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Research Article

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Inhibition of T Cell Responses by Activated Human CD8+ T Cells is Mediated by Interferon- γ and is Defective in Chronic Progressive Multiple Sclerosis

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Abstract

The autologous mixed lymphocyte reaction (AMLR) involves the activation of T cells by autologous antigen presenting cells. Cells are generated during the course of the AMLR that have suppressive properties *in vitro*. In the present study we investigated the induction of CD8+ T cells in the AMLR with suppressive properties and the mechanism by which these cells downregulate *in vitro* proliferative responses. Purified CD8+ but not CD4+ T cells activated in the AMLR in conditioned medium inhibited proliferation of autologous T cells by anti-CD3 or PPD. Nonactivated CD8+ T cells did not suppress. The CD8+ T cells activated in the AMLR in the presence of conditioned medium (CD8+ Tact) were CD11b negative and were noncytotoxic. The inhibitory effect of CD8+ Tact cells was completely abrogated by anti-IFN- γ antibody, but not by anti-IL-4, anti-IL-10, or anti-TGF- β antibody. The induction of CD8+ Tact cells in the AMLR was blocked by anti-IL-2 or by anti-GM-CSF antibody and the combination of these two recombinant cytokines could support the induction of suppressive CD8+ Tact cells. CD8+ Tact cells were defective in patients with chronic progressive multiple sclerosis (MS) as compared to patients with relapsing-remitting MS or normal controls. Our studies provide a basis for understanding the mechanism of suppression by human CD8+ T cells in terms of specific cytokines, and demonstrate the potential importance of these cells in a human autoimmune disease as their function is defective in patients with progressive MS. (*J. Clin. Invest.* 1995; 95:2711–2719.) Key words: autologous mixed lymphocyte reaction • immune regulation • cytokine • central nervous system • autoimmunity

Introduction

Although a great deal is known about the heterogeneity and function of CD4+ T cells, less is known about CD8+ lymphocytes, especially CD8+ T cells that are noncytotoxic. CD8+ T cells appear to play an important role in immunoregulation of autoimmune diseases. This was most recently demonstrated in

studies of autoimmune encephalomyelitis in CD8+ deficient mice or animals treated with anti-CD8 monoclonal antibodies (1, 2). CD8+ deficiency led to an increased incidence of relapses and a more severe disease course. Although the mechanism by which cells actively suppress immune responses is not well understood, increasing evidence suggests that in many instances suppression is mediated by inhibitory cytokines. A number of inhibitory cytokines are produced by T cells and CD8+ T cells have been reported in murine models to be mediators of immune suppression via the secretion of TGF- β (3) or IL-10 (4, 5). IFN- γ has also been reported to be a suppressive cytokine secreted by T cells (6) and has suppressive effects on the immune system as shown in IFN- γ deficient mice (7) and in graft vs. host responses (8, 9). Other investigators have reported an inhibitory effect of IL-4 produced by *Mycobacterium leprae*-specific CD8+ T cells in humans (10).

Multiple sclerosis (MS)¹ is a chronic inflammatory disease of the central nervous system (CNS). Although the etiology and the pathogenesis of the disease is unknown, there is increasing evidence suggesting that MS is a T cell-mediated autoimmune disease associated with a variety of immune abnormalities that can be demonstrated in the peripheral immune compartment (11, 12). One hypothesis regarding the disease is that antigen nonspecific dysregulation of the immune system results in recurrent episodes in which activated myelin specific T cells migrate into the CNS and result in demyelination. A number of antigen nonspecific immune abnormalities have been described in MS, particularly in patients with the chronic progressive form of the disease. These abnormalities include a decrease of Concanavalin A, anti-CD3, and AMLR induced T cell-mediated suppression (13–17), and an impaired autologous mixed lymphocyte reaction (AMLR) (18, 19).

Given this background, we initiated a series of experiments to further delineate the mechanisms by which human CD8+ T cells mediate suppression of immune responses *in vitro* and to investigate whether these mechanisms were defective in patients with multiple sclerosis. In the present report, we describe the generation of activated CD8+ T cells in the AMLR and have found that: (a) IFN- γ mediates inhibition of human T cell responses by activated CD8+ T cells; and (b) Chronic progressive MS patients show a defect in the generation of such cells.

Methods

Reagents. Monoclonal antibodies reactive with human leukocyte surface antigens CD3 (T3, IgG1), CD4 (T4, IgG1), CD8 (T8, IgG1), CD11b (Mo1, IgM), CD56 (NKH-1A, IgG1), CD20 (B1, IgG2a) and control

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1. Abbreviations used in this paper: AMLR, autologous mixed lymphocyte reactions; CNS, central nervous system, MS, multiple sclerosis; PPD, purified protein derivative of tuberculin; Tact, activated T cells; Tcon, control T cells.

mouse IgG and IgM were purchased from Coulter Immunology (Hialeah, FL). These antibodies were FITC or PE conjugated or unlabeled. FITC-conjugated F(ab')² goat anti-mouse Ig was obtained from Tago Inc. (Burlingame, CA). The following monoclonal antibodies were purified from ascitic fluid: anti-CD3 (OKT3, IgG2a), anti-CD14 (MY-4, IgG2b), and anti-CD16 (3G8, IgG1) and were kindly provided by C. Morimoto (Dana Farber Cancer Institute, Boston, MA). For cell separation they were used after ultracentrifugation and for functional and phenotypic analysis they were purified using Affi-Gel protein A-MAPS II kit (Bio-Rad Labs., Richmond, CA). Goat anti-human IL-2, IL-4, GM-CSF, and TNF- α neutralizing Ab (IgG) and chicken anti-human TGF- β neutralizing Ab (Ig) were purchased from R&D Systems Inc. (Minneapolis, MN). Control normal goat IgG was purchased from CALTAG Labs. (South San Francisco, CA) and control chicken Ig from R&D Systems Inc. Mouse anti-human IFN- γ neutralizing mAb (IgG2a) was obtained from Genzyme Corp. (Cambridge, MA) and control normal mouse IgG2a from Coulter Immunology. Mouse anti-human IL-10 neutralizing mAb (IgG1) was purchased from Biosource International (Camarillo, CA) and control mouse IgG1 from Fisher (Pittsburgh, PA). Human rIL-2 was purchased from Immunex (Seattle, WA) or Boehringer Mannheim Corp. (Indianapolis, IN). Human rGM-CSF was kindly provided by Dr. J. Griffin (Dana-Farber Cancer Institute, Boston, MA). Human rINF- γ was purchased from GIBCO BRL (Gaithersburg, MD). Rabbit complement was obtained from Cedarlane Lab. Ltd. (Ontario, Canada) and from Pel-Freez Biologicals (Rogers, AR). Human T-STIMTM is an IL-2 containing human lymphocyte-conditioned medium from Collaborative Biomedical Products (Bedford, MA) that contains factors in addition to IL-2 that promote human T cell activation. It consists of supernatant from PHA-activated human mononuclear cells from which the PHA is then removed. Tetanus toxoid was obtained in purified form from Massachusetts Public Health Laboratory (Boston, MA) and PPD was purchased from Statens Serum Institute (Copenhagen, Denmark). INF- γ was measured by an ELISA kit from Biosource International.

Subjects. A group of MS patients characterized clinically as relapsing-remitting ($n = 15$) or chronic-progressive ($n = 30$) were studied. Relapsing-remitting patients had an average expanded disability status scale (EDSS) of 2.3 and chronic progressive patients, 5.5. Patients had not received immunosuppressive therapy in the past or steroid treatment in the 6 mo before blood drawing. The average age of chronic progressive patients was 48 ± 9.23 , of healthy donors was 44 ± 14.2 , and of relapsing-remitting patients was 35 ± 6.15 .

Cell Preparation. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Monocytes were isolated by adherence to plastic petri dishes for 1 h at 37°C in a CO₂ incubator (Falcon no. 3003; Becton Dickinson Labware, Lincoln Park, NJ) precoated with human AB serum (Sigma Immunochemicals, St. Louis, MO) followed by detachment of adherent cells with a cell lifter (Costar Corp., Cambridge, MA) as described (20). Monocytes were greater than 95% CD11b⁺ and less than 1% CD3⁺, CD20⁺, and CD56⁺. CD4⁺ and CD8⁺ T cells were separated from nonadherent PBMC by positive magnet separation with Dynabeads M-450 CD4 Detachabead and Dynabeads M-450 CD8 followed by detachment of Dynabeads from positively selected cells (DYNAL, Inc.; Great Neck, NY). The positively separated CD4⁺ and CD8⁺ T cells were > 97% CD4⁺, > 95% CD3⁺, < 2% CD8⁺ and > 97% CD8⁺, > 90% CD3⁺, < 2% CD4⁺, respectively. For isolation of T cells or T cell subsets by negative selection unfractionated PBMC were first separated into E rosette positive cells (E⁺) with 5% sheep red blood cells (Microbiological Assoc. Inc. Rockville, MD), as previously described (21). For purification of T cells or T cell subsets by negative selection, E⁺ cells were first incubated with anti-CD14 (MY-4) and anti-CD16 (3G8) antibodies with or without anti-CD4 or anti-CD8 antibodies. Negative separation was carried out with goat anti-mouse IgG-coated immunomagnetic beads (Advanced Magnetic Inc., Cambridge, MA), at a 10:1 bead to target cell ratio. The T cells obtained were > 97% CD3⁺, < 2% CD56⁺, < 2% CD20⁺, < 1% CD14⁺,

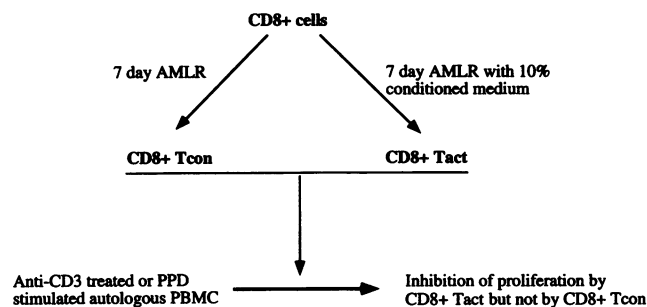


Figure 1. CD8⁺ T cells stimulated in the AMLR in the presence of conditioned medium inhibit anti-CD3- or PPD-induced proliferation (Schema).

< 5% CD11b⁺. For purification of CD4⁺ cells and CD8⁺ cells by negative selection additional anti-CD8 (21thy2D3) and anti-CD4 (19thy5D7) ascitic fluid was used (kindly supplied by C. Morimoto). Following negative selection, CD8⁺ cells were further enriched by C-dependent lysis with rabbit C (Pel-Freez Biologicals), as described (21). The purity of CD4⁺ and CD8⁺ T cells obtained by negative selection were: > 91% CD3⁺ (range 91–96%), > 90% CD4⁺ (range 90–94%), < 2% CD8⁺ and > 90% CD3⁺ (range 90–95%), > 82% CD8⁺ (range 82–90%), < 2% CD4⁺, respectively. Flow cytometric analysis was performed on an EPICS C flow cytometer (Coulter) according to standard procedures.

T cell activation in AMLR. AMLR cultures were performed in culture medium consisting of RPMI 1640 (Whittaker Bioproducts, Walkersville, MD) supplemented with 10% human AB serum (Sigma), 4 mM L-glutamine, 25 mM Hepes buffer, 50 U/ml penicillin, and 50 μ g/ml streptomycin (all from Whittaker Bioproducts). T cells or their subsets (1×10^5 cell/well) were cocultured with autologous irradiated (2,500 rad) monocytes (2×10^4 cell/well) with or without 10% conditioned medium (Human T-STIMTM) or selected cytokines in a total volume of 0.2 ml/well in 96-well flat-bottomed microtiter culture plates (Costar) at 37°C in a humidified atmosphere with 5% CO₂. For blocking experiments antihuman cytokine goat Ab (10 μ g/ml) or mouse mAb (5 μ g/ml) was added to wells at the beginning of the AMLR. On day 7 nonadherent cells were washed twice, resuspended in culture medium, and tested for their ability to inhibit T cell proliferation as described below. The separation of activated CD8⁺CD11b⁻ T cells was performed by incubation of 5×10^6 activated CD8⁺ T cells with 5 μ g/ml of anti-CD11b (Mo1, IgM) mAb for 30 min at 4°C followed by two washes and cell incubation in 50% rabbit complement (Cedarlane) at 37°C for 45 min. The purified activated CD8⁺CD11b⁻ T cells were > 95% CD3⁺, > 95% CD8⁺, < 2% CD4⁺, < 2% CD11b⁺.

Proliferation inhibition assay. For anti-CD3 induced proliferation, autologous purified T cells or autologous lymphocytes were treated with anti-CD3 (OKT3 at concentration of 1 μ g/ml) for 30 min at 4°C and washed two times. Anti-CD3-treated cells (1×10^5 cells/well) were mixed with autologous irradiated monocytes (2×10^4 cell/well) and placed in the wells of a conventional 96-well flat-bottomed microtiter culture plate (Costar) or in one series of experiments, in the top well of the transwell system (cat. no. 3413; Costar). Various numbers of AMLR-activated T cells (regulatory cells) were added directly to 96-well plates or to the bottom well of the transwell system and a 4-d proliferation assay was performed in the total volume of 0.2 ml (or 0.8 ml for transwell system). For the transwell an additional 5×10^4 autologous irradiated monocytes were also placed in the bottom well from the beginning of culture. For PPD, mumps or tetanus toxoid induced proliferation purified T cells or autologous resting lymphocytes were mixed with autologous irradiated monocytes (2×10^4 cell/well) and various numbers of AMLR-activated T cells (regulatory cells) and a 6-d proliferation assay was performed in 96-well round-bottomed microtiter culture plates (cat. no. 3799; Costar) with antigen (10 μ g/ml PPD, 5%

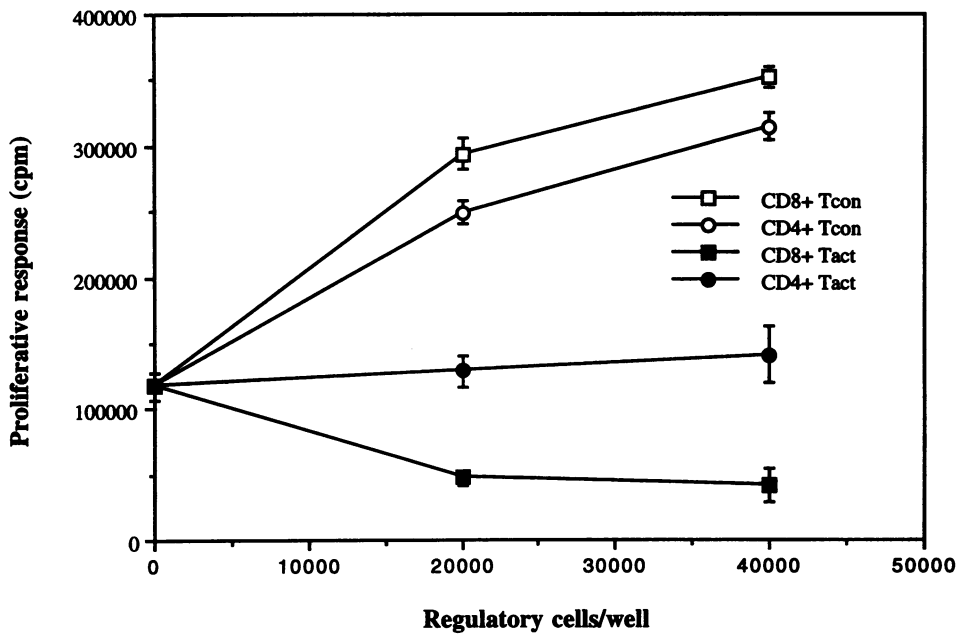


Figure 2. Activated CD8+ T cells inhibit anti-CD3-induced lymphocyte proliferative responses. CD4+ and CD8+ T cells were cultured in a 7-d AMLR without conditioned medium (*CD4+ Tcon* and *CD8+ Tcon*) or with 10% conditioned medium (*CD4+ Tact* and *CD8+ Tact*). The T cell subsets were then tested for their ability to suppress anti-CD3-induced proliferation of fresh autologous lymphocytes. The proliferative response (cpm) of responder cells grown with antigen-presenting cells alone = 480 ± 158 ; for CD8+ Tcon cells with antigen-presenting cells alone = 278 ± 66 ; and for CD8 Tact + antigen-presenting cells alone = 1039 ± 504 .

vol/vol mumps or 2.5 Lf Tetanus toxoid) in the total volume of 0.2 ml. For blocking experiments anti-human cytokine goat Ab ($10 \mu\text{g/ml}$) or mouse mAb ($5 \mu\text{g/ml}$) and the appropriate control Ig were added at the beginning of culture. For the last 16 h of culture, each well of 96-well plate or the top wells of transwell plates were pulsed with $1 \mu\text{Ci}$ of [^3H]dThd (New England Nuclear, Boston, MA) and then harvested on glass fiber filters. [^3H]dThd cell incorporation was measured by liquid scintillation counting. Percent of inhibition was calculated as $(1 - [\text{proliferation in the presence of regulatory cells}/\text{proliferation in the absence of regulatory cells}]) \times 100$. Results are shown as mean $\text{cpm} \pm \text{SD}$ of triplicate cultures from one experiment and are representative of at least three different experiments. Statistical significance was calculated using Student's *t* test.

Results

CD8+ T cells stimulated in the AMLR in the presence of conditioned medium inhibit anti-CD3 or antigen induced lymphocyte proliferation. CD8+ T cells purified by positive selection were stimulated in the AMLR in the presence or absence of conditioned medium (Human T-STIMTM), washed and then added to autologous T cells stimulated with anti-CD3 antibody. The scheme for this experiment is depicted in Fig. 1. As shown in Fig. 2, CD8+ T cells activated in the AMLR in the presence of conditioned medium (*CD8+ Tact*) significantly inhibited anti-CD3-induced proliferative responses of autologous T cells

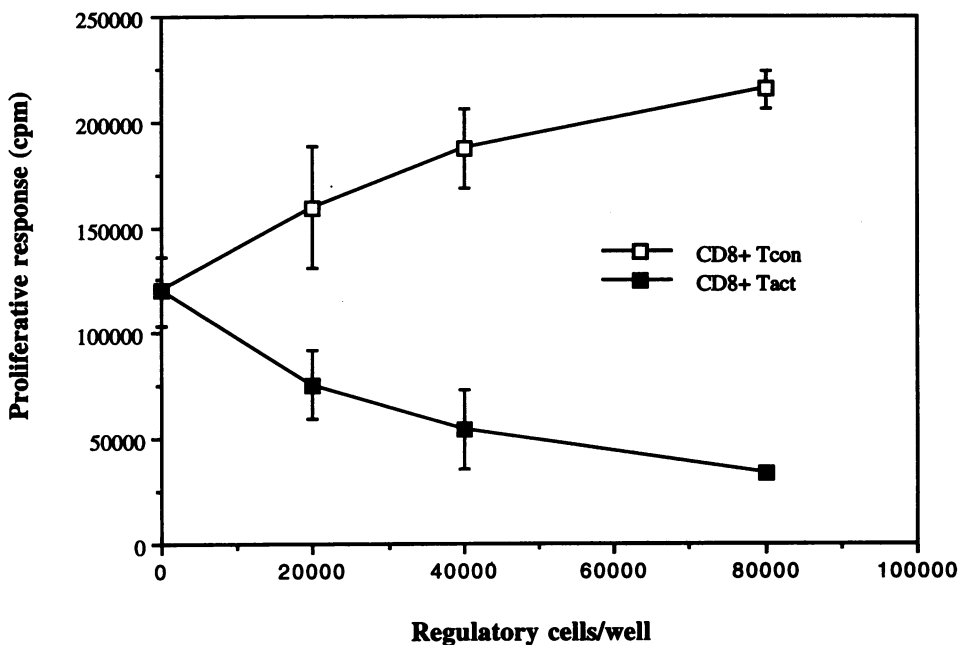


Figure 3. Activated CD8+ T cells inhibit PPD-induced proliferative responses. CD8+ T cells were cultured in a 7-d AMLR without conditioned medium (*CD8+ Tcon*), or with 10% conditioned medium (*CD8+ Tact*) and tested for their ability to suppress PPD induced proliferation of fresh autologous lymphocytes. The proliferative response (cpm) of CD8+ Tcon to PPD in the presence of antigen-presenting cells = 713 ± 202 ; for CD8+ Tact = 1454 ± 402 .

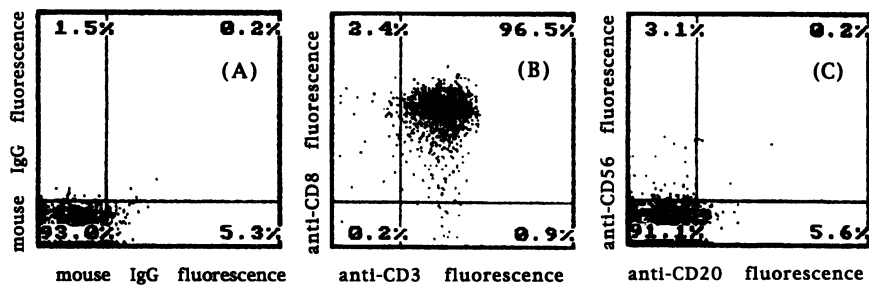


Figure 4. Phenotype of CD8+ Tact cells. The phenotype of CD8+ Tact cells from the 7-d AMLR was determined by direct staining. (A) Control PE-conjugated mouse IgG and FITC-conjugated mouse IgG; (B) PE-conjugated anti-CD8 mAb and FITC-conjugated anti-CD3 mAb; (C) PE-conjugated anti-CD56 mAb and FITC-conjugated anti-CD20 mAb.

whereas CD4+ cells stimulated in an identical fashion (CD4+ Tact) did not. Both CD4+ and CD8+ cells stimulated in the AMLR in the absence of conditioned medium (CD4+ Tcon, CD8+ Tcon) enhanced proliferative responses to anti-CD3. Freshly prepared, resting CD8+ T cells did not affect proliferation (not shown). CD8+ Tact cells also suppressed PPD-induced lymphocyte proliferation in a dose-dependent manner (Fig. 3) as well as mumps or tetanus toxoid-induced proliferation (not shown). Thus the inhibitory activity of CD8+ Tact cells was antigen-nonspecific. The CD8+ Tact cells were not cytotoxic (cytotoxicity less than 3%) to autologous or allogeneic monocytes or T cell blasts in a standard ^{51}Cr -release assay (20). FACS analysis of the CD8+ Tact cells showed them to be > 95% CD3+CD8+, < 2% CD56+, and < 2% CD20+ (Fig. 4). CD8+ Tact cells expressed activation markers CD25 and HLA-DR, and were larger than fresh CD8+ T cells or CD8+ cells stimulated in the AMLR without conditioned media. The percentage of CD11b+ cells in this population was less than 5%. Suppressor T cells have been reported to be both CD11b+ (22) and CD11b- (23). The removal of CD11b+ cells from the CD8+ Tact cell population did not affect the inhibitory activity in our system (not shown). To control for the effects of positive vs. negative selection of cells, we tested for suppression using both methods in the same experiment at 80,000 CD8+ Tact cells per well and obtained comparable results. Specifically, proliferation (CPM) of responder cells without regulatory cells = $122,830 \pm 16,420$; with CD8+ Tact cells obtained by positive selection = $33,415 \pm 3479$; with CD8+ Tact cells obtained by negative selection = $28,669 \pm 2428$.

IL-2 and GM-CSF induce CD8+ regulatory T cells in the AMLR. As shown in Fig. 1, human T cell conditioned medium

(Human T-STIMTM), a source of lymphokines produced by PHA-activated leukocytes, was required to induce regulatory CD8+ T cells in the AMLR. To determine which cytokines were required to induce CD8+ regulatory cells anti-human cytokine antibodies were added at the beginning of the AMLR. As shown in Fig. 5, anti-IL-2 and anti-GM-CSF Ab abolished the inhibitory effect of CD8+ Tact cells ($P < 0.01$). Although there is a suggestion that anti-IL-4 may reverse to some extent the inhibition by CD8+ Tact cells, this was not statistically significant or seen in repeat experiments ($n = 3$) whereas complete reversal by anti-GM-CSF or anti-IL-2 occurred in all experiments performed ($n = 3-5$). To determine whether these cytokines could induce CD8+ regulatory cells in the AMLR, T cells, CD4+, or CD8+ subsets were purified by negative selection and cultured in the AMLR with or without rIL-2 and rGM-CSF. Negative selection was used to control for any stimulatory effects that may have been generated by cross-linking of CD4 or CD8 by antibodies during positive selection. As shown in Figs. 6 and 7, only T cells or CD8+ T cells activated in AMLR with both rIL-2 and rGM-CSF suppressed proliferative responses, whereas CD4+ T cells did not. As with AMLR induced regulatory cells generated with conditioned medium, rIL-2 + rGM-CSF-induced T cells were > 95% CD3+, < 2% CD56+, < 2% CD20+, < 5% CD11b+ and noncytotoxic to autologous monocytes or T cell blasts (data not shown). 15 healthy individuals were tested for the generation of CD8+ Tact regulatory cells in the AMLR with conditioned medium, and in all instances regulatory cells were generated. The induction of CD8+ T cells with suppressive properties by rIL-2 + rGM-CSF cytokine was observed in three of six normal individuals tested and was reproducible in these individuals.

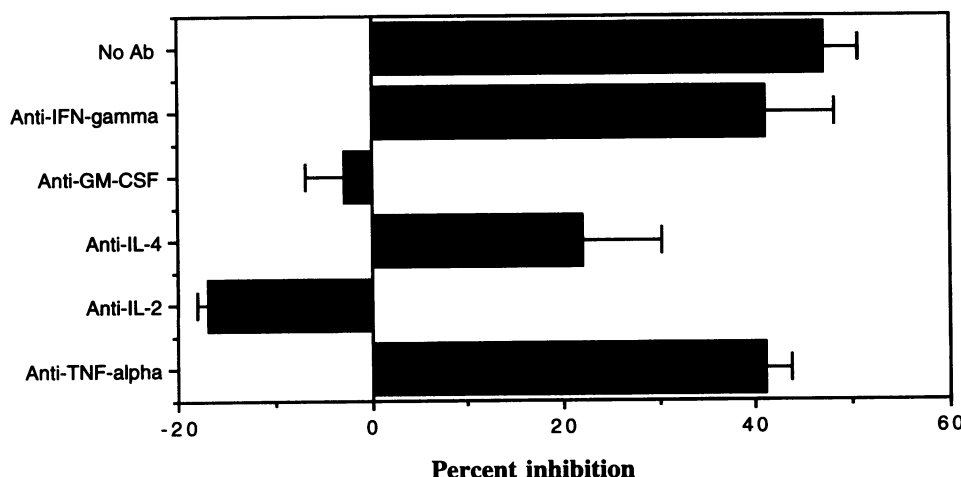


Figure 5. Anti-IL-2 and anti-GM-CSF Ab block induction of inhibitory activity in CD8+ Tact cells. CD8+ T cells were cultured in a 7-d AMLR with conditioned medium with or without various anti-cytokine Ab and tested for their ability to suppress anti-CD3-induced proliferation of fresh autologous lymphocytes at a concentration of 4×10^4 regulatory cells/well.

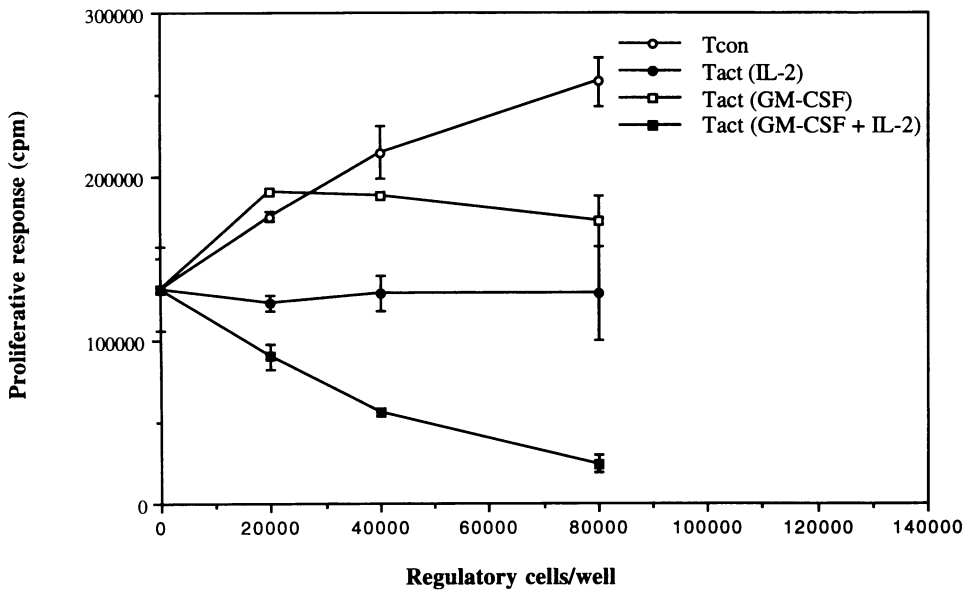


Figure 6. rIL-2 and rGM-CSF support induction of regulatory T cells in the AMLR. T cells were purified by negative selection and cultured in a 7-d AMLR without cytokines, (*Tcon*); with 20 U/ml of rIL-2, *Tact (IL-2)*; with 10 ng/ml of rGM-CSF, *Tact (GM-CSF)*; or with both IL-2 and GM-CSF; *Tact (GM-CSF + IL-2)*. These activated T cell subsets were then tested for their ability to suppress anti-CD3-induced proliferation of fresh autologous T cells.

Anti-IFN- γ blocks the suppressive effects of CD8+ Tact cells. To determine whether cell to cell contact was required for CD8+ Tact cells to inhibit proliferative responses, a transwell system was used in which anti-CD3-stimulated responder cells and CD8+ Tact cells were separated by a 0.4- μ m membrane. As shown in Fig. 8, significant suppression was observed in a dose-dependent fashion in the transwell system. In order to determine which cytokine(s) mediated the inhibitory effect, anti-cytokine antibodies were tested for their ability to abrogate suppression by CD8+ Tact cells of PPD responses. For these experiments, the effect of CD8+ Tact cells on PPD responses rather than OKT3 stimulation was measured since the OKT3 antibody is a mouse IgG2a. Anti-IFN- γ mAb is also a mouse IgG2a and thus would compete for the same Fc receptor on the surface of monocytes. As shown in Fig. 9, anti-IFN- γ mAb abolished the inhibitory effect of CD8+ Tact cells whereas no significant effect was observed with anti-IL-10, anti-IL-4, anti-TGF- β , or control mouse IgG2a. Anti-human IFN- γ mAb did not affect PPD-induced proliferation of lymphocytes cultured without CD8+ Tact cells.

IL-2 induces IFN- γ production by CD8+ Tact cells. We then investigated two pathways by which IFN- γ secretion can be stimulated, a pathway induced by stimulation of the TCR

and a pathway induced by IL-2. To investigate these two pathways, we incubated fresh CD8+ T cells, CD8+ Tcon cells or CD8+ Tact cells with rIL-2 or anti-CD3 mAb and then measured IFN- γ in the culture supernatants. As shown in Table I in the three normal subjects tested, rIL-2 induced significant amounts of IFN- γ production by CD8+ Tact cells (1882 \pm 680 pg/ml) whereas minimal amounts of IFN- γ were produced by CD8+ Tact cells stimulated by anti-CD3 (199 \pm 106 pg/ml), $P < 0.01$. CD4+ Tact cells also produced IFN- γ following IL-2 (428 \pm 100 pg/ml) or anti-CD3 (338 \pm 227 pg/ml) stimulation. The production of IFN- γ by IL-2- or anti-CD3-stimulated CD4+ Tcon cells was less than 100 pg/ml. The differences in production of IFN- γ by CD4+ Tact vs. CD4+ Tcon may explain the differential effect observed with these two populations in Fig. 2.

Chronic progressive multiple sclerosis patients have a defect in suppressive activity of CD8+ Tact cells. Defects in antigen-nonspecific suppression have been described in MS and primarily are seen in chronic progressive patients. We thus studied the immunoregulatory activity of purified CD8+ Tact cells generated in the AMLR from relapsing remitting and chronic progressive MS patients and controls. For these experiments, autologous monocytes and lymphocytes were frozen when cells were pre-

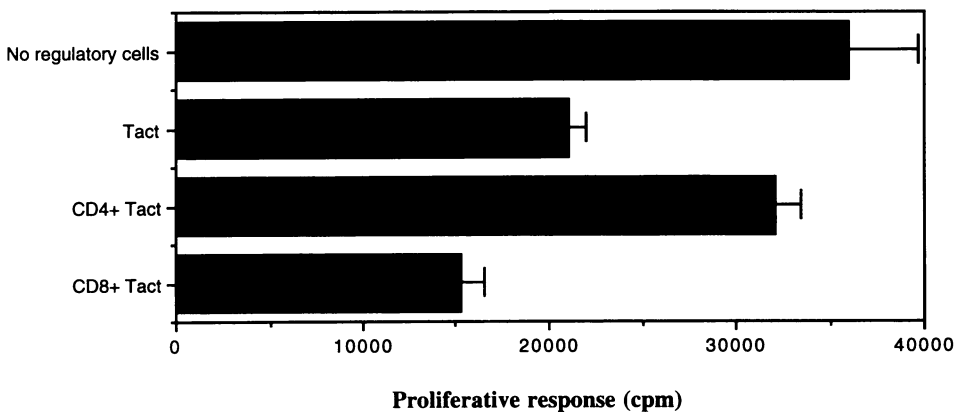


Figure 7. rIL-2 and rGM-CSF-activated CD8+ but not CD4+ T cells inhibit autologous T cell proliferative responses. T cells or T cell subsets were activated with both rIL-2 and rGM-CSF, and then tested for their ability to suppress anti-CD3-induced T cell proliferation at the concentration of 4×10^4 regulatory cells/well.

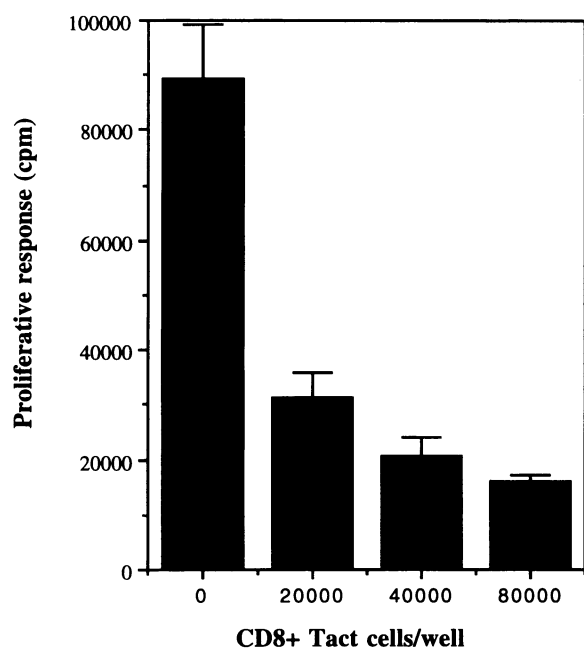


Figure 8. Inhibitory activity of CD8+ Tact cells in transwell culture. CD8+ Tact cells were placed in the bottom well of a transwell and autologous lymphocytes stimulated by anti-CD3 antibody in the top well. Proliferate responses in the top well were measured.

pared for the AMLR and then thawed for use as responder cells. As shown in Fig. 10, we found that CD8+ Tact cells from chronic progressive MS patients were defective in inhibiting anti-CD3–induced lymphocyte proliferation when compared with control donors, or relapsing remitting patients: average inhibitory activity = $54.1 \pm 15.6\%$ for normals, $15.9 \pm 47.3\%$ for relapsing-remitting patients and -79.4 ± 86.6 for chronic progressive MS patients ($P < 0.001$ control vs. CPMS; $P < 0.05$ control vs. RRMS; $P < 0.01$ RRMS vs. CPMS). These changes were seen equally in both male and female chronic progressive patients. We also calculated the average disability (EDSS) of the MS patients shown in Fig. 10 according to whether they inhibited proliferative responses. For MS patients ($n = 15$) with percent of inhibition > 0 , the average EDSS was 2.6 ± 0.6 ; for MS patients ($n = 18$) with percent of inhibition < 0 , the average EDSS was 5 ± 0.43 . The difference in EDSS is most probably related to the fact that

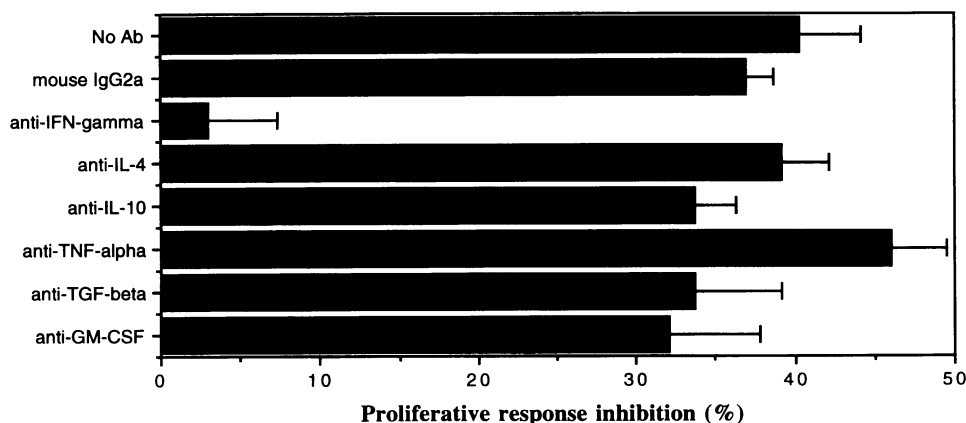


Figure 9. Anti-IFN- γ mAb abolishes the inhibitory effect of CD8+ Tact cells. CD8+ Tact cells were generated in a 7-d AMLR with 10% conditioned medium and then tested for their ability to suppress PPD-induced proliferation of fresh autologous lymphocytes in 96-well plates in the presence of anti-cytokine antibodies. Regulatory cells were added at a concentration of 2×10^4 regulatory cells/well.

Table 1. IL-2 Induces IFN- γ Production by CD8+ Tact Cells

Effector cells	Stimulation	IFN-gamma production			
		Exp. 1	Exp. 2	Exp. 3	Mean \pm SD
<i>pg/ml*</i>					
Fresh CD8+ T	None	< 16	< 16	< 16	< 16
Fresh CD8+ T	IL-2	< 16	< 16	< 16	< 16
Fresh CD8+ T	anti-CD3	17	< 16	< 16	< 16
CD8+ Tcon	None	17	94	94	68 ± 36
CD8+ Tcon	IL-2	110	71	59	80 ± 22
CD8+ Tcon	anti-CD3	76	73	62	70 ± 6
CD8+ Tact	None	112	98	39	83 ± 38.7
CD8+ Tact	IL-2	2357	2186	1103	$1882 \pm 680^\ddagger$
CD8+ Tact	anti-CD3	225	82	290	199 ± 106
CD4+ Tact	None	29	36	42	36 ± 5.3
CD4+ Tact	IL-2	479	502	287	428 ± 100
CD4+ Tact	anti-CD3	182	173	659	338 ± 227

* 10^5 fresh CD8+ T cells, or CD8+ Tcon, CD4+ Tact or CD8+ Tact cells generated in the AMLR were washed and then stimulated with OKT3 ($1 \mu\text{g/ml}$) or rIL-2 (50 U/ml) in the presence of 2×10^4 fresh autologous monocytes for 24 h and IFN- γ concentration in culture supernatants measured by ELISA. $^\ddagger P < 0.01$ compared to CD8+ Tact stimulated with anti-CD3 or unstimulated and $P = 0.02$ compared to CD4+ Tact stimulated with IL-2.

the defect in suppression is primarily seen in chronic progressive patients and such patients have a higher EDSS than relapsing-remitting patients. We then measured IL-2–induced IFN- γ production by CD8+ Tact cells generated in the AMLR in MS patients and found a significant decrease in IFN- γ production in chronic progressive MS when compared to healthy donors. As shown in Fig. 11, the average cytokine concentration in culture supernatants was $184 \pm 220 \text{ pg/ml}$ in progressive MS ($n = 12$) and $1733 \pm 703 \text{ pg/ml}$ in normal controls ($n = 6$) $P < 0.001$. To determine if IFN- γ added directly to PBMC from CP MS patients suppressed proliferation, recombinant IFN- γ was added to cultures. As shown in Fig. 12, anti-CD3–induced lymphocyte proliferation was inhibited by IFN- γ in a dose-dependent fashion.

Discussion

In the present study, we have found that stimulation of CD8+ T cells in the AMLR in the presence of conditioned medium

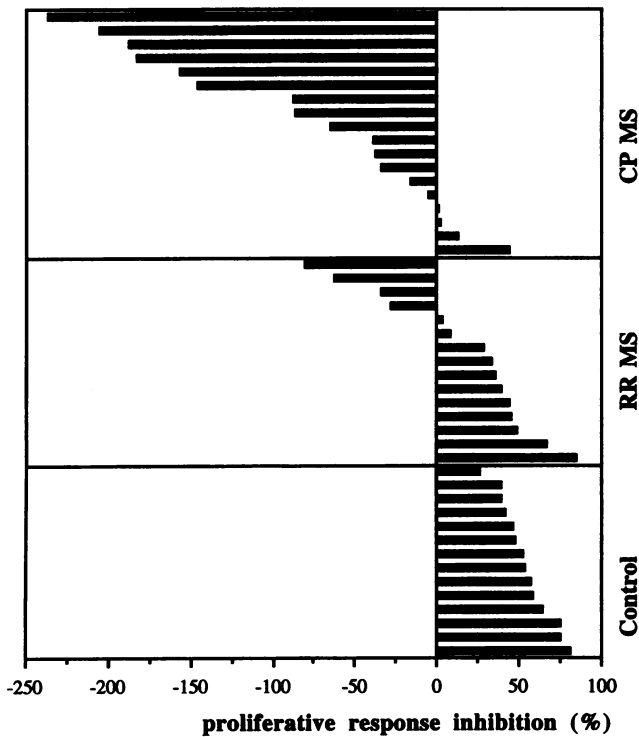


Figure 10. CD8+ Tact cell inhibition is defective in chronic-progressive MS. CD8+ Tact cells from healthy donors (*control*), relapsing-remitting (*RR*), and chronic-progressive (*CP*) MS patients were cultured in a 7-d AMLR with conditioned medium and then tested for their ability to suppress anti-CD3 induced proliferation of autologous lymphocytes in 96-well plates at the concentration of 4×10^4 regulatory cells/well.

inhibits anti-CD3- or PPD-induced proliferative responses of autologous lymphocytes in a dose-dependent fashion. These cells are noncytotoxic and their effect is mediated by IFN- γ . Yamada et al. have reported that the human CD8+ population can be divided into two subpopulations: CD11b+ and CD11b- (24). In our study the CD8+ Tact cells generated contained less than 5% CD11b+ cells and the removal of these CD11b+ cells did not affect the inhibitory activity of the CD8+ Tact cells. Takeuchi reported that CD8+ CD11b- cells generated in the allogeneic MLR suppressed pokeweed mitogen-induced IgG production (25). Others have reported that CD8+ cells induced by alloreactive CD4+ cells could suppress alloreactivity and were CD11b+ (22, 26).

Although the *in vivo* significance of the AMLR is not known, *in vitro* the AMLR predominantly generates suppression and there may be multiple mechanisms by which such suppression is induced. The effector function of such suppressor cells is not completely defined and may differ, depending on the system. The CD8+ regulatory cells generated in our system required the presence of conditioned medium and suppressed anti-CD3- and antigen-induced proliferation whereas in other systems CD8+ positive cells generated in the AMLR did not require conditioned medium and were shown to suppress pokeweed mitogen induced IgG synthesis (27). In this latter system, CD4+CD45RA+ cells were required to induce CD8+ suppressor cells and the mechanism by which suppression was mediated was not described. The relationship between the CD8+ Tact cells generated in our studies and AMLR-induced CD8 suppres-

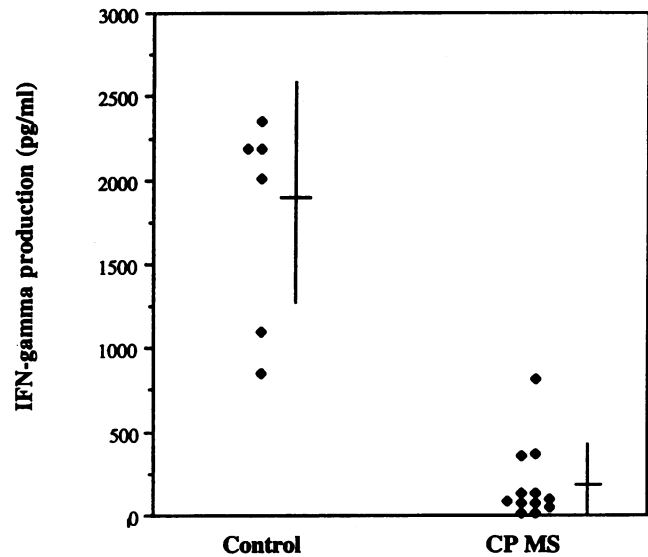


Figure 11. IFN- γ production by CD8+ Tact cells is defective in chronic-progressive MS. CD8+ Tact cells generated in the AMLR from healthy donors and chronic-progressive (*CP*) MS patients were tested for IL-2-induced IFN- γ production ($P < 0.001$, MS vs. control).

or cells described by Takeuchi et al. remains to be defined. Our studies provide a basis for further understanding CD8+ regulatory cells generated in the AMLR as they define activation requirements and effector function in terms of specific growth factors and cytokines.

There are different ways in which conditioned medium may function to allow the generation of CD8+ regulatory cells in

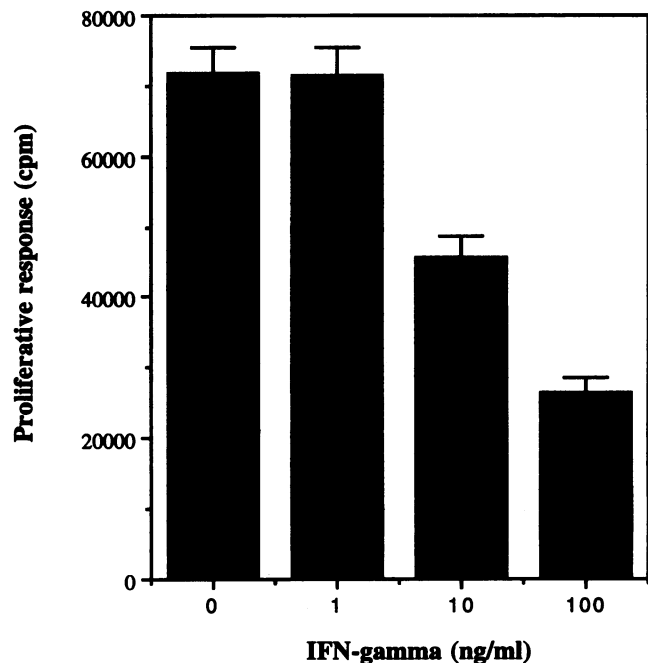


Figure 12. IFN- γ inhibits anti-CD3-induced proliferative responses. Anti-CD3-induced proliferation of fresh lymphocytes from CP MS was tested in the presence of different doses of IFN- γ added to cultures on day 0.

our system. Both IL-2 and GM-CSF were shown to play a role in activation of CD8+ regulatory cells. IL-2 is a classic T cell growth factor, whereas GM-CSF acts preferentially on antigen-presenting cells. GM-CSF increases MHC class II antigen expression and antigen-presenting function of antigen-presenting cells (28). GM-CSF also promotes differentiation of human dendritic cells (29). Thus, GM-CSF may function to enhance CD8+ T cell activation by antigen-presenting cells. CD4+ cells were not required to activate CD8+ T cells in our system, something which has not been shown before for the AMLR and which may be due to the addition of conditioned medium. It should be pointed out that we induced CD8+ regulatory cells with conditioned medium in all 15 donors tested, whereas IL-2 and GM-CSF induced CD8+ regulatory cells in three of six healthy individuals. In additional experiments we also found that 24 h (but not 4 h) supernatants from anti-CD3-treated PBMC also induced CD8+ Tact cells with suppressive function. Thus, in some instances, additional growth factors or culture conditions may be required. In another system, investigators have reported the induction of CD8+ suppressor cells which are generated in a pokeweed mitogen culture and suppress subsequent pokeweed mitogen-induced IgG secretion and proliferation (30). These investigators report that prostaglandin E₂ in combination with interferon- γ was required for differentiation of these CD8+ suppressor cells. They postulate that PGE₂ substitutes for monocytes and IFN- γ substitutes for CD4+ cells.

Although the mechanism by which suppressor T cells regulate immune responses has not been clearly defined (31) it now appears that a major mechanism involves the release of cytokines with suppressive properties. Such suppression may not require cell to cell contact and this has been shown in animal systems using a transwell system and has been termed "bystander suppression" (32). In this system, suppressor or regulatory cells from orally tolerized animals suppressed proliferative responses across the transwell by the release of cytokines such as TGF- β (3), and such suppression can be abrogated by anti-TGF- β antibody. T cell clones mediating this effect have also been described (9) and other suppressive cytokines that have been shown to suppress immune responses include IL-10 (4, 5) and IL-4 (10). TGF- β also appears to mediate natural suppressor activity of IL-2 activated lymphocytes (33, 34). In the present study, we have found that CD8+ Tact cells can mediate suppression across a transwell and anti-IFN- γ antibodies abrogate the suppression. It is known that IFN- γ has clear antiproliferative properties (7). Investigators have also reported that IFN- γ mediates suppression by antigen-specific T suppressor clones for BSA (6) and has an important role in immune suppression that develops in graft vs. host disease (9).

In addition to antigen or anti-CD3-induced IFN- γ production by T cells, IFN- γ can also be secreted by T cells or natural killer cells after activation with IL-2 or IL-12 (35–37). The antigen-mediated pathway is cyclosporin A sensitive whereas the IL-2 or IL-12 pathway is cyclosporin A resistant but requires unfixed HLA-DR+ accessory cells. In the present study we have found that CD8+ Tact cells secrete significant amounts of IFN- γ after IL-2 stimulation, but not after anti-CD3 stimulation. It was reported that IL-2-induced IFN- γ production by PBMC is decreased in MS patients approximately twofold (38). In our study, we found an even more significant defect of IFN- γ production by activated CD8+ T cells in CP MS. Of note is that some investigators have reported increased secretion of IFN- γ by fresh mononuclear cells from MS patients stimulated

with mitogen (39, 40). We have also found increased anti-CD3 induced IFN- γ production by fresh mononuclear cells in progressive MS (Balashov and Weiner, unpublished observation). The cells that produce increased IFN- γ in MS have not been defined. These observations appear separate from the specific defect of IL-2-induced IFN- γ production we have observed in CD8+ Tact cells generated in the AMLR.

Among its immunologic effects, IFN- γ may either enhance or suppress immune responses (41). IFN- γ is a potent activator of macrophages, induces class II MHC expression and the production of inflammatory cytokines such as IL-1. It can also act via macrophages to inhibit antigen-specific T cell proliferation perhaps through the release of nitric oxide radicals (42). The role of IFN- γ has also been studied in induction of experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis and has been used therapeutically to treat MS patients (43). In the EAE model, treatment of animals with IFN- γ reduces EAE and treatment with anti-IFN- γ monoclonal antibody exacerbates disease (44, 45). A similar effect of anti-IFN- γ monoclonal antibody was observed in the mouse model of experimental autoimmune uveoretinitis (46). Local administration of IFN- γ into the ventricular system of the CNS suppressed clinical signs of EAE (47). In vitro, IFN- γ acts on antigen presenting cells by enhancing their ability to activate T cell lines and acts on T cells themselves by inhibiting their response to antigen-pulsed antigen presenting cells. (48). In multiple sclerosis, however, intravenous treatment with IFN- γ resulted in exacerbation of disease within one month of infusions (43). Thus, if the defect in IL-2-induced IFN- γ release we have found in chronic progressive multiple sclerosis relates to the immunopathogenesis of the disease, we postulate that it relates to a defect of CD8+ cells to act locally and suppress T cell proliferative responses in the CNS or to inhibit generation of immune responses in the periphery. Our findings also raise the possibility that lymphocytes from MS patients are less sensitive to the inhibitory effect of IFN- γ .

A defect in antigen non-specific suppressor cell function in multiple sclerosis has been reported by a number of investigators, in which suppressor cells were stimulated with Concanavalin A, anti-CD3, or in the AMLR (13–17). Antel et al. reported that this defect primarily resides in the CD8+ population and found a decrease in suppression mediated by CD8+ T cell lines from patients with progressive MS (14). Our findings extend these results and provide basic information on the nature of the defect, although we have not directly tested CD8 cells from Concanavalin A-induced cultures as compared to the AMLR. Furthermore, at the present time we have no basis for knowing whether IFN- γ contributes to the previously described suppressor defects and whether they represent the same system. Of note is that we found that patients with relapsing remitting MS do not have this consistent defect. Why this defect develops in progressive MS and whether it is a primary or secondary immunologic finding is unknown. Nonetheless, the chronic progressive form of MS involves continual clinical progression without remission and the defect in CD8+ T cells we have described may account for the more malignant form of the disease.

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