+ CD57+
100
75
50
25
0
MS
MST
HC
E
F
p=.04*
p=.04*
% CD28- CD57-
% CD28+ CD57-

Figure 11 After 12 months of treatment, the CD8 GA response was predominantly $CD28^{-}CD57^{+}$ in MS patients.

CFSE loaded PBMC from untreated MS patients (MS), MS patients Treated with GA (MST), and Healthy controls (HC), were incubated for seven days with 50 μg/ml GA. Subsequently the cells were analyzed by flow cytometry. GA cultured PBMC are shown electronically gated for (A) $CD8^{hi}$ lymphocytes and (B) $CD8^{hi}$ $CFSE^{dim}$ lymphocytes. Numbers represent percent of events in each quadrant. (C-F) Scattergrams show the percent of events in the $CD8^{hi}$ $CFSE^{dim}$ lymphocyte gate of each quadrant of all samples that tested positive). (C) $CD28^{-}CD57^{+}$ (■), (D) $CD28^{+}CD57^{+}$ (▲), (E) $CD28^{-}CD57^{-}$ (▼), (F) $CD28^{+}CD57^{-}$(●). *indicates the result of a one tailed Mann Whitney test.

The percentage of $CD28^{-}CD57^{+}$ events was significantly increased in the population of treated patients vs. untreated MS patients (p=.004, 14 % ±4 vs. 51 % ±10). This indicates that chronic stimulation with GA leads to a $CD28^{-}CD57^{+}$ phenotype in $CD8^{hi}$ T cells. In addition, a concurrent decrease between the untreated vs. treated MS patients was observed uin both the $CD28^{-}CD57^{-}$ (37 % ±6 vs., 23 % ±7) and the $CD28^{+}CD57^{-}$ (46 % ±7 vs. 23 % ±9) populations (Figure 11E & F). The phenotype of GA-reactive CD8 T cells in healthy controls did not differ significantly from untreated MS patients (Figure 11C-F). Not surprisingly, the percent of responding $CD28^{-}CD57^{+}$ T cells was significantly higher in treated MS patients than in untreated healthy controls (51.2 % ±9.9 vs. 4.6 ±1.8, p=.006) (Figure 11C). Few events were observed in any double positive population (Figure 11D). As controls, I examined the response to anti-CD3, mumps and CMV. Stimulation with anti-CD3, which is indicative of the overall T cell response, demonstrated no significant changes in the percent of cells in each population between untreated MS patients, treated MS patients and healthy controls. GA treatment also had no effect on the level of $CD8^{hi}CD28^{-}CD57^{+}$ T cells proliferating in response to anti-CD3 in MS patients (Figure 12A-D). Stimulation with mumps antigen was included as a control to show the response to a typical recall antigen, as opposed to a chronic antigen response. The percent of responding $CD28^{-}CD57^{+}$ cells and $CD28^{+}CD57^{+}$was low in the recall response to mumps for treated MS patients (8.8 % ±5.2) (Figure 12E-F). The same percent was obtained for untreated MS patients and healthy controls (data not shown).

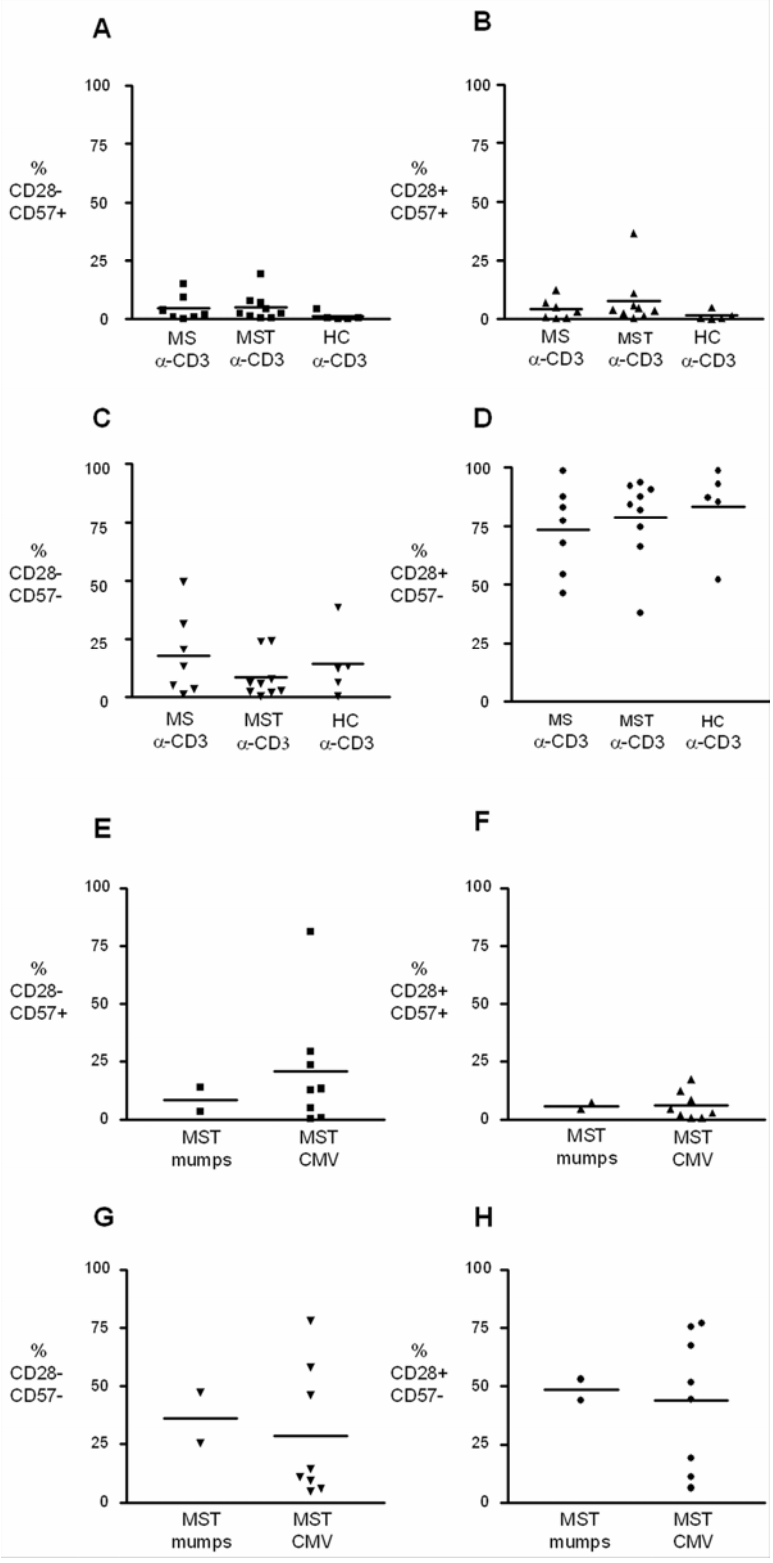
A
B
C
D
E
F
G
H
%
CD28-
CD57+
%
CD28+
CD57+
%
CD28-
CD57-
%
CD28+
CD57-
100
75
50
25
0
MS
α-CD3
MST
α-CD3
HC
α-CD3
MST
mumps
MST
CMV

Figure 12 Control Anti-CD3 and mumps responses are not $CD28^{-}CD57^{+}$.

CFSE loaded PBMC from untreated MS patients (MS), MS patients Treated with GA (MST), and Healthy controls (HC), were incubated for seven days with 1 μg/ml anti-CD3, inactivated mumps, or inactivated CMV. Subsequently the cells were analyzed by flow cytometry. Scattergrams show the percent of events in the $CD8^{hi}$ $CFSE^{dim}$ lymphocyte gate of each quadrant from all samples that tested positive as in figure 3. (A-D) response to anti-CD3. (A) percent of $CD28^{-}CD57^{+}$ events (■), (B) $CD28^{+}CD57^{+}$ (▲), (C) $CD28^{-}CD57^{-}$ (▼), and (D) percent of $CD28^{+}CD57^{-}$ events (●). (E-H) Response to inactivated mumps and inactivated CMV (E) $CD28^{-}CD57^{+}$, (F) $CD28^{+}CD57^{+}$, (G) $CD28^{-}CD57^{-}$, (H) $CD28^{+}CD57^{-}$.

This demonstrates that the $CD28^{-}CD57^{+}$ response is not seen in a normal memory recall response, and may only develop in response to chronic stimulation. CMV was also included as a positive control because it causes a chronic viral infection and is known to induce a $CD8^{+}CD28^{-}CD57^{+}$ response (Lenkei and Andersson 1995; Wang, Moss et al. 1995). When CFSE loaded cells were not stimulated with antigen, they did not proliferate (Figure 13A), demonstrating that GA treatment had no effect on the overall expression of CD28 and CD57 on total $CD8^{hi}$ T cells. The percentage of $CD28^{-}CD57^{+}$ events in unstimulated $CD8^{hi}$ T cells was the same as the percentage of $CD28^{-}CD57^{+}$ events in the $CD8^{hi}$ proliferating fraction of GA stimulated PBMC from untreated MS patients (Figure 11C MS vs. Figure 13C MS), and untreated healthy controls. This indicates that the $CD8^{hi}CD28^{-}CD57^{+}$ T cells observed to proliferate in healthy controls and untreated patients may be due to background levels of $CD8^{hi}CD28^{-}CD57^{+}$ T cells. In GA treated MS patients however, a greater percentage of $CD28^{-}CD57^{+}$ events were observed in the proliferating fraction in response to GA than in unstimulated $CD8^{hi}$ T cells (51.2 % ±9.9 vs. 16.1 % ±3.9); (Figure 11C MST vs. Figure 13C MST), and this difference was significant ($p=.001$). This indicates that in GA-treated MS patients, GA-reactive $CD8^{hi}CD28^{-}CD57^{+}$ T cells are generated.

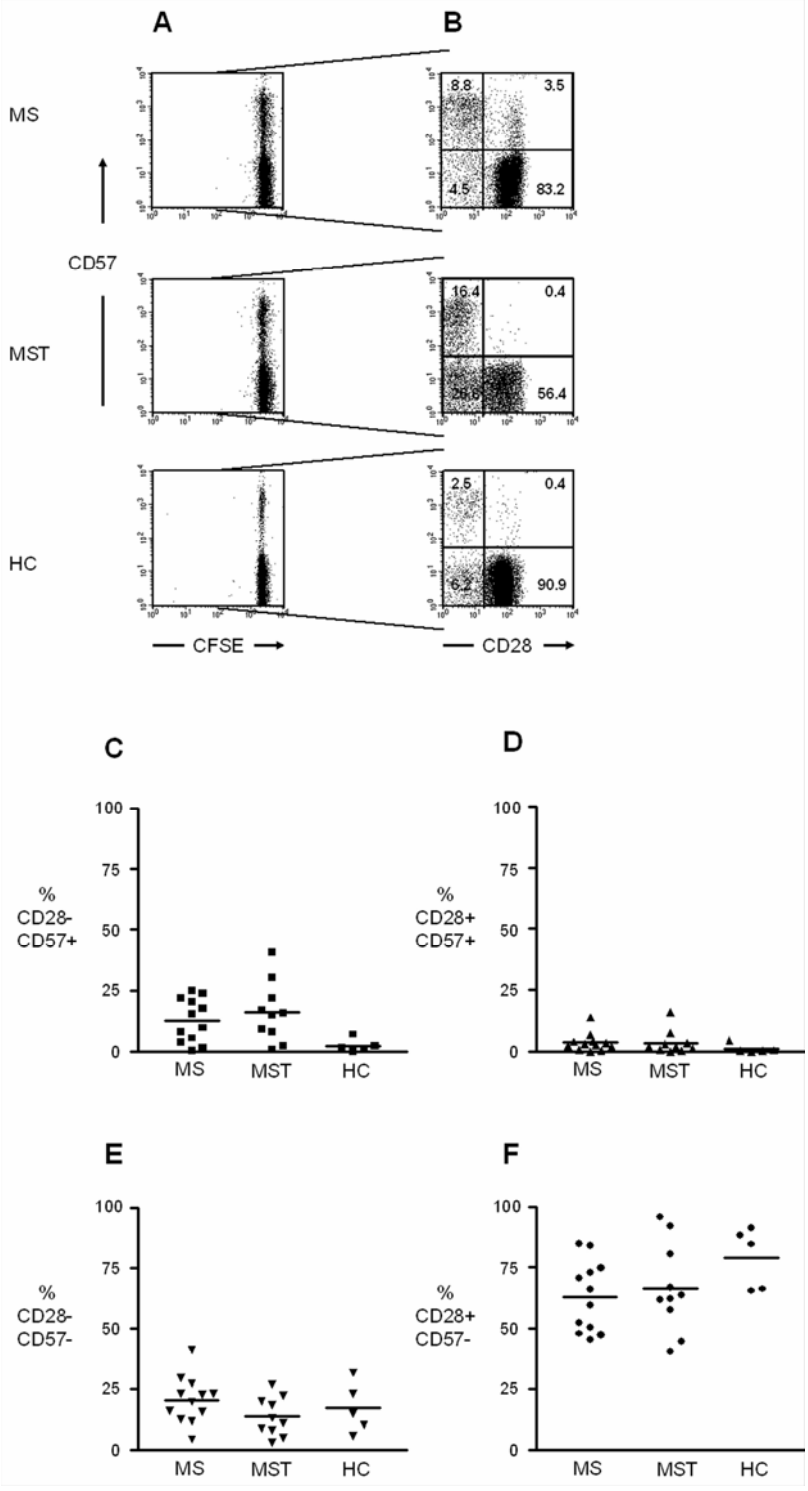
A
B
MS
MST
HC
CD57
8.8
3.5
4.5
83.2
16.4
0.4
28.6
56.4
2.5
0.4
6.2
90.9
CFSE
CD28
C
D
E
F
% CD28- CD57+
% CD28+ CD57+
% CD28- CD57-
% CD28+ CD57-
100
75
50
25
0
MS
MST
HC

Figure 13 GA treatment does not change the level of CD28 and CD57 expression on all CD8 T cells.

CFSE loaded PBMC from GA treated (MST), untreated (MS), and healthy controls (HC), were incubated for seven days with no antigen. Subsequently the cells were analyzed by flow cytometry. (A&B) PBMC are shown electronically gated for $CD8^{hi}$ lymphocytes. Numbers represent percent of events in each quadrant. (C-F) Scattergrams show the percent events in the $CD8^{hi}$ lymphocyte gate of each quadrant of all samples tested. (C) $CD28^{-}$ $CD57^{+}$ (■), (D) $CD28^{+}CD57^{+}$ (▲), (E) $CD28^{-}CD57^{-}$ (▼), and (F) $CD28^{+}CD57^{-}$(●).

These data indicate that chronic stimulation with GA results in a population of $CD8^{hi}CD28^{-}CD57^{+}$ T cells, which accounts for the majority of the T cell response to this complex antigen.

C. The $CD8^{hi}CD28^{-}CD57^{+}$ response develops over time

We demonstrated that PBMC from treated MS patients respond to rechallenge with GA *in vitro* with a $CD8^{hi}CD28^{-}CD57^{+}$ phenotype (Figure 11). I then examined the response of six GA treated patients before initiation of treatment and every 4 months thereafter (Figure 14). The $CD8^{hi}CD28^{-}CD57^{+}$ GA-reactive response is initially low, then starts to increase after 8 months of treatment (Figure 14A), and appears to plateau after 18 months. This is in contrast to the $CD28^{+}CD57^{-}$ population that begins as the largest percentage and begins to decline after 8 months of treatment. The $CD28^{-}CD57^{-}$ population did not change significantly, but did show some decline after 2 years of treatment. It is noteworthy that all patients studied displayed an increase in the percent of $CD28^{-}CD57^{+}$ T cells in the proliferating $CD8^{hi}$ compartment. The absolute response in the three populations examined displayed similar kinetics (data not shown). Using the CFSE assay, I was able to determine the number of divisions each cell had undergone (Figure 14B). I was then able to calculate the initial precursor frequency of GA-reactive $CD8^{hi}$ cells. This assay is more sensitive than a limiting dilution analysis because it will detect cells that only divide a few times. However, like a limiting dilution analysis, it may underestimate the frequency as it does not take into account apoptosis.

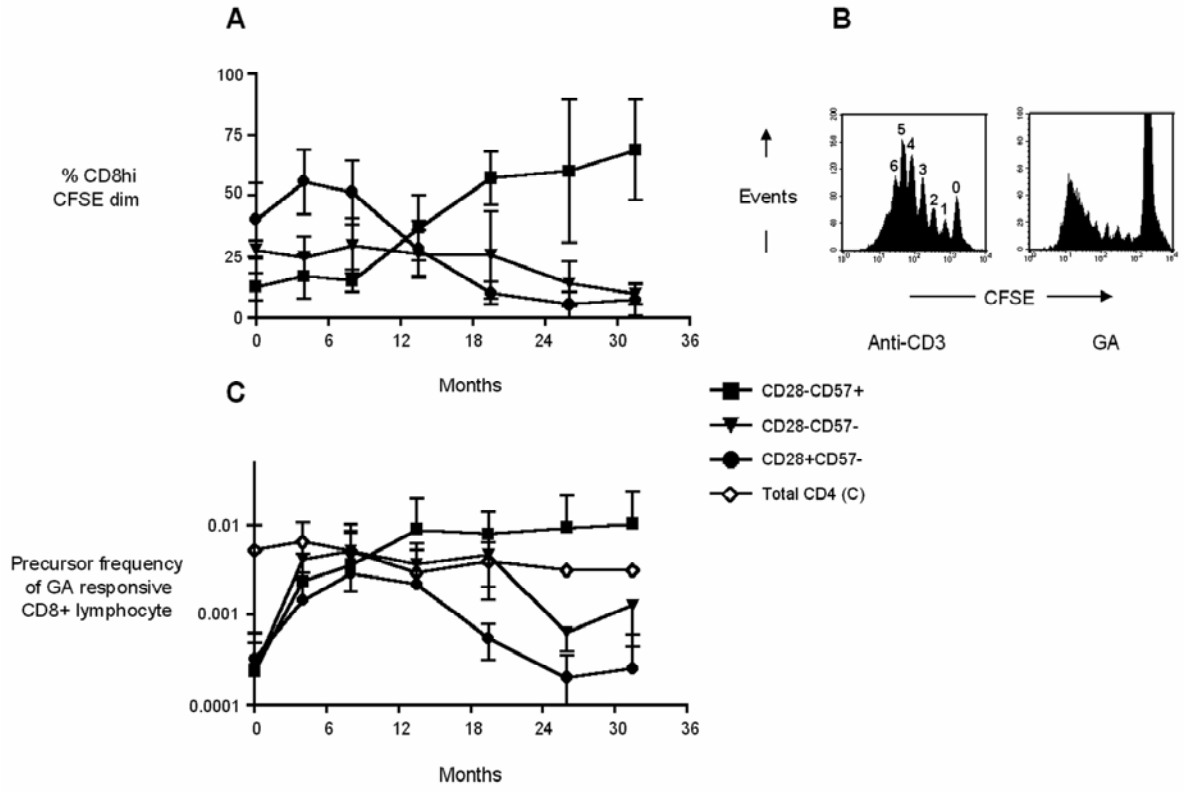

Figure 14 The $CD8^{hi}$ $CD28^{-}CD57^{+}$ response only develops after chronic stimulation.

Average of 6 GA-treated MS patients is shown (0 month time point on x axis denotes pre-treatment). (A) Three populations of $CD8^{hi}$ cells are shown $CD28^{-}CD57^{+}$ (■), $CD28^{-}CD57^{-}$ (▼), and $CD28^{+}CD57^{-}$ (●). The subsets are shown as a percent of the live $CD8^{hi}$ $CFSE^{dim}$ lymphocyte gate. Error bars represent the SEM. (B) CFSE allows the determinations of the number of divisions a cell has undergone *in vitro*. (C) The precursor frequency was calculated from this data and is shown. Total CD4 T cells are also shown (◊). Error bars represent one SD.

It may also over estimate the frequency, due to apoptosis. Initially, the frequency of all populations increased, however the $CD57^-$ populations subsequently declined and only $CD28^-CD57^+$ $CD8^{hi}$ T cells remained elevated after 2 years (Figure 14C). In addition, the precursor frequency of $CD8^{hi}CD28^-CD57^+$ GA reactive T cells was higher than the frequency of $CD4^+$ GA reactive T cells after 8 months. These data indicate that chronic stimulation with GA results in a population of $CD8^{hi}CD28^-CD57^+$ T cells, which accounts for the majority of the T cell response to this complex antigen. My data indicate the adoption of the $CD28^-CD57^+$ phenotype by $CD8^{hi}$ T cells is due to stimulation *in vivo*. To rule out the possibility that the adoption of this phenotype is due to *in vitro* influences from other cell types, I mixed

sorted $CD8^+$ cells from a patient treated for one year with CD8 depleted PBMC from the same patient after only two months of treatment and stimulated the cells with 50 μg/ml GA (Figure 15). If the $CD28^-CD57^+$ phenotype results from altered antigen presentation *in vitro*, I would expect to observe a largely $CD28^+CD57^-$ responding population. However, the proliferating $CD8^+$ T cells adopted a $CD28^-CD57^+$ phenotype (Figure 16A & B), indicating that the changes observed in the $CD8^+$ response resulted from changes in the $CD8^+$ T cells *in vivo*. The reverse experiment was also performed. Namely, CD8 T cells from a patient treated for 2 months were mixed with non-CD8 T cells from the same patient treated for 2 months or 1 year. When 2 month CD8 T cells were mixed with 2 month non CD8 T cells a typical 2 month response was observed (Figure 16C). When 2 month CD8 T cells were mixed with 1 year non-CD8 T cells substantial proliferation in response to GA was not

observed (Figure 16D). The proliferative response from the 1 year CD8 and 2 month-non-CD8 T cells was the most robust. Thus when 2 month non CD8 T cells and 1 year CD8 cells are used robust proliferation is observed, and when 1 year non-CD8 T cells and 2 month CD8 T cells faint proliferation is observed.

After demonstrating that $CD57^+$ T cells arise from GA treatment, I wanted to determine what function, if any, these cells may be playing *in vivo*. Previously, it has been reported that viral specific $CD28^-CD57^+$ T cells contain perforin (Mollet, Sadat-Sowti et al. 1998; Sze, Giesajtis et al. 2001) and are cytotoxic (Mollet, Sadat-Sowti et al. 1998; Weekes, Wills et al. 1999; Sze, Giesajtis et al. 2001). To examine if GA reactive T cells were potential Cytotoxic T Lymphocytes (CTL) as well, their content of perforin was examined. While this question could be asked with a 7 day incubation period, it is possible that the cells in question would change phenotype *in vitro* during this time. To overcome this obstacle, I wanted to examine the GA response with as short an *in vitro* incubation as possible. Indeed, when incubated *in vivo,* CD8 T cells lose their perforin content when they are incubated with GA or with no antigen (Figure 17). Here PBMC were incubated with 50 μg/ml GA or with no antigen. The cultures were incubated for 1, 2, or 4 days, examined for perforin expression, and plots were then gated for $CD8^{hi}$ expression. On day 1, perforin expression was high with most perforin expression occurring in $CD28^-$ T cells. On day 2, this expression decreases and by day four, it decays substantially regardless of whether antigen was added to the culture.

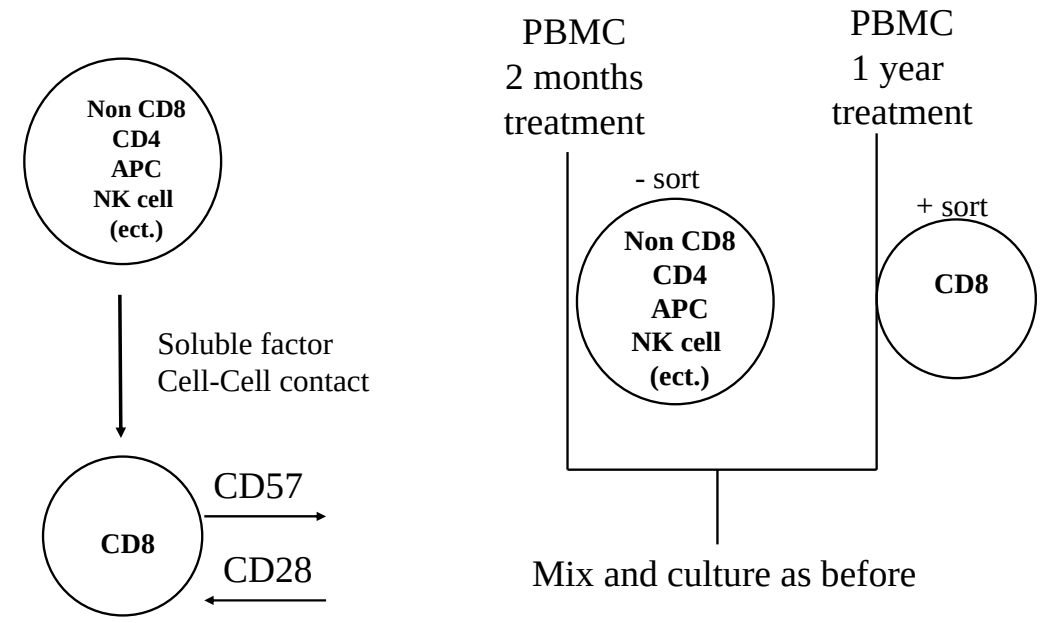

Figure 15 CD8 T cells may be altered *in vitro* or *in vivo*.

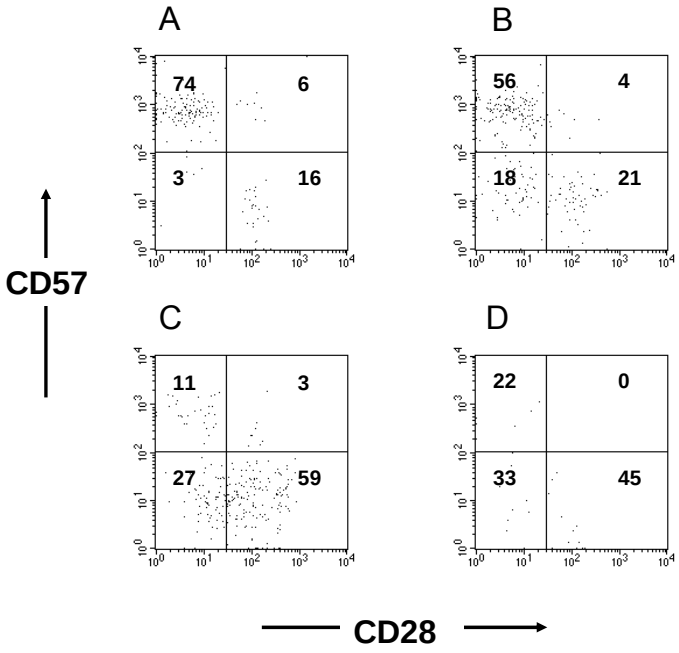

Figure 16 $CD8^{hi}$ T cells from patients trated for one year respond to GA rechallenge with a $CD28^{-}CD57^{+}$ phenotype regardless of the origin of non-CD8 T cells.

CD8 T cells were sorted by positive selection from PBMC 2 months or one year after initiation of treatment from the same patient. (A) 1 year CD8 T cells were mixed with 1 yr non-CD8 T cells. (B) 1yr CD8 T cells were mixed with 2 month non CD8 T cells. (C) 2 month CD8 T cells were mixed with 2 month non-CD8 T cells. (D) 2 month CD8 T cells were mixed 1 year non CD8 T cells. Cultures were then incubated for seven days with 50 μg/ml GA. Subsequently the cells were analyzed by flow cytometry. Plots are electronically gated for live $CD8^{hi}$ $CFSE^{dim}$ lymphocytes and normalized to show the same number of events (D was not normalized due to few events). Numbers represent percent of events in each quadrant. An experiment using purfied cells from MS patient is shown. Experiment was repeated twice with unpurified cells with similar results.

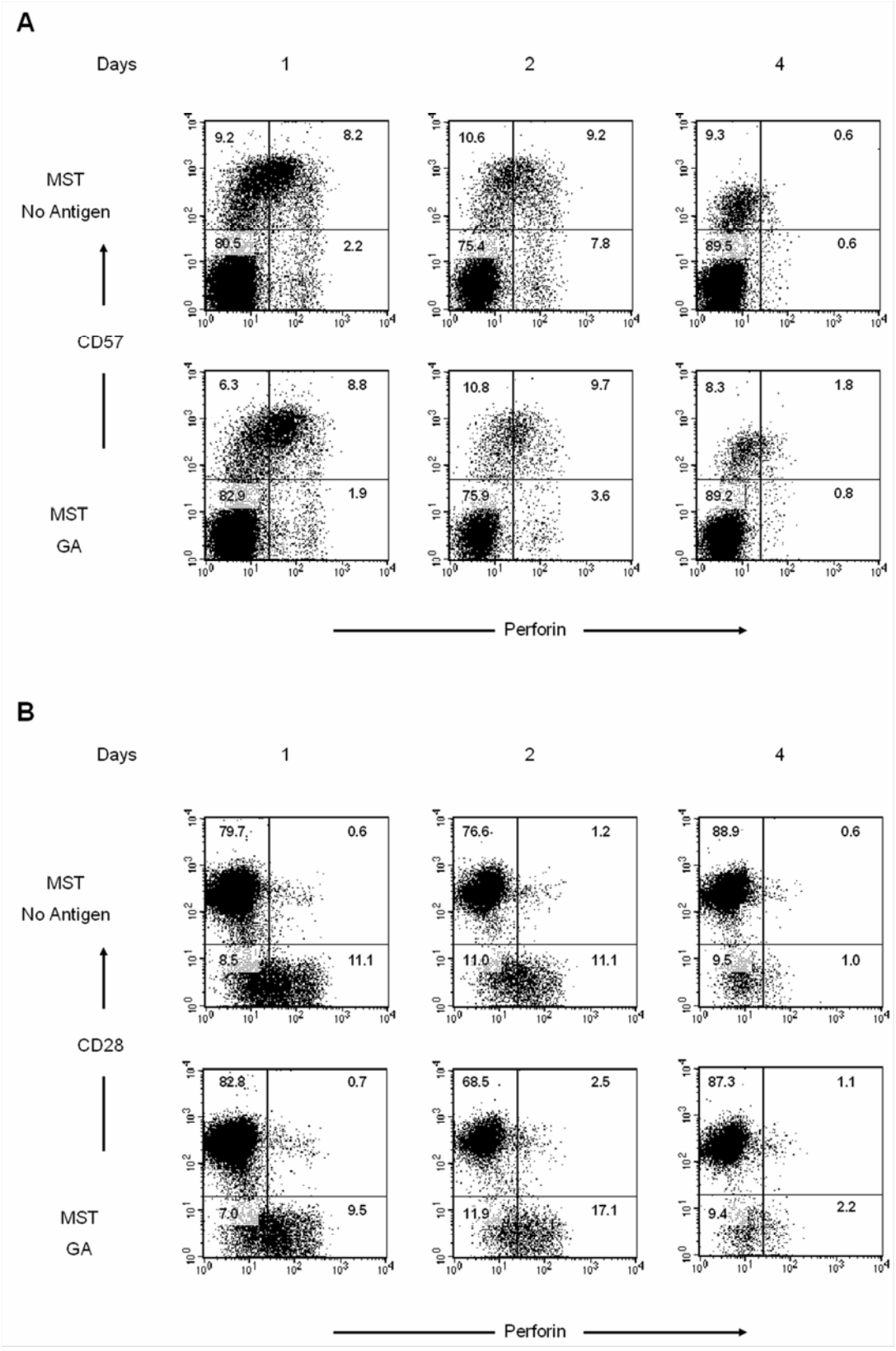
A
Days
1
2
4
MST
No Antigen
CD57
MST
GA
9.2
8.2
80.5
2.2
10.6
9.2
75.4
7.8
9.3
0.6
89.5
0.6
6.3
8.8
82.9
1.9
10.8
9.7
75.9
3.6
8.3
1.8
89.2
0.8
Perforin
B
Days
1
2
4
MST
No Antigen
CD28
MST
GA
79.7
0.6
8.5
11.1
76.6
1.2
11.0
11.1
88.9
0.6
9.5
1.0
82.8
0.7
7.0
9.5
68.5
2.5
11.9
17.1
87.3
1.1
9.4
2.2
Perforin

Figure 17 $CD8^{hi}perforin^{+}$ cells diminish by 4 days.

PBMC from a treated patient (MST) were cultured for the indicated number of days with no antigen or 50 μg/ml GA. Subsequently the cells were analyzed by flow cytometry. Events are shown electronically gated for $CD8^{hi}$ lymphocytes. Numbers represent percent of events in each quadrant. (A) Plots of CD57 vs. perforin expression are shown. (B) Plots of CD28 vs. perforin expression are shown. This experiment was performed on 1 MS patient.

To overcome this obstacle, I wanted to use another marker of T cell activation. $CD57^+$ T cells upregulate CD25 (IL-2Rα) after stimulation with anti-CD3 (Figure 18)(Scheuring, Sabzevari et al. 2002). Next I investigated optimal incubation times. When PBMC were incubated with anti-CD3, a large population of $CD4^+CD28^+$ T cells upregulated CD25 within one day (Figure 19A). Some $CD4^+$ T cells expressed CD25 without activation as expected. $CD28^+CD8^+$ T cells upregulated CD25 in one day and expression was maximal on day 5. This data indicates that CD25 expression can be detected in one or two days on $CD28^+$ T cells. When $CD28^-$ T cells were examined, both $CD4^+$ and $CD8^+$ T cells upregulated CD25 in one day and this expression was maintained, although at somewhat diminished levels for seven days. Subsequently, the response of PBMC from a patient not treated with GA were examined to determine if CD25 upregulation could be detected in an antigen specific manner. As shown in Figure 20, $CD28^+$ T cells bearing either the CD4 or CD8 receptor upregulated CD25 on day one of incubation and this expression reached a local maximum on day 2. $CD28^-$ T cells from this untreated patient did not upregulate CD25 (Data not shown). From this data, it was decided to determine if the $CD28^-CD57^+$ phenotype described previously could be detected using CD25 as a marker of activation on day 2. When PBMC were incubated for 2 days with GA, I observed a population of $CD8^{hi}CD28^-CD57^+$ T cells that upregulated CD25 in response to GA (75.8 % ±6.2) which was absent in untreated patients (7.6 % ±4.1) (p=.014) (Figure 21). As before, this population is diminished in response to anti-CD3. This experiment demonstrated that chronic stimulation with GA leads to $CD8^{hi}CD28^-CD57^+$ responsive cells by methods that require only 2 days of *in vitro* culture,

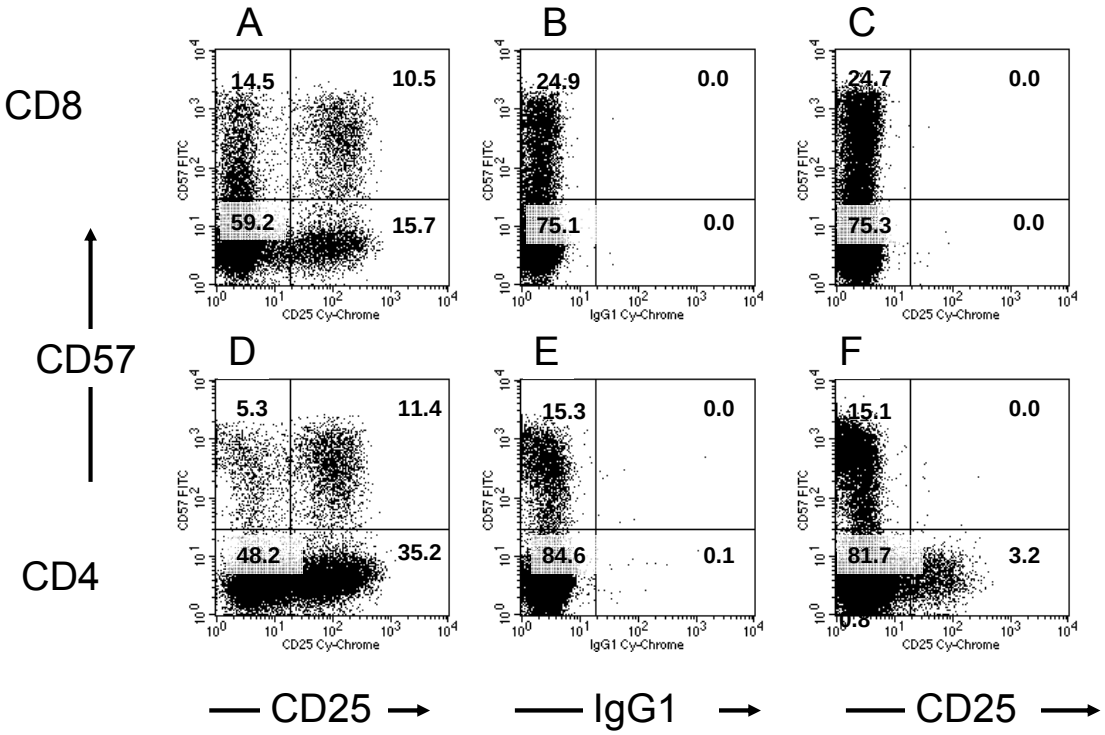

Figure 18 CD57^{+} T cells upregulate CD25.

PBMC were stimulated for 7 days with (A, B, D, E) anti-CD3 or (C, F) no antigen. The cells were then analyzed by flow cytometry. (A-C) gated on CD8^{hi} T cells. (D-F) Gated on CD4^{+} T cells This experiment was repeated on 3 MS patinets.

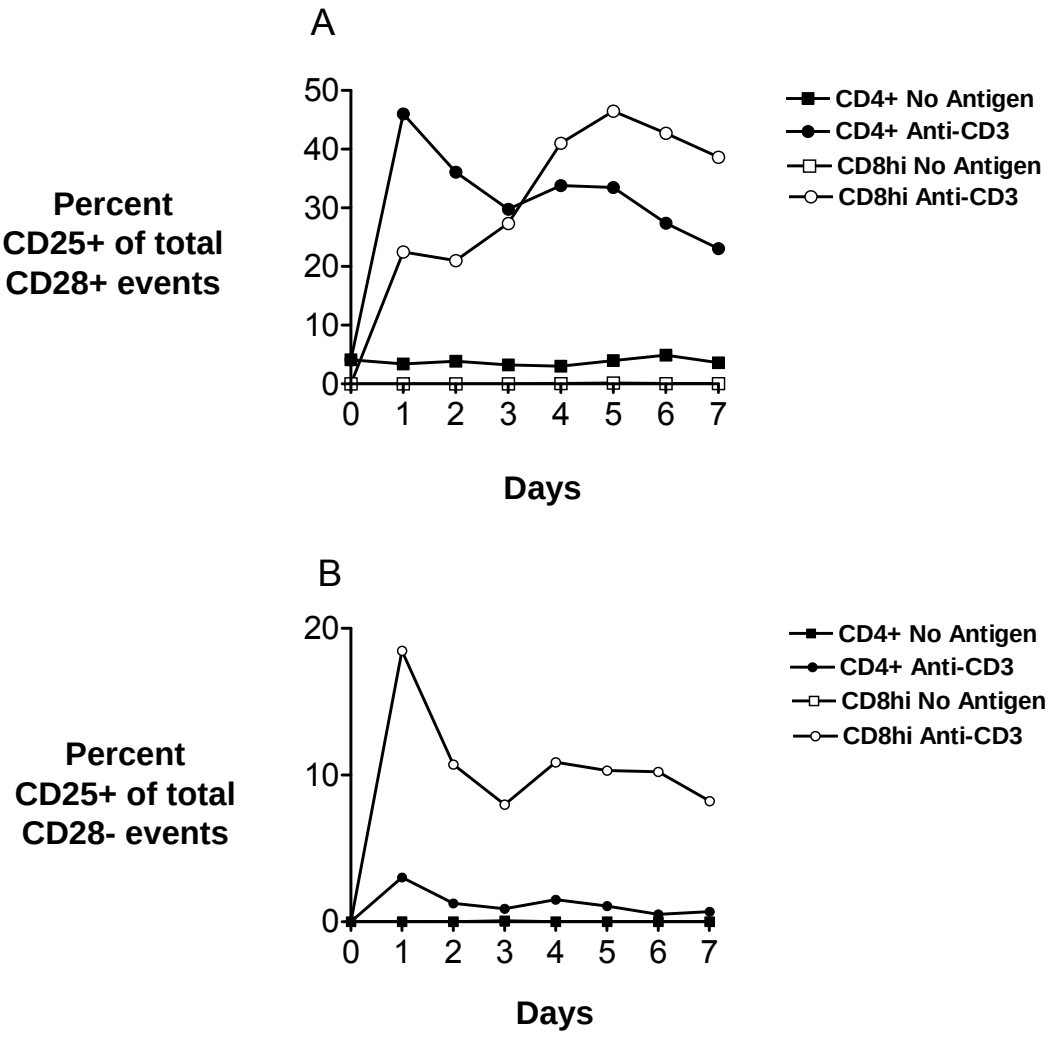

Figure 19 $CD28^-$ and $CD28^+$ T cells up regulate CD25 one day after stimulation with anti-CD3.

PBMC were stimulated with anti-CD3 or with no antigen for the indicated number of days and analyzed by flow cytometry. (A) Percent of $CD25^+$ events in the $CD28^+$ fraction. (B) Percent of $CD25^+$ T cells in the $CD28^-$ fraction. This experiment was performed 2 times with samples from an MS patient.

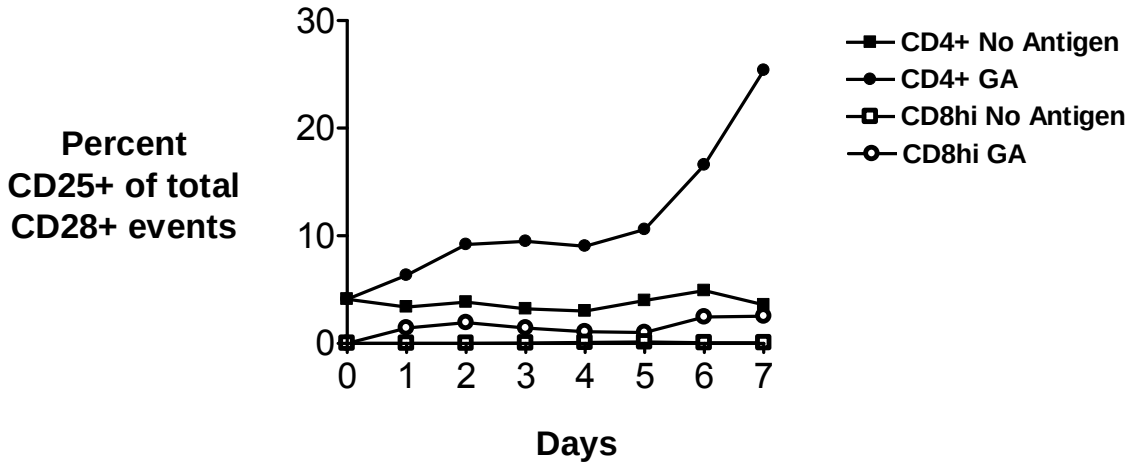

Figure 20 T cells up regulate CD25 two days after GA stimulation.

PBMC from an untreated patient were stimulated with GA or with no antigen for the indicated number of days and analyzed by flow cytometry. Cells were electronically gated for $CD28^{+}$ and $CD4^{+}$ expression or, $CD28^{+}$ and $CD8^{hi}$ expression. This experiment was performed 2 times.

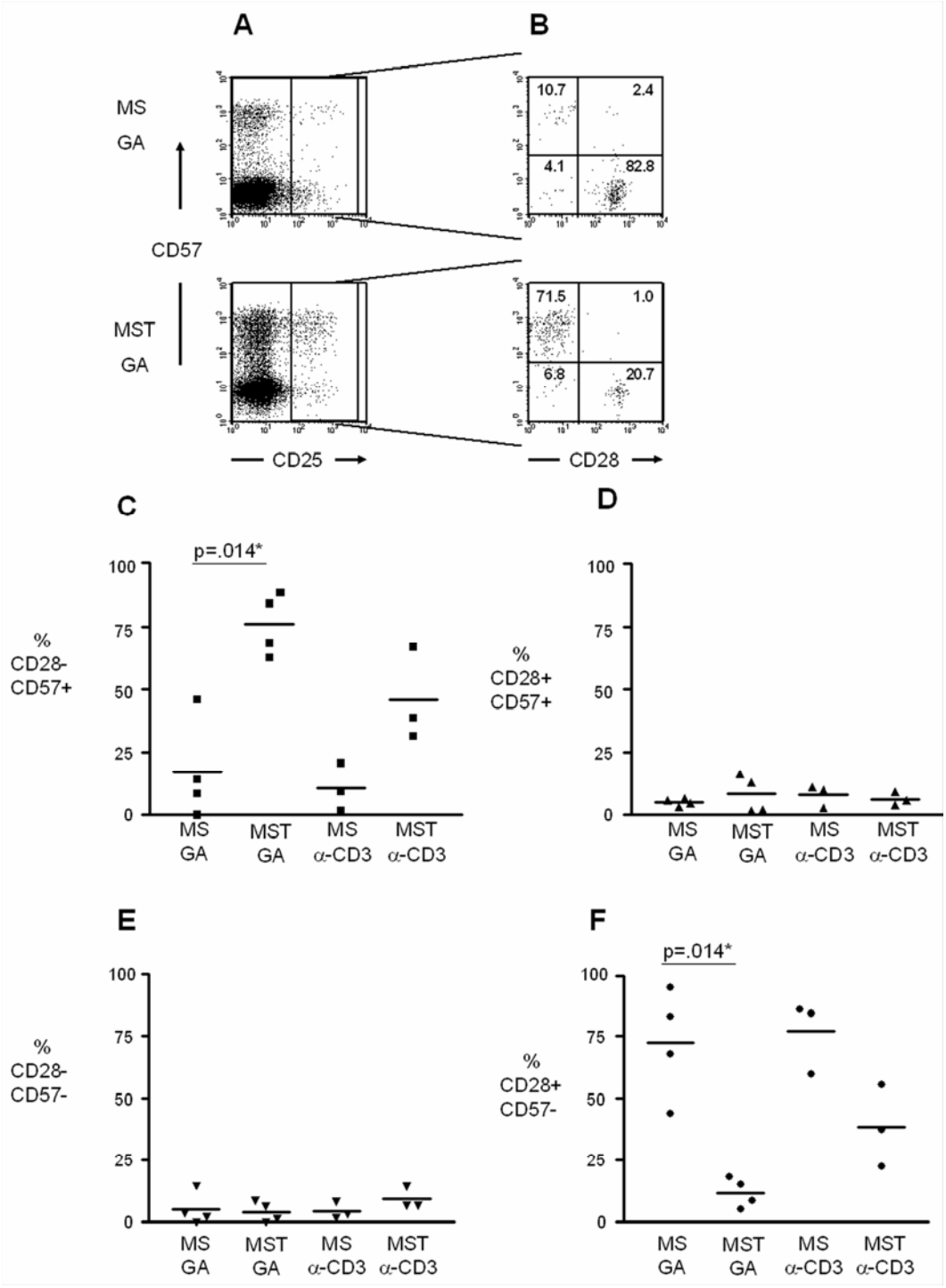
A
B
MS
GA
CD57
MST
GA
CD25
CD28
10.7
2.4
4.1
82.8
71.5
1.0
6.8
20.7
C
D
E
F
p=.014*
p=.014*
% CD28- CD57+
% CD28+ CD57+
% CD28- CD57-
% CD28+ CD57-
100
75
50
25
0
MS GA
MST GA
MS α-CD3
MST α-CD3

Figure 21 After 12 months of treatment, $CD8^{hi}$ $CD28^{-}CD57^{+}$ T cell responses can be detected in short term (2 day) assays by CD25 upregulation.

PBMC from untreated MS patients (MS), and MS patients Treated with GA (MST), were incubated for seven days with were incubated for two days with 50 μg/ml GA or anti-CD3. Subsequently the cells were analyzed by flow cytometry. Plots are electronically gated for (A) $CD8^{hi}$ lymphocytes and (B) $CD8^{hi}$ $CD25^{+}$ lymphocytes. Numbers represent percent of events in each quadrant. (C-F) Scattergrams show the percent events in the $CD8^{hi}$ $CD25^{+}$ lymphocyte gate of each quadrant of all samples that tested positive. (C) $CD28^{-}$ $CD57^{+}$ (■), (D) $CD28^{+}CD57^{+}$ (▲), (E) $CD28^{-}CD57^{-}$ (▼), (F) $CD28^{+}CD57^{-}$(●). *indicates the result of a one tailed Mann Whitney test.

and provided a better estimation of the characteristics these chronically stimulated cells possessed *in vivo*.

D. Chronically stimulated CD8 T cells express perforin

Previously, it has been reported that viral specific $CD28^{-}CD57^{+}$ T cells contain perforin (Mollet, Sadat-Sowti et al. 1998; Sze, Giesajtis et al. 2001) and are cytotoxic (Mollet, Sadat-Sowti et al. 1998; Weekes, Wills et al. 1999; Sze, Giesajtis et al. 2001). To examine if GA reactive T cells were potential CTL as well, perforin expression of $CD8^{hi}$ T cells that upregulated CD25 after 2 days incubation with GA were examined. The response of a typical treated and untreated patient is shown (Figure 22A). I observed a small population of $CD57^{+}$ cells in untreated patients and a large population of $CD57^{+}$ cells in treated patients (Figure 22B-E). Most of the $CD57^{+}$ cells in both the untreated and treated patients expressed perforin. An increase in GA-reactive $CD8^{hi}$ $perforin^{+}$ $CD57^{+}$ cells was observed in the treated MS patients (19% ±8 vs. 61 % ±9, p=.018). Large percentages of $CD8^{hi}$ $perforin^{+}CD57^{+}$ T cells were not observed in response to anti-CD3. This indicates that cytotoxic $CD8^{+}CD57^{+}$ cells are generated in response to chronic stimulation. Recent studies in our group suggest that GA-reactive T cells can indeed lyse GA-pulsed targets (Tennakoon, Mehta et al. 2006). It should be pointed out that CD57 is not always co-expressed with perforin (Figure 17A). Lastly, I observed a small increase in the percentage of $CD57^{+}Perforin^{+}$ T cells induced by anti-CD3 stimulation in GA treated patients (Figure 22C). This difference was not observed when proliferation was used as a readout (Figure 11C).

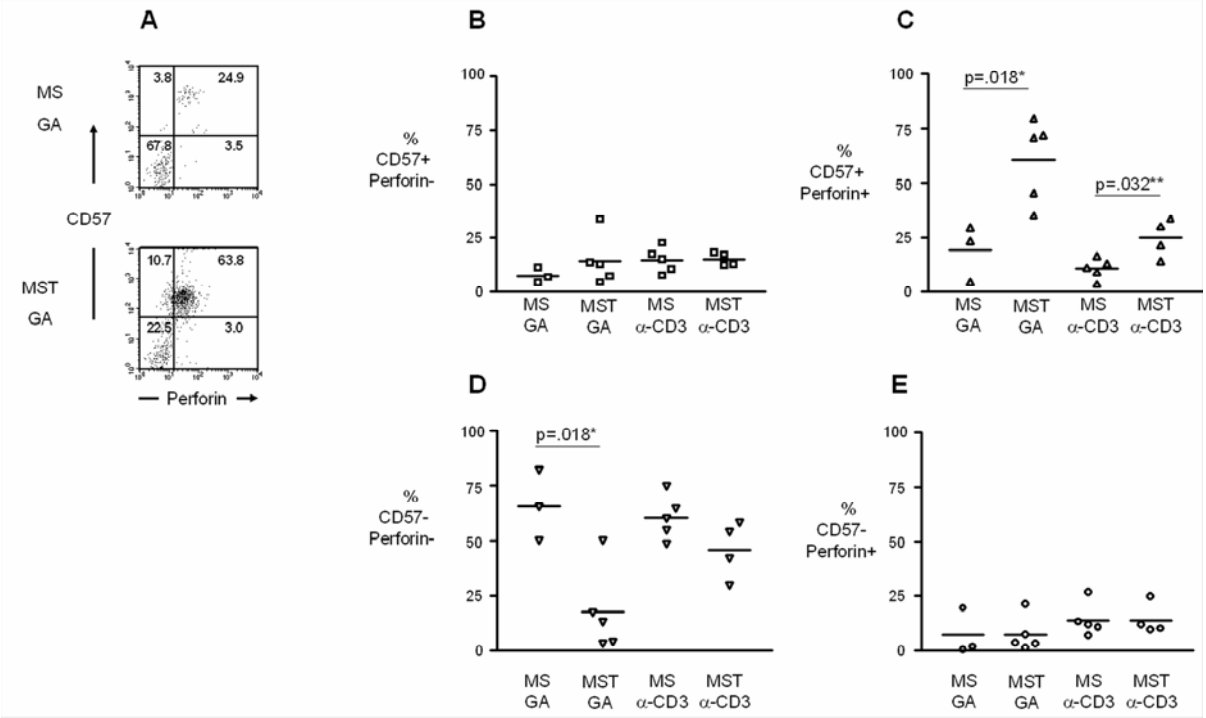

Figure 22 After 12 months of treatment, $CD8^{hi}$ $CD57^{+}$ T cells responding to GA by CD25 upregulation express perforin.

PBMC from untreated MS patients (MS), and MS patients Treated with GA (MST), were incubated for seven days with 50 μg/ml GA or anti-CD3. Subsequently the cells were analyzed by flow cytometry. (A) Plots are electronically gated for $CD8^{hi}$ $CD25^{+}$ lymphocytes. Numbers represent percent of events in each quadrant. (B-E) Scattergrams show the percent events in the $CD8^{hi}$ $CD25^{+}$ lymphocyte gate of each quadrant of all samples that tested positive. (B) $CD57^{+}$ $Perforin^{-}$ (□), (C) $CD57^{+}Perforin^{+}$ (△), (D) $CD57^{-}Perforin^{-}$ (▽), (E) $CD57^{-}Perforin^{+}$ (○). *indicates the result of a one tailed Mann Whitney test. ** indicates the result of a two tailed Mann Whitney.

This indicates that GA treatment may generate a population of GA reactive $CD57^{+}Perforin^{+}$ T cells that do not survive *in vitro* culture.

III. Independent confirmation that the $CD28^{-}CD57^{+}$ results from chronic stimulation

Although I have demonstrated that the change to CD8 T cells occurs *in vivo*, it could be argued that it does not result from antigenic stimulation, but from some other effect of GA. This possibility is unlikely since antibodies that block HLA class I inhibited the proliferation of CD8 T cells to GA (Karandikar, Crawford et al. 2002). Nevertheless, since GA is a random polymer, it is difficult to rule out the possibility of GA also acting in a non-antigen specific manner. To independently confirm that chronic stimulation of T cells results in the development of $CD28^{-}CD57^{+}$ T cells, I used DNA mutations as a marker of chronic stimulation. This was done since cells may develop a small number of mutations with every cell division. Specifically, I stimulated CFSE loaded PBMC with anti-CD3 in the presence of 6-thioguanine (2-amino-6-mercaptopurine). 6-thioguanine is toxic to T cells that have an intact hprt gene and only allows $hprt^{-}$ cells to divide (Albertini 1985). GA treated MS patients, untreated MS patients, and untreated healthy controls were all examined. When this was done, most subjects displayed an increase in $CD57^{+}$ T cells when 6-thioguanine was added to the culture (Figure 23A), although, not all subjects displayed an increase in $CD57^{+}$ T cells. However this was not found to be significant.

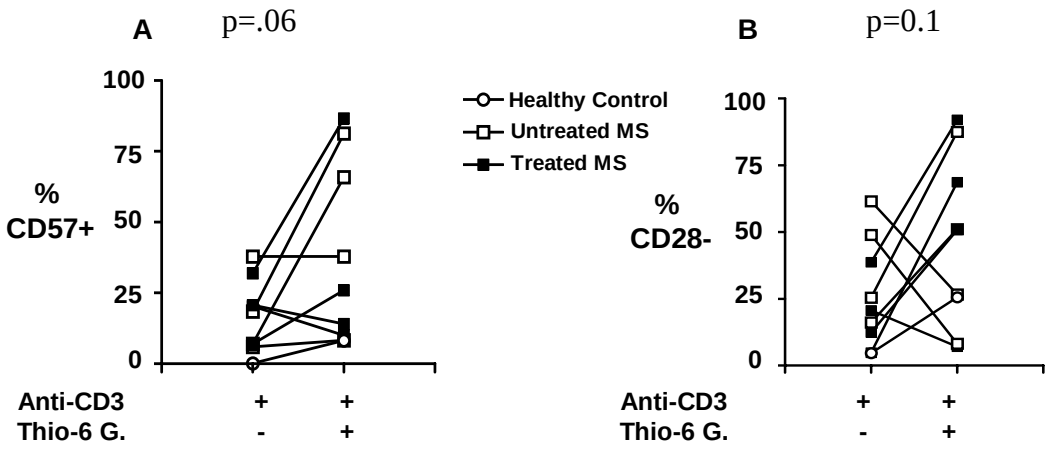

Figure 23 Examination of CD57 and CD28 expression on $HPRT^-$ $CD8^+$ T cells.

CFSE loaded PBMC were cultured with anti-CD3 with and without 6-thioguanine for 7 days. Subsequently, the cells were analyzed by flow cytometry. (A) The percent of $CD57^+$ or (B) $CD28^-$events in the live $CD8^{hi}$ diving ($CFSE^{dim}$) lymphocyte gate is shown for, 1 healthy control, 4 untreated MS patients, and 4 MS patients treated with GA for one year. P values are from paired t tests comparing all samples assayed.

No difference was observed among treated MS patients, untreated MS patients, and untreated healthy controls. I also examined CD28 expression. The majority of subjects appeared to display an increase in $CD28^-$ T cells when their PBMC were incubated with 6-thioguanine (Figure 23B). Although again, some subjects were observed to have a smaller population of $CD28^-$ T cells when incubated with 6-thioguanine. When a two tailed paired student t test was performed, significance could not be confirmed.

IV. Myelin-reactive chronically stimulated T cells

Since I have determined that chronic stimulation results in a $CD28^-CD57^+$ phenotype in MS patients, I next wanted to examine if myelin-reactive T cells could be observed with this phenotype in MS patients. As shown (Figure 24) when PBMC from 12 MS patients and 9 age matched healthy controls were loaded with CFSE and stimulated with myelin peptides, I was able to detect CD8 and CD4 T cell proliferation after a seven day incubation (Crawford, Yan et al. 2004). A strategy of using multiple peptides was adopted to increase the frequency of responding T cells to detectable levels. To determine if myelin-reactive T cells from MS patients display markers indicative of a memory or a chronically stimulated phenotype, I examined CD28 and CD57 expression on the proliferating fraction of T cells. I observed $CD8^{hi}CD57^+$ proliferation in all of the MS patients that displayed a $CD8^{hi}$ response. 2 paatients had a robouts response, 6 patients had a mild response and the rest did not respond. An example of each is shown if Figure 25A.

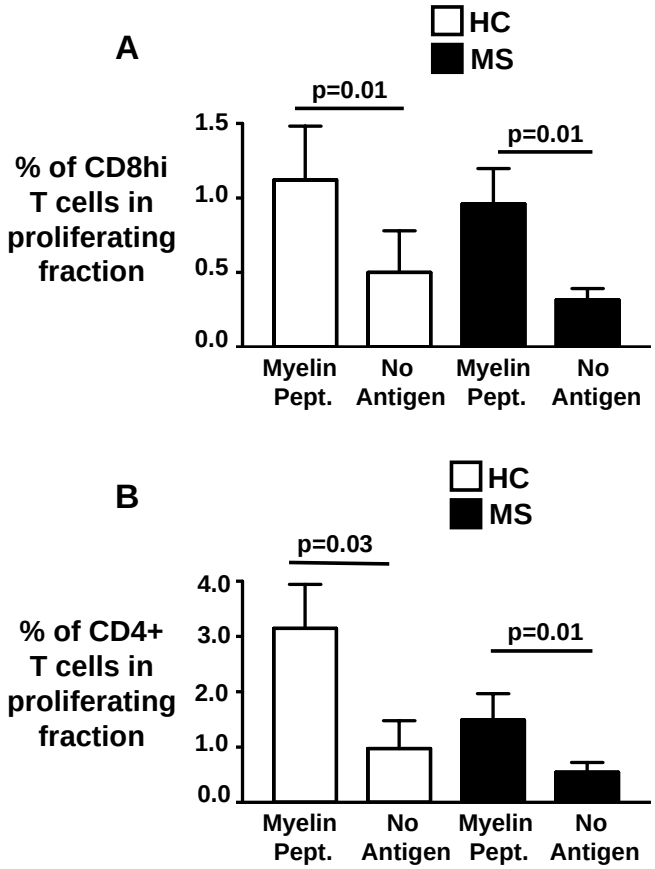

Figure 24 CD4 and CD8 T cells from MS patients and healthy controls proliferate in response to myelin antigens.

PBMC from 12 MS patients (MS) and 9 age matched healthy controls (HC) were loaded with CFSE and incubated for seven days with the panel of myelin peptides. Subsequently the cells were analyzed by flow cytometry (■) = MS, (□) = HC. (A) Percent of $CD8^{hi}$ T cells in the proliferating fraction of events for each condition indicated. (B) Percent of $CD4^{+}$ T cells in the proliferating fraction of events of each condition indicated. P values are the result of a one tailed Mann Whitney test.

Little $CD57^+$ proliferation was detected in the proliferating fraction from healthy controls (Figure 25B). Thus myelin-reactive CD28-CD57+ T cells are found in MS patients but not in healthy contols. This is the same phenotype that was previously shown to predominate after chronic stimulation. I also examined $CD4^+$ T cell responses in a similar manner (Figure 26). A slight increase in the level of $CD4^+CD28^-CD57^+$ T cells may exist in MS patients, but the majority of the $CD4^+$ response to myelin antigens was $CD28^+CD57^-$ in both MS patients and healthy controls (Figure 26). When a response to CMV was examined, I observed $CD28^-CD57^+$ T cells in both the healthy controls and MS patients (Figure 27A). These data also indicate that $CD28^-CD57^+$ T cells from healthy controls can proliferate in response to CMV (Figure 27A, far left open bar). When a recall response to mumps was examined, the majority of the response was $CD57^-$ in both MS patients and healthy controls (Figure 27B). Furthermore, the recall response to mumps from MS patients was predominantly $CD28^-$ $CD57^-$. Thus, $CD8^{hi}CD28^-CD57^+$ T cells are the predominant T cells responding to myelin antigens, but this is not a feature of all T cells from MS patients.

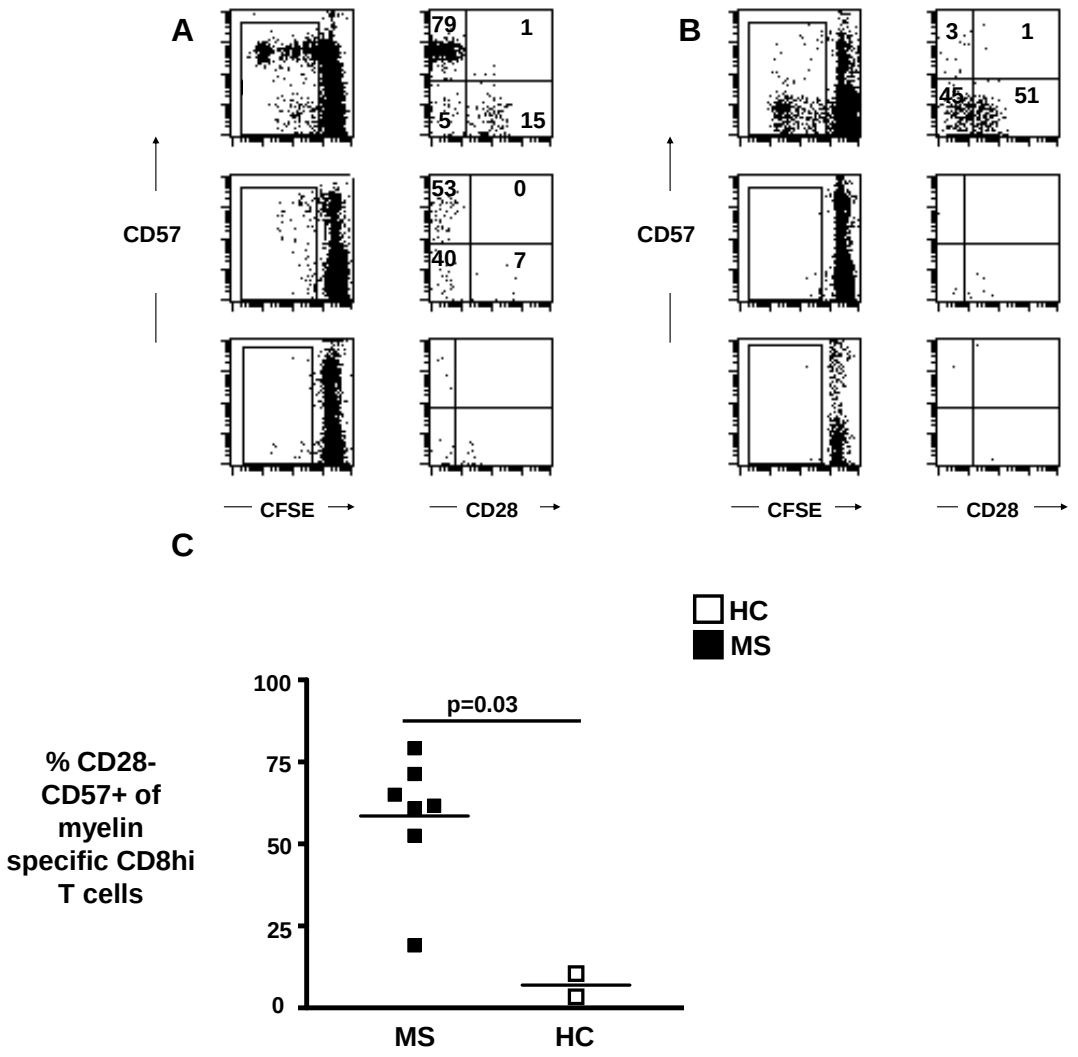

Figure 25 Myelin reactive $CD28^{-}CD57^{+}CD8^{hi}$ T cells can be found in MS patients but not healthy controls.

PBMC from 12 MS patients and 9 healthy controls were loaded with CFSE and incubated for seven days with the panel of myelin peptides and analyzed by flow cytometry. Representative examples are shown of the CFSE dilution of $CD57^{+}$ T cells for (A) CD8 MS and (B) CD8 HC. (C) Scattergram showing the percent of $CD28^{-}CD57^{+}$ events in the live $CD8^{hi}$ $CFSE^{dim}$ lymphocyte gate of samples that responded by proliferation. *indicates the result of a one tailed Mann Whitney test.

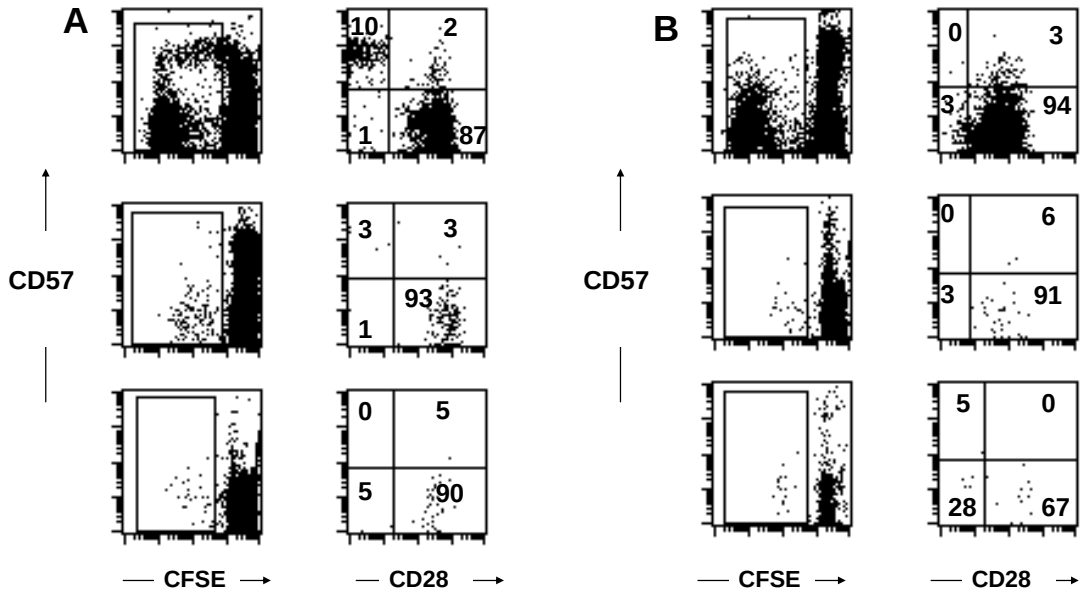

Figure 26 Myelin reactive $CD4^+$ T cells.

PBMC from 12 MS patients and 9 healthy controls were loaded with CFSE and incubated for seven days with the panel of myelin peptides and analyzed by flow cytometry. Representative examples are shown of the CFSE dilution of $CD57^+$ T cells for (A) $CD4^+$ MS and (B) CD4 HC.

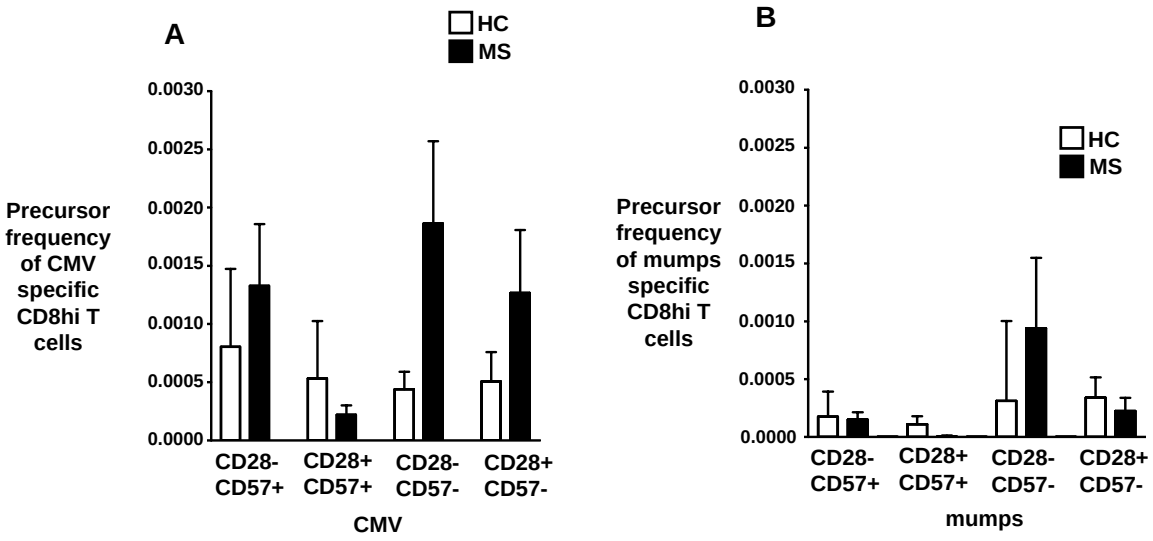

Figure 27 MS patients can mount a predominantly $CD28^{-}CD57^{-}$ effector response and HC can mount a $CD57^{+}$ response.

PBMC from MS patients and healthy controls were loaded with CFSE and incubated for seven days with (A) CMV (MS n = 6, HC n = 5) or (B) mumps antigen (MS n = 4, HC n = 3) and analyzed by flow cytometry. Mean precursor frequencies were calculated for CMV or mumps reactive $CD8^{hi}$ T cells.

Next, I wanted to determine other characteristics of the myelin-reactive $CD8^{hi}CD28^{-}CD57^{+}$ T cells. I wished to use as short an incubation time as possible. Since a response of 4 in 10,000 is close to the minimum level detectable by flow cytometry without proliferation, I again employed CD25 upregulation as a marker of T cell activation. When two day assays were performed, a detectable level of CD25 upregulation was observed in patients previously determined to have a high frequency of myelin-reactive T cells (Figure 28A). The upregulation of CD25 in response to no antigen (top) or myelin peptides (bottom) is shown for $CD8^{hi}$ T cells (Figure 28A). A majority of these responding T cells were $CD28^{-}CD57^{+}$ and expressed perforin (Figure 28B & C), a molecule used in the cytolytic pathway. No upregulation of CD25 on CD4 T cells was detected by this method (data not shown). I also observed a high frequency of myelin-reactive T cells by ELISPOT (Figure 28D), indicating a high frequency of responding cells can be detected by an alternate method. $CD8^{hi}CD28^{-}CD57^{+}$ T cells have also been demonstrated to release TNF-α, IFN-γ, and possess cytotoxic ability (Mollet, Sadat-Sowti et al. 1998; Weekes, Wills et al. 1999; Sze, Giesajtis et al. 2001). To confirm that these T cells have a cytotoxic phenotype, I stimulated PBMC from MS patients for seven days with myelin peptides and sorted out the myelin-reactive $CD8^{hi}CD28^{-}CD57^{+}$ and $CD8^{hi}CD28^{+}CD57^{-}$ T cells. mRNA was extracted, amplified, and RT-PCR was performed on these samples. When this was done, myelin-reactive $CD8^{hi}CD28^{-}CD57^{+}$ T expressed more granzyme B transcripts and more IFN-γ transcripts than $CD8^{hi}CD28^{+}CD57^{-}$ T cells (Figure 29A, B). Granzyme B is a molecule used in the cytolytic pathway. This

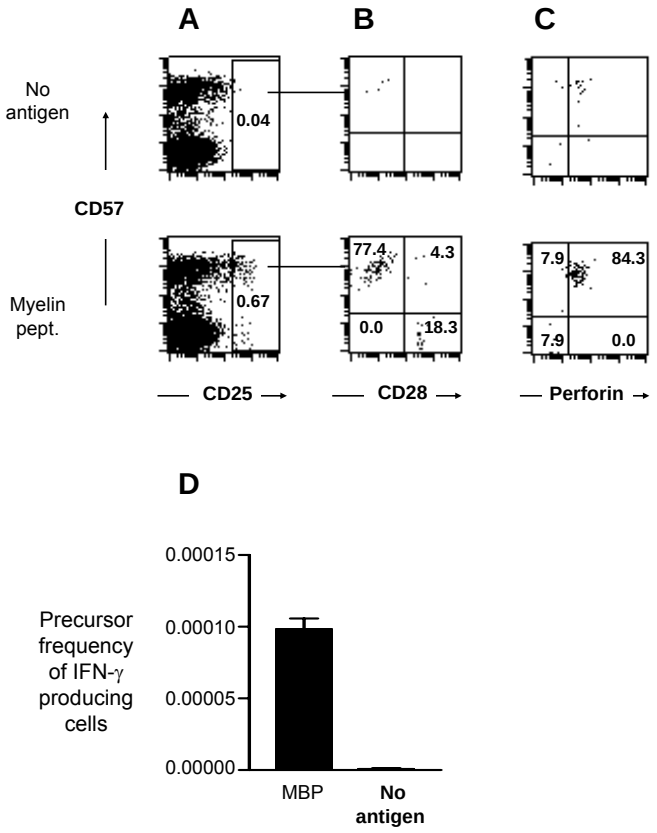

Figure 28 Myelin reactive T cells are detectable by CD25 (IL-2Rα) upregulation after two day *in vitro* incubation with myelin peptides.

PBMC from MS patients were incubated for two days with no antigen (top row) or myelin peptides (bottom row). Subsequently, the cells were analyzed by flow cytometry. (A) Plot is electronically gated for $CD8^{hi}$ lymphocytes. (B & C) Plots are gated for $CD8^{hi}CD25^{+}$ lymphocytes. Numbers represent percent of events in each quadrant. CD25 upregulation was detected in 2 patients previously determined to have a high frequency by proliferation. 5 patients were assayed in total. (D) Frequency IFN-γ secreting cells in PBMC after a 36 hour stimulation with MBP peptides, as determined by ELISPOT. ELISOPT was repeated on 3 MS patients.

indicates these autoreactive T cells have a similar phenotype to previously studied $CD8^{hi}CD28^{-}CD57^{+}$ T cell populations. High levels of CXCR3 and not CCR4 have been observed on the CD8 T cells in the CSF of MS patients (Balashov, Rottman et al. 1999). We observed relatively lower levels of CCR4 mRNA transcripts than CXCR3 transcripts (Figure 29C & D). However no difference in mRNA expression of CCR4, CXCR3 or TNF-α was identified when $CD8^{hi}CD28^{+}CD57^{-}$ $CD8^{hi}$ T cells were compared to $CD28^{-}CD57^{+}$ $CD8^{hi}$ T cells (Figure 29C-E). $CD57^{+}$ T cells from MS patients released IFN-γ when stimulated with PMA and ionomycin (Figure 30A). When restimulated with PMA and ionomycin for 12 hours T cells responding to myelin peptides by CD25 upregulation released IFN-γ (Figure 30 B & C top). PBMC not originally stimulated with myelin peptides did not up regulate CD25 appreciably after 12 hours of stimulation with PMA and ionomycin (Figure 29F, G bottom). This indicates myelin-reactive T cells are at least capable of releasing IFN-γ, but whether they do so *in vivo* cannot be examined. Granzyme B and IFN-γ expression supports a cytotoxic potential for myelin reactive $CD8^{hi}CD28^{-}CD57^{+}$ T cells in MS patients.

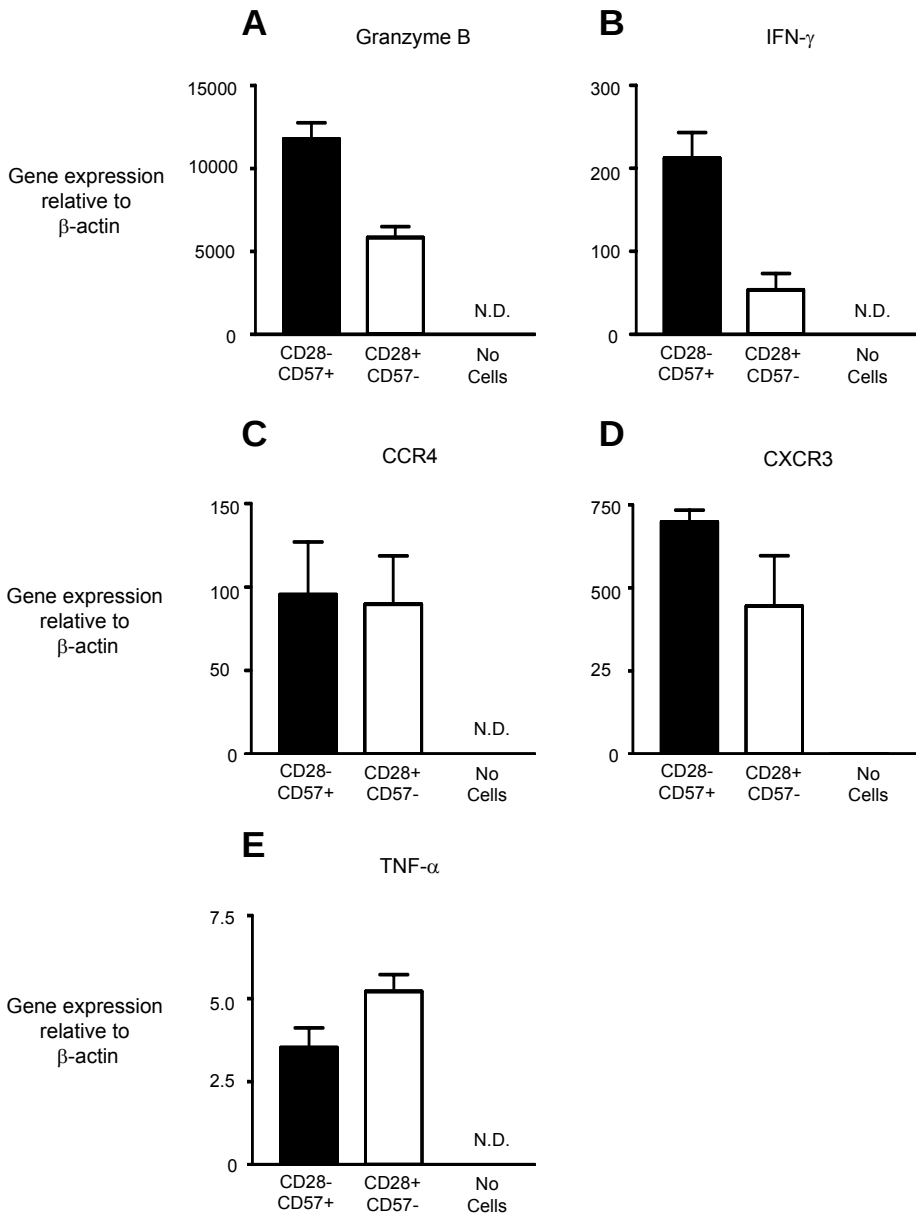

Figure 29 Autoreactive $CD8^{hi}CD28^{-}CD57^{+}$ contain granzyme B and IFN-γ mRNA. Expression of (A) granzyme B, (B) IFN-γ, (C) CCR4, (D) CXCR3, and (E) TNF-α mRNA from sorted myelin-reactive T cells was examined by real time PCR, and normalized to β actin expression. n = 2.

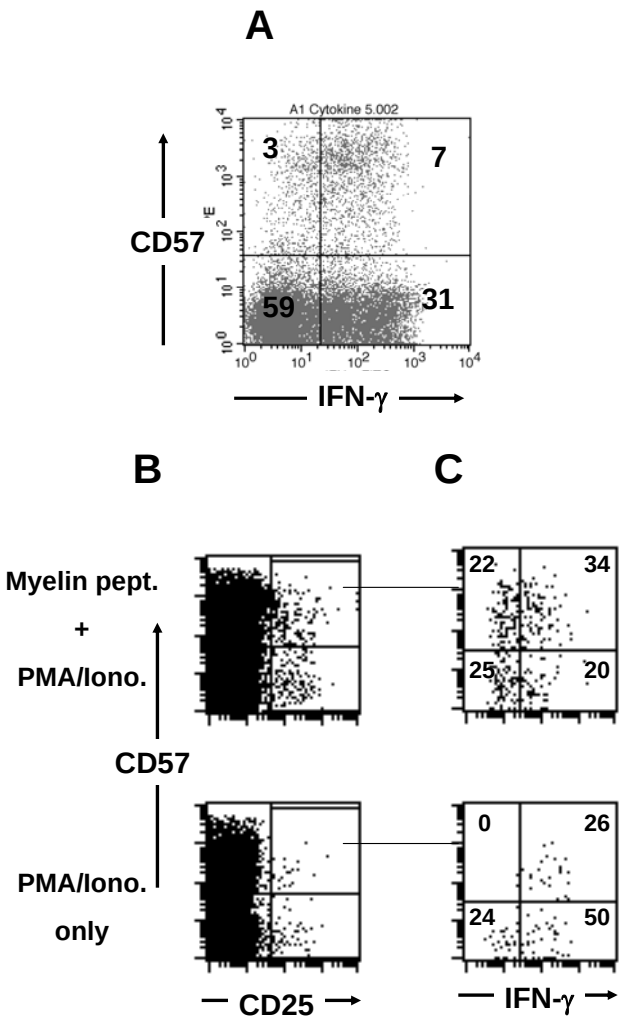

Figure 30 $CD57^{+}CD8^{hi}$ T cells release IFN-γ when stimulated with PMA ionomycin.

(A) PBMC from an MS patient was simulated with PMA and ionomycin for 12 hours. The cells were then analyzed by flow cytometry. $CD8^{hi}$ events are shown. Representative example of 4 MS patients is show. (B) PBMC stimulated with myelin peptides for seven days also upregulate CD25 and (C) release IFN-γ when restimulated with PMA and ionomycin for the final 12 hours of culture. Representative example of two experiments is shown.

CHAPTER FOUR DISCUSSION

I. Introduction and summary of GA results

In an effort to understand how autoreactive T cells may initiate an autoimmune process which results in MS like symptoms, much research has been conducted on animal models of MS such as EAE. This research has been very productive, and has given us a greater understanding of the range of possible mechanisms that may underlie the disease process in MS. However from a theoretical point of view the only way to know if it these models faithfully recapitulate MS, is to examine MS patients. Furthermore, one aspect of MS is unlikely to be recapitulated in animal models, and this is the maintenance of disease over several decades. In this thesis, I have demonstrated some of the changes that may occur as T cells are repeatedly stimulated in MS patients. This investigation also allowed the hypothesis that autoreactive T cells in MS patients are repeatedly stimulated in MS patients to be examined. T cells with a chronically stimulated phenotype were of interest as they may play a role in other situations besides MS. Some properties adopted by T cells that were chronically stimulated may be similar to the properties of T cells from subjects of advanced age. This work will be relevant to any situation where chronic stimulation of T cells occurs. This includes but is not limited to rheumatoid arthritis, some tumors and persistent viral infections that are not initially cleared by the immune system but are kept in check by a T cell response.

A. Chronic Stimulation leads to a $CD28^{-}CD57^{+}$ phenotype.

Initially a series of experiments were set up that examined two hypotheses. One is that repeated stimulation of T cells results in a $CD28^{-}CD57^{+}$ phenotype. The second is that CD8 GA-reactive T cells are chronically stimulated when MS patients undergo GA treatment. These two hypotheses could lead to circular arguments however. This was avoided by independently verifying the first hypothesis, and the fact that some evidence to support the second has already been established. To test the first hypothesis, I decided to use MS patients treated with daily injections of GA. This system is advantageous because I have samples from patents before they initiate therapy and every 4 months thereafter. This allowed us to precisely monitor the time patients have responded to GA. This is important as responses may change overtime. For example initially, the response may be a robust memory response, this may change to a less potent response after chronic treatment, and finally to clonal exhaustion and a minimal response. Thus, studies on T cells in humans that are chronically infected with viruses may generate conflicting results because of the uncontrolled variable of time. This effect could always be lessened by studying a larger number of patients, but it cannot be formally removed. Studies on animals are informative, but as discussed above, the only way to know for sure they recapitulate the human condition is to examine humans. Another advantage is that since GA is composed of multiple specificities, the response it generates is robust. There are however, a few draw backs to this approach. One is GA itself, which is a random polymer. Because of this, GA may have many effects that are difficult to eliminate. For example, it may bind to and affect T cells

directly, or it may directly affect another cell type that indirectly effects a change on T cells. A second concern is that this study will only be conducted on MS patients as healthy controls will not be given this drug. This may limit the generality of the findings. There is little reason to suppose that MS patients will have altered responses to GA as opposed to healthy controls and in any case, knowing the effect on the MS population is informative for the purposes of this study.

When the response to GA was examined in MS patients before and after daily injection, I observed a response that changed from one predominantly $CD28^{-}CD57^{-}$, and $CD28^{+}CD57^{-}$ to one that was predominantly $CD28^{-}CD57^{+}$ in the absence of the confounding effects of a viral infection. This phenotype was also observed by methods which did not rely on proliferation and required only 2 days of culture *in vitro*, thus providing a reasonable estimation of the characteristics of these cells *in vivo*. Using this system, $CD28^{-}CD57^{+}$ T cells were also shown to express a low amount of perforin, similar to T cells that are known to be viral reactive (Appay, Dunbar et al. 2002). This suggests that these T cells may be similar in nature to T cells that develop in response to chronic viral infections. When the response to GA was followed over time I only observed the development of this phenotype after several months of treatment.

The possibility remained however that GA had a direct effect on the CD8 T cells and the $CD28^{-}CD57^{+}$ phenotype was due to this direct effect and not chronic stimulation. Furthermore GA could have an effect on some other cell type besides CD8 T cells such as an antigen presenting cell that then has an effect on the CD8 T cell and causes it to change

phenotype. To minimize this possibility, I mixed sorted $CD8^+$ cells from a patient treated for one year with CD8 depleted PBMC from the same patient after only two months of treatment, and stimulated the cells with GA. If the $CD28^-CD57^+$ phenotype results from altered antigen presentation *in vitro*, I would expect to observe a largely $CD28^+CD57^-$ responding population. However, the proliferating $CD8^+$ T cells adopted a $CD28^-CD57^+$ phenotype, indicating that the changes observed in the $CD8^+$ response resulted from changes in the $CD8^+$ T cells *in vivo*. This experiment suggests the change in the CD8 T cell does not happen *in vitro* but occurs *in vivo*. It does not however, demonstrate that this *in vivo* change is the direct effect of GA. These data are consistent with our hypotheses namely, that repeated stimulation of T cells results in a $CD28^-CD57^+$ phenotype and that CD8 GA specific T cells are chronically stimulated when MS patients undergo GA treatment. To independently confirm that chronic stimulation of T cells results in the development of $CD28^-CD57^+$ T cells, and avoid circular arguments, I used DNA mutations as a marker of chronic stimulation. This was done since cells may develop a small number of mutations with every cell division. Specifically, I stimulated CFSE loaded PBMC with anti-CD3 in the presence of 6-thioguanine (2-amino-6-mercaptopurine). 6-thioguanine is toxic to T cells that have an intact hprt gene and only allows $hprt^-$ cells to divide (Albertini 1985). GA treated MS patients, untreated MS patients, and untreated healthy controls were all examined. As shown in Figure 23, when this was done most subjects displayed an increase in $CD57^+$ T cells when 6-thioguanine was added to the culture. A trend toward more $CD57^+$ T cells was observed but this did not reach statistical significance. When I examined CD4 T cell

responses from GA treated patients, I did not observe the development of a $CD28^{-}CD57^{+}$ phenotype. Thus for $CD4^{+}$ T cells neither of our hypotheses were supported by the data. One side benefit to uncovering this information is that it presents several possible mechanisms whereby GA may be having an effect on the course of MS.

II. Possible mechanism of action of GA

Although $CD28^{-}CD57^{+}$ T cells have been described as suppressor or regulatory cells (Mollet, Sadat-Sowti et al. 1998; Najafian, Chitnis et al. 2003), they also possess cytotoxic ability (Mollet, Sadat-Sowti et al. 1998; Weekes, Wills et al. 1999; Sze, Giesajtis et al. 2001). These two possibilities are not mutually exclusive as cytotoxic T cells may suppress themselves or other cells by releasing soluble factors or by killing. Indeed, it has been reported that cytotoxic $CD57^{+}$ T cells regulate themselves by releasing a soluble factor (Sadat-Sowti, Debre et al. 1991; Sadat-Sowti, Debre et al. 1994; Mollet, Sadat-Sowti et al. 1998; Mollet L 1999; Wang, Lehner et al. 1994). Alternatively, it may be that the $CD8^{+}CD28^{-}CD57^{+}$ T cells induced by GA release this factor *in vivo* which inhibits the cytotoxic ability of myelin-reactive $CD8^{+}CD28^{-}CD57^{+}$ T cells observed in Figure 25. This may then ameliorate the severity of MS. In this model, GA reactive CD8 T cells may only inhibit proliferation in the presence of GA, and this would explain why a significant change in $CD8^{+}CD28^{+}CD57^{-}$ reactive T cell proliferation was not observed in treated MS patients (data not shown).

There is mounting evidence that CD8 cells can suppress CD4 cells that express Qa-1 via cytotoxicity (Jiang and Chess 2000; Hu, Ikizawa et al. 2004). Although I used CD57 as a typical NK cell marker and as a marker of chronically stimulated T cells, $CD57^+$ T cells have been reported to express other NK cell markers such as CD94 (Tarazona, DelaRosa et al. 2000; Scheuring, Sabzevari et al. 2002; Bigouret, Hoffmann et al. 2003) which is the ligand for Qa-1 and its human homologue HLA-E. This presents the possibility that these $CD8^+CD57^+$ cells are able to inhibit HLA-E expressing CD4 T cells. If these cells were to inhibit myelin-reactive T cells *in vivo*, this could represent another mechanism of action of GA. Interestingly, in mice this mechanism has been shown to preferentially inhibit Th1 responses (Jiang, Braunstein et al. 2001). If these cells are able to inhibit Th1 responses through killing, it would explain the Th2 bias observed after administration of GA (Duda, Schmied et al. 2000; Chen, Gran et al. 2001; Dhib-Jalbut, Chen et al. 2003). Alternatively, memory CD8 T cell responses have been shown to be dependent on CD4 T cell help, and these CD8 T cells may be eliminating the CD4 T cells required to maintain an anti-myelin response (Matloubian, Concepcion et al. 1994; Janssen, Lemmens et al. 2003; Shedlock and Shen 2003; Sun and Bevan 2003).

III. Origin of $CD28^-CD57^+$ T cells

We have shown that the $CD28^-CD57^+$ phenotype predominates after repeated stimulation of CD8 T cells *in vivo*. There are two hypotheses of where $CD28^-CD57^+$ T cells originate. One is the possibility that the $CD28^-CD57^+$ population derives from a previously

small $CD8^{+}CD28^{-}CD57^{+}$ cell population. The other is that $CD28^{-}CD57^{+}$ T cells are derived from a $CD28^{+}CD57^{-}$ or similar population. When I examined the precursor frequency of the different subpopulations in Figure 14C initially all populations started out low and all populations increased about one log in frequency after initiation of GA treatment. This data could be interpreted as suggesting that these populations arise from separate lineages. However, several considerations argue against this interpretation. It may be the case that not all CD8 T cells responding to GA are naïve. Although GA was designed to resemble MBP, I detected no TCR cross reactivity between these antigens in humans (data not shown). Nevertheless, not all of the T cells responding to GA are necessarily naïve and some could have already converted to a $CD28^{-}CD57^{+}$ phenotype. This is supported by the fact that the percentage of $CD8^{hi}CD28^{-}CD57^{+}$ T cells in unstimulated PBMC (Figure 13C) was the same the percentage of $CD8^{hi}CD28^{-}CD57^{+}$ T cells in the proliferating fraction of GA stimulated PBMC from untreated MS patients (Figure 4C MS vs. Figure 13C MS). The same was true for untreated healthy controls (Figure 4C, HC). This indicates that the $CD8^{hi}CD28^{-}CD57^{+}$ T cells observed to proliferate in healthy controls and untreated patients may be due to background levels of $CD8^{hi}CD28^{-}CD57^{+}$ T cells. When the CDR3 region of TCR from $CD28^{-}$ and $CD28^{+}$ CD8 T cells were examined, the same sequence could be found in both $CD28^{-}$ and $CD28^{+}$ T cells (Mugnaini, Spurkland, et al. 1998; Mugnaini, Egeland et al. 1999). This indicates that one population arises from the other, and my data would indicate the $CD28^{-}$ population arises from the $CD28^{+}$ population.

IV. Myelin

Since my data are consistent with the observation that chronic stimulation does indeed lead to the development of $CD8^{hi}CD28^{-}CD57^{+}$ T cells, I used this result to test the hypothesis that T cells play a role in MS. I reasoned that if this is the case, myelin-reactive T cells would be chronically stimulated in MS patients and would adopt a $CD28^{-}CD57^{+}$ phenotype. To test this, I stimulated PBMC from MS patients and age matched healthy controls with a panel of myelin peptides and examined the $CD8^{hi}$ T cells that responded. Using age matched controls, was important as the level of $CD28^{-}CD57^{+}$ T cells has been shown to increase with age (Merino, Martinez-Gonzalez et al. 1998). While much informative research has been conducted trying to determine which autoantigens T cells in MS patients may be responding, in this study I was uninterested in this question, and since the frequency of responding T cells could be expected to be near or below the level detectable by this method, I decided to use multiple peptides in these assays. Utilizing a flow cytometric approach, which reduces the bias towards rapidly dividing T cells inherent in past studies, I observed a population of autoreactive $CD28^{-}CD57^{+}$ CD8 T cells in some MS patients. This indicates these may be the memory T cells previously observed and these autoreactive T cells are replication impaired due to extensive *in vivo* proliferation. $CD8^{hi}CD28^{-}CD57^{+}$ T cells have also been demonstrated to release TNF-α, IFN-γ and possess cytotoxic ability (Mollet, Sadat-Sowti et al. 1998; Weekes, Wills et al. 1999; Sze, Giesajtis et al. 2001). Our data indicate the autoreactive $CD8^{hi}CD28^{-}CD57^{+}$ T cells observed in MS patients contain granzyme B, TNF-α, and IFN-γ mRNA transcripts; and released IFN-γ

when restimulated with PMA and ionomycin. This indicates these autoreactive T cells have a similar phenotype to previously studied $CD8^{hi}CD28^{-}CD57^{+}$ T cell populations. In addition, high levels of CXCR3 and not CCR4 have been observed on the CD8 T cells in the CSF of MS patients (Balashov, Rottman et al. 1999). I also observed relatively lower levels of CCR4 mRNA transcripts than CXCR3 transcripts.

a) Down regulation of CD57

On average, $CD28^{-}CD57^{+}$ T cells made up 58.7 % ±6.3 percent of the response, though this may be an underestimation. As shown in Figure 5, when $CD8^{+}CD28^{-}CD57^{+}$ T cells were sorted, it appeared that some previously $CD57^{+}$ T cells down regulated CD57 when stimulated. It is possible that these previously $CD57^{+}$ T cells downregulated CD57 during a seven day incubation. In support of this I found a higher frequency of GA reactive T cells when I examined them at two days (Figure 21) as opposed to seven days (Figure 11). This held true when mumps, anti-CD3 or CMV activated T cells were examined as well. This indicates that the percentage of $CD28^{-}CD57^{+}$ T cells that are auto reactive in MS patients may be higher than the 58.7 % ±6.3 percent that was observed. Although this difference could also be due to differential proliferation in the intervening 5 days, or cells that respond by $CD25^{+}$ upregulation may be more likely to be $CD57^{+}$ than cells that respond by proliferation.

b) Alternative possibilities

While I may have uncovered the population of T cells that mediates destruction in MS patients there are a number of reasons why this may not be the case. For instance the T cells that proliferated in this assay may not have been antigen-specific. It has been demonstrated that some previously activated T cells are able to divide in the presence of activating cytokines in the absence of TCR mediated stimulation (Geginat, Sallusto et al. 2001; Geginat, Lanzavecchia et al. 2003). A second cell type may have been activated by the peptide mix, subsequently secreted cytokines and induced CD8 T cells to proliferate. This would not however, explain why the T cells proliferating in MS patients are $CD28^{-}CD57^{+}$. It has been shown that CMV positive individuals have higher levels of $CD57^{+}$ T cells than CMV negative individuals. So it is possible that CMV or some similar virus induces MS and initiates this disease and causes MS patients $CD57^{+}$ T cells to respond in this way. This however is unlikely for several reasons. When I examined the general level of $CD57^{+}CD28^{-}CD8^{hi}$ T cells, I did not observe higher levels in MS patients. Third, in Figure 27, I examined the level of mumps reactive T cells in MS patients and they were predominantly $CD57^{-}$, although I did observe some $CD57^{+}$ T cells. Second healthy controls were shown to contain similar levels of CMV reactive $CD8^{hi}CD57^{+}$ T cells.

V. High frequency of myelin-reactive T cells in MS patients

Methods that rely on proliferation have revealed low frequencies of myelin-reactive T cells in MS patients and healthy controls (Jingwu, Medaer et al. 1992; Joshi, Usuku et al.

1993). Methods that do not rely on proliferation, such as ELISPOT, have demonstrated frequencies as high as 1 $x10^{-4}$ (Pelfrey, Rudick et al. 2000; Hellings, Baree et al. 2001; Van der Aa, Hellings et al. 2003). In addition, the myelin-reactive T cells in MS patients, but not healthy controls, have been shown to be less dependent on CD28 costimulation, indicating they have been previously activated and have properties of a memory response (Lovett-Racke, Trotter et al. 1998; Scholz, Patton et al. 1998). This discrepancy could be explained by the *in vitro* apoptosis of autoreactive T cells. However, when apoptosis through CD95 stimulation was blocked, the frequency of T cells proliferating against myelin antigens was reported to be 1.3 $x10^{-6}$ in PBMC (Zang, Kozovska et al. 1999), well short of the frequencies observed by ELISPOT (Pelfrey, Rudick et al. 2000; Hellings, Baree et al. 2001; Van der Aa, Hellings et al. 2003), The frequency of $CD8^{hi}CD28^{-}CD57^{+}$ I observed (4.0 $x10^{-4}$ HC, and 8.1 $x10^{-4}$ MS) in CD8 T cells was consistent with the level observed by ELISPOT (Pelfrey, Rudick et al. 2000; Hellings, Baree et al. 2001; Van der Aa, Hellings et al. 2003), In addition, the precursor frequency of viral-reactive T cells I observed by this method (14 x 10^{-4} MS, 10 x 10^{-4} HC, mumps; and 46 x 10^{-4} MS, 23 x 10^{-4}CMV in total CD8) is consistent with the level of viral reactive T cells observed by ELISPOT (5 to 3 x 10^{-4}) in PBMC (Ott, Berner et al. 2004). The frequency of CMV reactive T cells as determined by tetramer staining was even greater and ranged from 0.21% to 3.79% of total CD8 T cells.

VI. Why anti-CD4 does not ameliorate MS

Autoreactive CD4 T cell have been implicated in the pathogenesis of MS. This is supported by the association of MHC class II gene DR to MS susceptibility, and similarities between MS and its animal model EAE. In most forms of EAE, $CD4^+$ T cells are required for initiation and maintenance of disease. The definitive study to determine if MS is similar to EAE in this respect would be to deplete CD4 T cells from patients with established disease and determine if this lessens or stops the accumulation of disability. When this was done, it did not improve MS patients outcomes (van Oosten BW 1997), suggesting $CD4^+$ T cells may not play a role in the later stages of disease. Furthermore, drugs that suppress proliferation of $CD4^+$ T cells such as cyclophosphamide, azathioprine, and mitoxantrone have modest effects on the course of MS (Yudkin, Ellison et al. 1991) (1991). Based on this data, it has been suggested that MS is not mediated by autoreactive $CD4^+$ T cells (Sriram S 1997). Our observation of the existence of a high frequency of myelin-reactive replication deficient $CD8^{hi}$ T cells in MS patients can explain this observation, although $CD4^+$ T cells may be required to provide T cell help, especially during initiation of the immune response (Janssen, Lemmens et al. 2003).

a) Supporting evidence that CD8 T cells play a role in MS

Previously, it has been shown that effector CD8 T cells are capable of mediating MS-like autoimmunity (Huseby, Liggitt et al. 2001; Sun, Whitaker et al. 2001). Moreover, oligodendrocytes express class I MHC molecules, but not class II, which makes CD8 involvement in the pathogenesis of MS more plausible. In addition, CD8 T cells have been observed in the brain tissue of MS patients (Babbe, Roers et al. 2000; Jacobsen, Cepok et al. 2002; Skulina, Schmidt et al. 2004). In these studies, healthy controls were not examined and it has not been determined if CD8 T cells exist in normal brain, or are myelin-reactive. This leaves open the possibility that CD8 T cells are incidental to the disease process (Scotet, Peyrat et al. 1999). Here I show myelin reactive CD8 T cells may have a phenotype of chronic stimulation in MS patients as compared to healthy controls, suggesting they play a role in the disease process. This also suggests that treatments which attempt to inhibit proliferation of myelin-reactive $CD8^{hi}$ T cells may be ineffective due to the reduced proliferative ability of these T cells.

VII. Function of CD57 and why it may be pathogenic

CD57 is defined by the binding ability of the mouse IgM antibody HNK-1 (Abo and Balch 1981), The HNK-1 epitope is present on an N- linked oligosaccharide (Kanda, Yamawaki et al. 1995). Interestingly, CD57 has been reported to function as a neuronal

homophilic adhesion molecule, and is expressed on myelin proteins. While I have shown that CD57 is expressed on T cells as a result of chronic stimulation, the exact function of CD57 when expressed on T cells is less clear. As discussed previously, it has been reported to function as a neuronal homophilic adhesion molecule, and is expressed on myelin proteins (Shy, Gabel et al. 1986; Mikol, Gulcher et al. 1990; Filbin and Tennekoon 1991; Field, Wing et al. 1992; Filbin and Tennekoon 1993; Needham and Schnaar 1993). It has also been reported to bind to L- and P selectin (Needham and Schnaar 1993) and its expression can be induced on brain microvascular endothelial cells by IL-1β (Kanda, Yamawaki et al. 1995). One could envision a scenario whereby the upregulation of CD57 on $CD8^{hi}$ cells aids their trafficking into the CNS and subsequent binding to myelin proteins. In this scenario, the CD8 T cells may only become pathogenic after chronic stimulation and this would fit with epidemiological observations discussed earlier. When I examined the levels of $CD8^{hi}CD28^{-}CD57^{+}$ T cells in the PBMC of MS patients directly *ex vivo* I did not detect increased percentages as compared to healthy controls. However when the percent of $CD28^{-}CD57^{+}$ T cells in an anti-CD3 and GA response was slightly elevated in MS patients. Furthermore when PBMC were incubated for 7 days without antigen a greater percentage of $CD8^{hi}CD28^{-}CD57^{+}$ T cells was found in MS patients than in healthy controls, and this was significant ($p=.02$ two tailed Mann Whitney; $p=.02$ two tailed t test) (Figure 13C). This presents the possibility that $CD8^{+}CD28^{-}CD57^{+}$ T cells are more resistant to apoptosis in MS patients. This is an interesting hypothesis that should be tested further, however it should be noted that the healthy controls were on average slightly younger than the untreated MS patients (37y vs.

44). Lastly, IL-6 has been reported to bind the CD57 epitope. In resting human lymphocytes, the majority of CD57 is expressed on the IL-6 coreceptor gp130 (Cebo, Durier et al. 2002). IL-6 expression has been shown to be associated with MS (Woodroofe and Cuzner 1993; Maimone, Guazzi et al. 1997) and necessary for the development of experimental autoimmune encephalomyelitis (EAE) (Eugster, Frei et al. 1998), an animal model of MS.

VIII. Rheumatoid arthritis

One possibility is that MS is the result of an underlying CD4 or CD8 lymphopenia. In this scenario, a reduction in the number of CD4 or CD8 T cells leads to the homeostatic expansion of the remaining CD4 or CD8 T cells, to replace the T cell deficit. This expansion leads to the accumulation and activation of autoreactive T cells, which subsequently traffic to the brain and cause disease. This may be a general mechanism that leads to autoimmunity and certain MHC such as HLA DR15 may lead to the MS where other MHC lead to other organ specific diseases or systemic autoimmunity.

Rheumatoid arthritis, another organ specific autoimmune disease, has been hypothesized to result from homeostatic T cell proliferation (Wagner, Koetz et al. 1998). RA patients display increased numbers of $CD28^-$ lymphocytes when the total population of peripheral blood $CD4^+$ T cells was examined (Schmidt, Goronzy et al. 1996). I hypothesized homeostatic proliferation generates effector T cells which are myelin-specific and cause MS, and as in RA when this happens higher levels of $CD28^-CD57^+$ cells are found. However, I

found no difference in the level of CD28 and CD57 expression on T cells from the peripheral blood of MS patients when compared to healthy controls of either $CD4^+$ or $CD8^{hi}$ T cells. If my data is assumed to be representative of all MS patients, and represents what is in the CSF as well, then it disproves the second hypothesis but not the first. This data does not disprove the first hypothesis, but does not support it either. The CD4 data is variable and this has been observed by others and, probably reflects the "true values". When I incubated PBMC for 7 days in Figure 13, I did observe a small increase in the level of $CD28^-CD57^+$ T cells between MS patients and healthy controls. This difference was significant (p=.0235 two tailed Mann Whitney). This difference was also apparent in the CD8 response to GA in Figure 11. In Figure 10, I observed more $CD4^+$ $CD28^-CD57^+$ T cells dividing in MS patients, but did not observe it Figure 12 when I examined CD8 T cells. Thus my data is ambiguous. One trivial explanation is that the MS patients are slightly older than the healthy controls (44y vs. 37y) and levels of $CD28^-CD57^+$ T cells increase with age. Another is that there is a small difference that is difficult to detect. A small difference has been reported by another groups (Hug, Korporal et al. 2003) (Thewissen, Linsen et al. 2005). Alternately, there may be a difference in resistance to apoptosis, or spontaneous proliferation in MS patients and HC. This does not support the hypothesis, but would not disprove it either. This possibility generates a new hypothesis that $CD28^-CD57^+$ T cells do not undergo apoptosis in MS patients and become pathogenic.

IX. Overall significance of work

In this thesis, I have examined the effects of chronic stimulation on CD4 and CD8 T cells from MS patients and healthy controls. In addition to MS, these cell types were of interest as they may play a role in other situations as well. Some properties adopted by chronically stimulated T cells may be similar to the properties of T cells from subjects of advanced age. This work will be relevant to any situation where chronic stimulation of T cells occurs. This includes but is not limited to rheumatoid arthritis other autoimmune diseases, some tumors, and persistent viral infections that are not initially cleared by the immune system but are kept in check by a T cell response.

My data indicates that $CD4^{+}CD28^{+}CD57^{-}$ T cells proliferate most robustly so these cells should have a competitive advantage. So it could be expected that these cells will predominate in long term cultures. Their predominance in long term TCL may reflect this proliferative advantage and not their importance to the process of MS pathology.

X. Future directions

A. Examine CSF for presence of $CD8^{+}CD28^{-}CD57^{+}$ T cells

My hypothesis is that $CD28^{-}CD57^{+}$ T cells play a role in MS. If this is the case, then these T cells should be present in the Central Nervous System (CNS) of MS patients especially during relapse. Evidence to test this could be collected in several ways. One is to examine the T cells found in the Cerebral Spinal Fluid (CSF) of MS patients and healthy

controls. Another is to examine the brains of MS patients upon autopsy and determine if $CD57^+CD3^+$, or $CD57^+CD8^+$ cells are found in the lesions caused by MS.

B. Deplete $CD57^+$ T cells in MS patients

Another way to test this hypothesis would be to deplete the $CD57^+$ T cells in MS patients, or administering a CD57 blocking antibody. This application would suffer two problems. One NK cells would be blocked or inhibited. Another is that antibodies that block the HNK-1 epitope have been shown to inhibit hippocampus dependent memory formation when injected directly into the hippocampus of mice (Strekalova, Wotjak et al. 2001). A way around this would be to administer anti-CD8 and anti-CD57 antibodies that act together to kill any cell both antibodies are bound to but not any other cell type. While these approaches are possible, it would be more practical to target other molecules that correlate with this phenotype that are only expressed on $CD8^+CD28^-CD57^+$ T cells.

C. Determine other markers

CD28 is a major costimulatory marker and its permanent loss could signal major changes in phenotype and function. Because of this, the expression of many molecules may be altered in $CD28^-CD57^+$ T cells. If this is indeed the case, that could be answered with a mRNA subtraction technique such as gene chip array. Subsequently, the differential expression of genes could be confirmed with real time PCR.

D. Show $CD28^{-}CD57^{+}$ CD8 T cells kill GA pulsed cells

While other investigators have show $CD28^{-}CD57^{+}$ T cells are cytotoxic, I have shown $CD28^{-}CD57^{+}$ T cells express perforin and the level CD8 T cell killing of GA pulsed targets correlates positively with the level of $CD28^{-}$ T cells in patient samples, I have not directly demonstrated they cytotoxic potential of GA-reactive $CD8^{hi}CD28^{-}CD57^{+}$ T cells. This could be accomplished by sorting out CD28-$CD28^{+}$ and $CD57^{-}$ $CD57^{+}$ T cells from a GA treated patient and determining directly the ability of these T cells to lyse GA pulsed targets. While it would be interesting to determine if myelin-reactive T cells are able to lyse myelin pulsed targets, the frequency of myelin-reactive T cells would be very low and this experiment is probably impossible to perform on primary cells with techniques currently available. While this experiment would be demanding, it would also allow the expression of inhibitory and activating receptors on the surface of these myelin-reactive T cells to be examined, as discussed below.

a) Expression of KIR and KAR

Like NK cells, cytotoxic CD8 T cells express receptors that modulate their cytotoxic ability. If a predominance of inhibitory molecules are expressed or engaged, the cell will not lyse its target. If however a predominance of activating receptors are expressed or engaged, the cell will lyse its target. The expression of these molecules could be assayed using real

time PCR and surface protein expression. This could be done on both GA reactive and myelin reactive CD8 $CD28^{-}CD57^{+}$ T cells and the results compared. In the case that GA reactive $CD8^{+}CD28^{-}CD57^{+}$ T cells prove to be cytotoxic, this would give a method to gage the cytotoxic ability of these T cells.

b) Chemokines and Chemokine receptors

T cells utilize various receptors and ligands called chemokines to home to specific tissues. It is thought that T cells that home to the brain and play a role in MS may express a specific pattern of these molecules, although the exact expression pattern that correlates with homing to the brain has not been determined. However, MIP-1α, IP-10, RANTES, and I-TAC have been reported to be expressed in the lesion of MS patients (Sorensen, Tani et al. 1999). CXCR3, the ligand of IP-10, has also been demonstrated to be expressed on T cells infiltrating into MS lesions and its expression is increased on T cells in the CSF. Expression of CXCR3 on $CD8^{+}CD28^{-}CD57^{+}$ would support the hypothesis that these cells play a role in MS.

c) Block CD57 with an HNK-1

CD57 may be involved in T cell adhesion. To test this, I would restimulate PBMC from GA treated patients with GA in the presence of antibodies that block CD57 from binding. If this decreases the percentage of $CD57^{+}$ T cells in the proliferating fraction, this

would be consistent with the hypothesis it blocks binding. If this experiment is successful, synapse formation could be assayed in the presence and absence of CD57 blocking antibody. Since CD57 has been shown to be involved in binding L-selectin and P selectin (Needham and Schnaar 1993; Kanda, Yamawaki et al. 1995), it may promote rolling in laminar flow as opposed to synapse formation. This ability of T cells with a high proportion of CD57 expressing cells could be assayed for their ability to roll in the presence and absence of CD57 blocking antibodies. Due to the fact that P0 (a protein expressed on the surface of Schwann cells) binds in a homophilic manner only when two interacting P0 molecules are glycolosated with the HNK-1 epitope (Filbin and Tennekoon 1991), CD57 may promote T cell-T cell interactions which may be involved in regulatory functions. If this is the case, blocking CD57 may enhance proliferation in response to GA restimulation.

XI. Conclusions

These studies present new data supporting the possibility that autoreactive T cells play a role in MS, and they provide new insight into the nature of this response. Although this understanding may not lead directly to a new therapy or cure it does open up several avenues of investigation that may lead in that direction. One, it is possible that a chronically stimulated response is substantially different from a normal memory response and many markers will be available to target this response and it may be possible to target these cell types individually, by either a blocking antibody or depleting these cells directly. Although this could be accomplished with CD57, this approach would suffer two problems. First, NK

cells would be blocked or inhibited. Another is that antibodies that block the HNK-1 epitope have been shown to inhibit hippocampus dependent memory formation when injected directly into the hippocampus of mice (Strekalova, Wotjak et al. 2001). A way around this would be to administer anti-CD8 and anti-CD57 antibodies that act together to kill any cell both antibodies are bound to but not any other cell type.

Nevertheless in addition to uncovering data that indicate CD28-CD57+ T cells are chronically stimulated pre-senescent cells I have shown a population of $CD8^{hi}$ myelin-reactive T cells may exist in MS patients with this phenotype. In addition to the characteristics I have demonstrated, upregulation of CD57, downregulation of CD28, release of IFN-γ, expression of perforin, and expression of CD25 when activated, these cells will likely display other characteristics of pre-senescent T cells as well. This includes an inability to divide, even in the presence of IL-2 (Perillo, Naeim et al. 1993), reduced expression of p21(CIP1) (Herbig, Jobling et al. 2004), and an inability to upregulate hsp70 (Effros, Zhu et al. 1994). However, senescent T cells also gain some functions and thus are not anergic. They may release TNF-α and IL-6 (Effros 2003), and importantly may still possess cytotoxic abilities (Perillo, Naeim et al. 1993). In addition they may be resistance to apoptosis, and have increased levels of Bcl2 (Campisi 2001) (Spaulding, Guo et al. 1999), although as we have seen this is controversial. Thus, an ongoing autoimmune response may differ substantially from an acute immune response, not because of autoimmunity but because of chronic stimulation driving T cells to pre-senescence. While the chronic stimulation may have resulted from a break in tolerance, a break in tolerance may also result from chronic

stimulation. This has been demonstrated to happen in response to homeostatic proliferation (King, Ilic et al. 2004) and as I have hypothesized may in other ways as well.

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Vitae

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