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CD8$^+$ Myelin Peptide-Specific T Cells Can Chemoattract CD4$^+$ Myelin Peptide-Specific T Cells: Importance of IFN-Inducible Protein 10

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The demyelination process that occurs in the central nervous system (CNS) of patients with multiple sclerosis (MS) is due, in part, to an inflammatory response in which CD4$^+$ and CD8$^+$ T cells and macrophages infiltrate white matter. While it is thought that the inflammatory and demyelination process in MS is the product of Th1-associated cytokines secreted by CD4$^+$ myelin protein-specific T cells present in the CNS, the mechanisms that are responsible for the recruitment and maintenance of these myelin-reactive CD4$^+$ T cells in the CNS have not been elucidated. We have shown previously that CD8$^+$CTL that recognize peptides derived from sequences of the myelin proteolipid protein (PLP) presented by HLA class I molecules can be generated in vitro, and that these PLP-specific CD8$^+$CTL secrete the proinflammatory chemokines macrophage-inflammatory protein-1α and -1β, IL-16, and IP-10. In this study, we demonstrate that soluble products of these PLP-specific CD8$^+$CTL can chemoattract CD4$^+$ T cells that are specific for a myelin basic protein peptide and a PLP peptide, and that the majority of this chemotactic activity is mediated by IFN-inducible protein 10. These results demonstrate that PLP-specific CD8$^+$ T cells can play a role in the recruitment and retention of myelin-derived peptide-specific CD4$^+$ T cells, and indicate that they may play a proinflammatory role in the pathogenesis of MS. ***The Journal of Immunology,*** **1998, 160: 444–448.**

Multiple sclerosis (MS)[2] is the principal demyelinating disease in humans and is postulated to be caused by autoimmune mechanisms (1–3). The pathogenesis of MS involves the demyelination of axons within the central nervous system (CNS). The demyelination process appears to be the consequence of an inflammatory response in which CD4$^+$ and CD8$^+$ T cells and macrophages infiltrate white matter (1–3). One popular view is that the inflammatory and demyelination process in MS is the product of Th1-associated cytokines secreted by CD4$^+$ myelin protein-specific T cells present in the CNS (1–3). Some studies have indicated that there are elevated levels of myelin protein-specific CD4$^+$ T cells in the peripheral blood and cerebrospinal fluid of MS patients compared with normal controls (4, 5). However, the mechanisms that are responsible for the recruitment and maintenance of these myelin-reactive CD4$^+$ T cells in the CNS have not been elucidated.

We have shown previously that CD8$^+$ T cells can also recognize myelin protein-derived peptides presented by HLA class I molecules (6, 7). CD8$^+$CTL responses could be induced in vitro to myelin proteolipid protein (PLP)-derived peptides PLP80–88, presented by HLA-A2 (6), and PLP45–53, presented by HLA-A3 (7). PLP peptide-specific CD8$^+$CTL lines established in vitro from MS patients were shown to secrete a variety of proinflammatory mediators: macrophage inflammatory protein-1α (MIP-1α), MIP-1β, IL-16, and IP-10 (8). Interestingly, each of these CD8$^+$ T cell products is a chemokine that has been shown to be capable of chemoattracting CD4$^+$ T cells.

MIP-1α and MIP-1β are C-C chemokines that are synthesized by T cells, B cells, and monocytes (9), with the greatest levels being synthesized by the CD8$^+$ T cell subset (10). Both MIP-1α and MIP-1β have been shown to chemoattract CD4$^+$ T cells (11, 12). IL-16 (lymphocyte chemoattractant factor) (13) is produced by CD8$^+$ T cells and is chemotactic for CD4$^+$ T cells as well as CD4$^+$ monocytes and eosinophils (13). IP-10 is a C-X-C chemokine (14) that has been shown to be a chemoattractant for activated CD4$^+$ T cells, and enhances the adhesion of activated T cells to endothelial cells (15).

We hypothesize that myelin PLP peptide-specific CD8$^+$ T cells can actually chemoattract myelin peptide-specific CD4$^+$ T cells by secretion of one or more of these chemoattractant substances. In this study, we directly test this hypothesis in vitro, and show that such chemotactic activity for myelin peptide-specific CD4$^+$ T cells is contained in soluble products of these CD8$^+$ T cell lines, and further show that the majority of the chemotactic activity is mediated by IP-10.

Materials and Methods

Synthetic peptides

Peptides were synthesized and purified by HPLC, as previously described (16). Peptides MBP87–106 (VVHFFKNIVTPRTPPPSQGK) (17), PLP40–60 (TGTEKLIETYFSKNYQDYEYL) (18), PLP45–53 (KLI ETYFSK) (7), PLP80–88 (FLYGALLLA) (6), and human cytomegalovirus gB 613–628 (PSLKIFIAGNSAYEYV) (19) were dissolved in stock solutions of PBS/50% DMSO at 1 mg/ml.

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[2] Abbreviations used in this paper: MS, multiple sclerosis; CNS, central nervous system; IP-10, interferon-inducible protein 10; MBP, myelin basic protein; MIP, macrophage-inflammatory protein; PLP, proteolipid protein.

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Generation and characterization of T cell lines

The generation of CD8$^+$CTL lines specific for PLP80–88 plus HLA-A2 and PLP45–53 plus HLA-A3 by our laboratory was previously described using PBL from two MS patients (6,7). These CTL lines are designated PLP80–88.1–3 and PLP45–53.1–4. The CD4$^+$ T cell line GD22 specific for MBP87–106 was generated from the PBL of an MS patient, as previously described (17). Two CD4$^+$ T cell lines specific for PLP40–60, designated PLP40–60.1 and PLP40.60.2, were derived from the PBL of an MS patient, as described (18).

Generation and quantitation of chemokines

CD8$^+$CTL lines were used for chemokine generation by thawing frozen stocks and stimulating them for 5 to 7 days with peptide-pulsed PBL (6, 7). CTL lines were cultured in 96-well round-bottom plates at 5×10^4 cells/well. APCs were HLA-A2- or HLA-A3-transfected Hmy2.C1R cells (20) that were treated with mitomycin C (200 μg/ml; Sigma Chemical Co., St. Louis, MO) and pulsed (1 μM) with the appropriate peptides for 2 h at 37°C. APCs were then washed three times with PBS and added to wells at 10^5 cells/well. Culture medium was Iscove's modified Dulbecco's medium (Life Technologies, Gaithersburg, MD) with 5% heat-inactivated FCS. Supernatants were collected 48 h later and stored at −70°C until assayed. Supernatants obtained from mitomycin C-treated Hmy2.C1R cells cultured alone had no detectable chemokines, as detected in sandwich ELISA assays or in chemotaxis assays (see below).

Quantitation of chemokines was performed by sandwich ELISA. Each CTL line was tested in at least two independent experiments. MIP-1α and MIP-1β assays were performed as described by the manufacturer (R&D Systems, Minneapolis, MN). IL-16 ELISA assays were performed as previously described (13). IP-10 ELISA assays were performed with biotinylated and nonlabeled polyclonal goat anti-human IP-10 (R&D Systems) similar to previously described techniques (15). The levels of sensitivity of the ELISA assays were as follows: IP-10 = 50 pg/ml; IL-16 = 1 pg/ml; MIP-1α and MIP-1β = 15 pg/ml.

The capacity of supernatants from CD8$^+$ T cells to chemoattract CD4$^+$ T cells was quantitated by a chemotaxis assay using Transwell plates (Costar, Cambridge, MA). Transwell plates (Costar 3421) contained polycarbonate inserts with 5-μm pores. Transwells with inserts were loaded with 1 ml of Iscove's modified Dulbecco's medium with 0.25% human serum albumin (chemotaxis assay medium) for 1 h at 37°C, 5% CO_2. Wells were then aspirated and loaded with 0.8 ml of supernatants or control medium and incubated for 20 min at 37°C, 5% CO_2. CD4$^+$ T cells (5×10^5 in chemotaxis assay medium) in 100 μl were added to the insert well, and the Transwell plates were incubated at 37°C, 5% CO_2 for 1 h. Cells present in the lower chamber were then collected and counted using a FACScan (Becton Dickinson, San Jose, CA) for 1 min with a constant flow rate of 67 μl/min. Each sample was counted three times, and the results are expressed as the average and SD of the three counts. Results reported as percentage of cell migration were calculated as the mean percentage of cell migration of indicator cells relative to the migration in the presence of chemotaxis assay medium alone (normalized to 100% in all experiments). Statistical comparisons of the significance of cell migration data were performed by Student's *t* test, assuming unequal variances.

In experiments in which chemokine-specific neutralizing antisera were used to inhibit the chemotactic activity present in supernatants, 0.8 ml of supernatants plus antisera were mixed and added to the Transwell plates and incubated for 30 min at 37°C, 5% CO_2. Indicator cells were then added to the inserts, and the Transwell plates were incubated for 1 h at 37°C, 5% CO_2. The chemokine-specific neutralizing antisera were as follows: anti-IP-10 = goat anti-human rIP-10 (15), neutralization capacity = 5 μg/ml, neutralizes 1,000 pg/ml IP-10, used at 50 μg/ml; anti-IL-16 = rabbit anti-human rIL-16 (13), neutralization capacity = 5 μg/ml, neutralizes 8,000 pg/ml IL-16, used at 5 μg/ml; anti-MIP-1α = goat anti-human rMIP-1α (R&D Systems), neutralization capacity = 5 μg/ml, neutralizes 8,000 pg/ml, used at 5 μg/ml; anti-MIP-1β = anti-human rMIP-1β (R&D Systems), neutralization capacity = 5 μg/ml, neutralizes 20,000 pg/ml, used at 5 μg/ml; and anti-mouse IgG = goat anti-mouse IgG (21), used at 50 μg/ml.

Human rIP-10 was purchased from PeproTech (Rocky Hill, NJ). Chemokine receptor 5-specific mAb 5C7 (22) was obtained through AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health from LeukoSite (Cambridge, MA).

Results

Specificity of myelin protein-specific CD4$^+$ T cell lines

The specificity of the CD4$^+$ T cell line GD22 for MBP87–106 presented by HLA-DR2 has been previously reported (17). Presentation of PLP40–60 by HLA-DR4 to CD4$^+$ T cell lines has also been reported (18). We have generated two such PLP40–60 CD4$^+$ T cell lines (designated PLP40–60.1 and PLP40–60.2) and have determined that each can secrete IFN-γ when stimulated with APC pulsed with PLP40–60, but not with an irrelevant peptide (human cytomegalovirus gB 613–628) or no peptide (data not shown). Neither T cell line could secrete IL-4 or IL-10 when stimulated with PLP40–60 (data not shown), indicating that these T cell lines express the Th1 phenotype.

Table I. *Chemokine production by CD8$^+$ PLP-specific T cell lines*[a]

T Cell Line	IP-10 (pg/ml)	IL-16 (pg/ml)	MIP-1α (pg/ml)	MIP-1β (pg/ml)
PLP80-88.1	130	63	2505	590
PLP80-88.2	60	51	2299	3240
PLP80-88.3	10,900	32	300	650
PLP45-53.1	10,300	31	1550	2420
PLP45-53.2	5,700	41	1410	3130
PLP45-53.3	17,200	42	1198	2130
PLP45-53.4	8,300	5	2674	1690

[a]Results are means of duplicate determinations.

Chemokine production by CD8$^+$ PLP-specific CTL lines

The characterization of CD8$^+$CTL lines that are specific for PLP80–88, presented by HLA-A2, and PLP45–53, presented by HLA-A3, has been previously reported (6, 7). Three PLP80–88-specific CTL lines (designated PLP80–88.1–3) and four PLP45–53-specific CTL lines (designated PLP45–53.1–4) were selected and expanded for these studies. Supernatants from each CTL line were generated by stimulation with peptide-pulsed APC for 48 h and assayed for the presence of IP-10, IL-16, MIP-1α, and MIP-1β by sandwich ELISA. The results (Table I) show that each of these CTL lines secreted detectable quantities (generally in the pg/ml range) of each of these chemokines, although the levels varied widely. IL-16 was produced in far less amounts compared with IP-10, MIP-1α, or MIP-1β. No detectable chemokines were produced by APCs cultured alone (data not shown).

CD4$^+$ T cell chemotaxis induced by products of CD8$^+$ PLP-specific CTL lines

Secretory products of PLP80–88- and PLP45–53-specific CTL lines were assayed for their ability to induce chemotaxis of CD4$^+$ T cell lines GD22 (MBP87–106) and PLP40–60.1 and .2. Representative results are presented in Figure 1.

Each of the CD8$^+$PLP-specific CTL lines produced substances that could induce migration of the CD4$^+$MBP peptide-specific T cell line GD22 (Fig. 1*A–D*) and the PLP40–60.1 (Fig. 1*E–G*) and PLP40–60.2 (Fig. 1, *H* and *I*) T cell lines. The activity present in undiluted supernatants induced cell migration of greater than 180% of control, with most CD8$^+$PLP-specific CTL lines producing 300% cell migration. The chemotactic activity was dose dependent, as shown by the effect of diluting each of the supernatants (data not shown). These results demonstrate that CD8$^+$PLP-specific CTL lines secrete factors that can induce chemotaxis of CD4$^+$ MBP and PLP peptide-specific T cells.

Identification of CD8$^+$ T cell products that are chemotactic for CD4$^+$ T cells

Neutralizing antisera that are specific for IP-10, IL-16, MIP-1α, and MIP-1β were assayed for their ability to inhibit the chemotactic activity produced by representative CD8$^+$ T cell lines. The

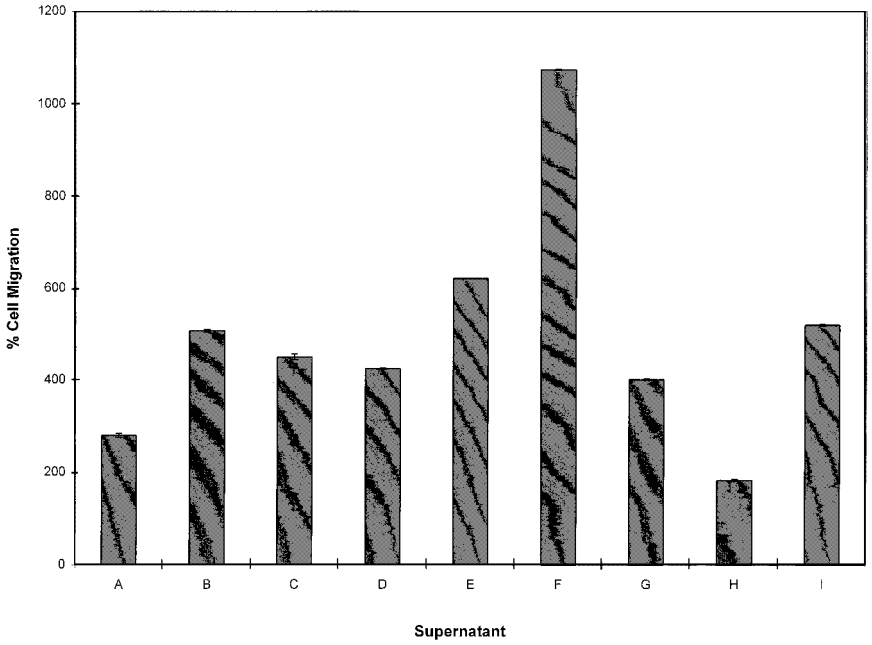

FIGURE 1. Soluble products of PLP-specific CD8+ T cells are chemotactic for MBP- and PLP-specific CD4+ T cells. The PLP-specific CTL lines used for supernatant generation are: *A*, PLP80–88.1; *B*, PLP80–88.2; *C*, PLP45–53.1; *D*, PLP45–53.2; *E*, PLP80–88.3; *F*, PLP45–53.3; *G*, PLP45–53.4; *H*, PLP80–88.1; and *I*, PLP45–53.2. In *A–D*, the MBP87–106 CD4+ T cell line GD22 was used as the indicator cell for chemotaxis; in *E–G*, the PLP40–60 CD4+ T cell line PLP40–60.1 was used; and in *H* and *I*, PLP40–60.2 was used as the indicator cell line. Values on the *y*-axis represent the mean percentage of cell migration of triplicate determinations in one experiment induced by undiluted supernatants, and the error bars are the SD of these triplicates.

Table II. *Neutralization of PLP80-88.3 chemotactic activity*[a]

Neutralizing Antisera	Exp. 1, Inhibition of Cell Migration (%)	Exp. 2, Inhibition of Cell Migration (%)	Exp. 3, Inhibition of Cell Migration (%)
Anti-IP-10	57 ($p = 0.00017$)[b]	36 ($p = 0.0008$)	59 ($p = 0.002$)
Anti-IL-16	21 ($p = 0.0045$)	21 ($p = 0.007$)	17 ($p = 0.029$)
Anti-MIP-1α	0	6 ($p = 0.47$)	10 ($p = 0.41$)
Anti-MIP-1β	5 ($p = 0.06$)	11 ($p = 0.25$)	7 ($p = 0.21$)

[a]CD4+ T cells in experiments 1 and 2 were PLP40-60.1 and in experiment 3 were GD22. Percent cell migration values in the absence of added antibodies were: experiment 1, 1116%; experiment 2, 381%; and experiment 3, 677%.
[b]*p* values represent comparisons to % cell migration in the absence of antibody.

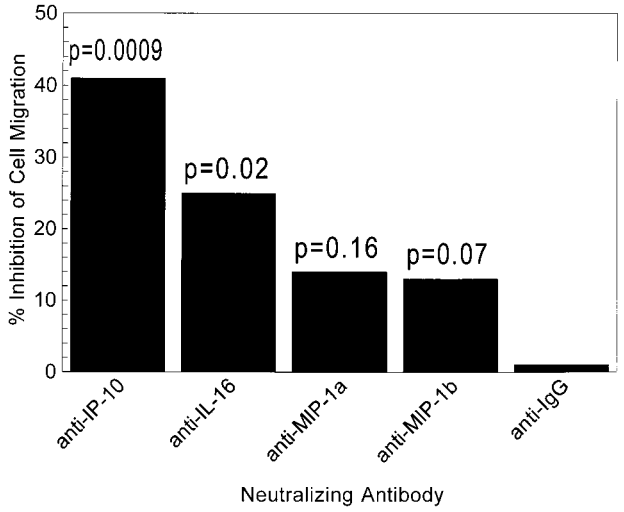

FIGURE 2. Ab neutralization of the chemotactic activity of soluble mediators secreted by PLP45–53.4. The supernatant from PLP45–53.4 was incubated with the indicated Abs. The indicator CD4+ T cell line GD22 specific for MBP87–106 was then added, and chemotactic activity was quantitated. Results are expressed as percentage of inhibition of cell migration. *p* values represent comparisons with percentage of cell migration in the absence of Abs.

neutralizing capacity of each of the antisera is provided in *Materials and Methods*. Each of the antisera was used in the chemotaxis assay at neutralizing concentrations that exceeded the concentration of the relevant chemokine in the supernatant. The results of three experiments are shown in Table II. Little or no inhibition of chemotactic activity was produced by anti-MIP-1α or anti-MIP-1β. Anti-IL-16 produced 17 to 21% inhibition, while anti-IP-10 inhibited 36 to 59% of the chemotactic activity. These results demonstrate that the majority of the chemotactic activity for CD4+ T cells that is produced by the CD8+CTL line PLP80–88.3 is mediated by IP-10.

Similar analyses of the identity of the chemotactic agent present in the supernatant of CD8+CTL line PLP45–53.4 were performed. One representative experiment of three performed is shown in Figure 2 using MBP-specific GD22 CD4+ T cells as indicator cells. The results are remarkably similar to the results obtained with the PLP80–88.3 CTL line: anti-IP-10 inhibited by 41%, followed by anti-IL-16 with 25%, followed by anti-MIP-1α and anti-MIP-1β with 14% and 13% inhibition, respectively. Again, these results indicate that the majority of the chemotactic activity for the CD4+ T cells produced by these CD8+ T cells is mediated by IP-10.

The inability to attribute any significant chemotactic activity for the CD4+ T cells to either MIP-1α or MIP-1β was not due to a lack of expression of an appropriate cell surface chemokine receptor on these T cells, because they showed positive cell surface staining for chemokine receptor 5 using the 5C7 mAb (22) (data not shown).

Direct demonstration that IP-10 can induce chemotaxis of CD4+ myelin peptide-specific T cells

Purified human rIP-10 was assessed for its ability to induce chemotaxis of PLP40–60.1 CD4+ T cells. The results (Fig. 3) demonstrate that rIP-10 induced detectable chemotactic activity of PLP40–60.1 T cells in the range of 100 to 10 ng/ml. Similar levels of percentage of cell migration were produced by undiluted supernatants from CD8+ T cell lines PLP80–88.3, PLP45–53.1, and PLP45–53.4 (Fig. 3). Similar results were observed for induction of chemotactic activity by MBP-specific GD22 CD4+ T cells (data

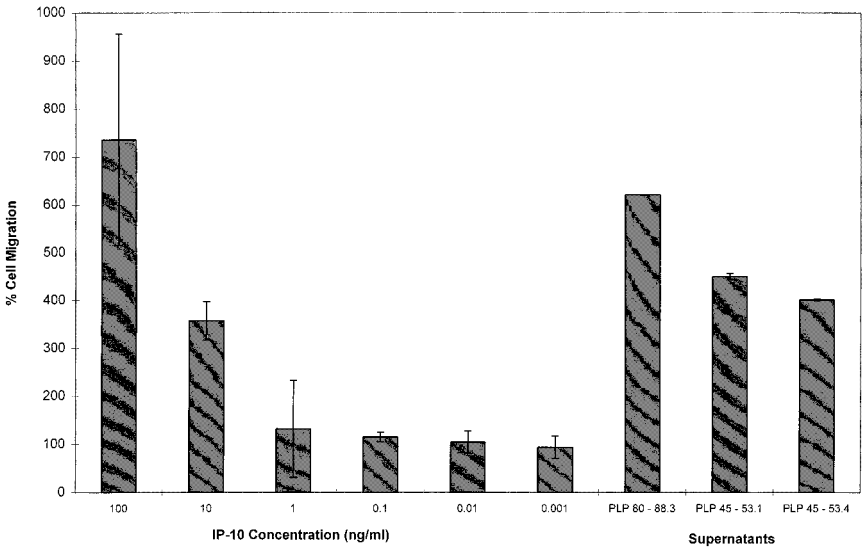

FIGURE 3. IP-10 can directly induce chemotaxis of $CD4^+$ myelin peptide-specific T cells. PLP40.60.1 T cell line was tested in the chemotaxis assay with the indicated concentrations of human rIP-10 or the indicated undiluted supernatants.

not shown). These results directly demonstrate that IP-10 alone can induce chemotaxis of $CD4^+$ myelin peptide-specific T cell clones.

Discussion

The major findings described in this study are that PLP peptide-specific $CD8^+$CTL lines produce soluble mediators that can chemoattract $CD4^+$ myelin-derived peptide-specific T cells, and that the majority of this chemotactic activity is mediated by IP-10. Unlike most other C-X-C chemokines, IP-10 has no demonstrable activity on neutrophils, but specifically targets lymphocytes (9, 15). The receptor for IP-10 has been shown to be present on activated, but not resting T cells (23). IP-10 has been shown to be a chemoattractant for activated $CD4^+$ T cells in vitro, and to induce adhesion of activated T cells to HUVEC (15). In vivo, IP-10 has been shown to promote migration of human T lymphocytes into the peripheral tissues of human PBL-SCID mice (24), and IP-10 has been shown to be present at sites of delayed-type hypersensitivity responses (25). IP-10 was also the chemokine that was generally present at the highest concentration in our $CD8^+$ T cell supernatants relative to IL-16, MIP-1α, and MIP-1β. However, there are significant problems associated with such quantitative comparisons due to different sensitivities of the ELISA assays used to quantitate these chemokines.

The observation that anti-IL-16 Abs could produce partial inhibition of $CD4^+$ T cell chemotaxis is consistent with the documented capacity of IL-16 to be chemotactic for human $CD4^+$ T cells (13). The failure to inhibit any significant chemotaxis with anti-MIP-1α and anti-MIP-1β antisera may be due to a requirement for the presence of extracellular matrix proteins, which were not in our chemotaxis assay, to facilitate optimal T cell movement induced by these two chemokines (11).

There is a wide variety of evidence to suggest that myelin peptide-reactive $CD4^+$ T cells are involved in the pathogenesis of MS (1–3), and certain studies have shown that there are elevated levels of myelin protein-specific T cells in the peripheral blood and cerebrospinal fluid of MS patients compared with normal controls (4, 5). However, it remains unclear where these $CD4^+$ T cells initially are stimulated by Ag and how they are recruited into and maintained within the CNS. $CD4^+$ T cells recognize peptide Ags presented by class II MHC molecules (26), but the myelin-producing human adult oligodendrocytes do not express class II MHC molecules, even when stimulated by IFN-γ (27). In contrast, $CD8^+$ T cells recognize peptide Ags presented by class I MHC molecules (28), and these class I molecules are expressed constitutively on adult oligodendrocytes (27). Thus, one possible scenario for the initial recruitment of myelin protein-specific $CD4^+$ T cells is that both $CD4^+$ and $CD8^+$ T cells are stimulated by myelin peptides and/or mimicry peptides (29) outside the CNS. Following a variety of insults to and/or infections of the CNS, which result in temporary breakdown of the blood-brain barrier, primed $CD8^+$ T cells then circulate through the CNS and their TCRs are engaged by myelin protein-derived peptide Ags that are presented by the class I molecules on oligodendrocytes. It has been demonstrated recently that myelin peptide-specific $CD8^+$ T cells can specifically recognize oligodendrocytes (30). These activated myelin peptide-specific $CD8^+$ T cells then secrete a variety of chemokines and cytokines (30), including IP-10, which can chemoattract activated myelin peptide-specific $CD4^+$ T cells into the CNS. A subset of these activated $CD4^+$ T cells may secrete TNF-α, which can directly injure oligodendrocytes (31).

The classical view of the functions of $CD8^+$ T cells is that they are either cytotoxic or suppressor cells (32). Recent results (33, 34), along with those in the present study, that have demonstrated that $CD8^+$ T cells secrete a large arsenal of cytokines and chemokines that have long-range biologic effects indicate that this classical view needs to be expanded significantly to include proinflammatory functions for $CD8^+$ T cells.

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