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Functional characterization of mouse spinal cord infiltrating CD8⁺ lymphocytes

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Abstract

Understanding the immunopathogenesis of neuroimmunological diseases of the CNS requires a robust method for isolating and characterizing the immune effector cells that infiltrate the spinal cord in animal models. We have developed a simple and rapid isolation method that produces high yields of spinal cord infiltrating leukocytes from a single demyelinated spinal cord and which maintains high surface expression of key immunophenotyping antigens. Using this method and the Theiler's virus model of chronic demyelination, we report the presence of spinal cord infiltrating acute effector CD8⁺ lymphocytes that are CD45^{hi}CD44^{lo}CD62L[−] and a population of spinal cord infiltrating target effector memory CD8⁺ lymphocytes that are CD45^{hi}CD44^{hi}CD62L[−]. These cells respond robustly to ex vivo stimulation by producing interferon γ but do not exhibit specificity for Theiler's virus in a cytotoxicity assay. We conclude that target-derived lymphocytes in a mouse model of chronic spinal cord demyelination may have unique functional specificities.

Keywords

CD8⁺ T cells; multiple sclerosis; interferon γ ; cell analysis; lymphocytes; flow cytometry; qRT-PCR

1. Introduction

Understanding the cells and molecules that mediate immunopathology in the central nervous system (CNS) is key to the rational discovery and design of therapeutic interventions for such diseases as multiple sclerosis (MS) (Ransohoff et al., 2003). An important component of this discovery process is a reproducible and sensitive method for isolating immune cells from the CNS in a manner that preserves phenotype and functionality. Using the Theiler's

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Competing Interests

The authors declare that they have no competing interests.

Author Contributions

CD executed all experiments, participated in experimental design and interpretation, and helped to draft the manuscript. CLH participated in experimental design and interpretation and helped to draft the manuscript. Both authors read and approved the final manuscript.

murine encephalomyelitis virus (TMEV) model of MS (Rodriguez, 2007), we have developed a rapid and simple method to isolate an enriched population of leukocytes from the demyelinated spinal cord, a tissue that is complicated by the high proportion of lipid-rich myelin. In contrast to previously published methods (Huang et al., 2001; Johnson et al., 1999; Klein et al., 2005; Lane et al., 2000), our preparation produces infiltrating cells exclusively from the spinal cord of a single animal, rather than pooled brain and spinal cord cells or cells pooled from multiple animals. In addition, our method isolates infiltrating cells from the chronically demyelinated spinal cord, rather than the acutely infected CNS (Huang et al., 2001; Klein et al., 2005). The spinal cord infiltrating leukocytes (SCILs) isolated by our non-enzymatic procedure are competent for ex vivo manipulation and for analysis of phenotypic markers by flow cytometry and qRT-PCR.

2. Materials and Methods

Animals and disease model

Male B10.D1-H2q/SgJ (B10Q) mice were produced in our breeding colony. These mice are of the H-2^q MHC haplotype. Animals were infected intracranially at 4–6 weeks of age with 2×10^5 PFU of the Daniel's strain of TMEV, as previously published (Deb and Howe, 2008). This susceptible strain develops characteristic spinal cord demyelination between 21 and 45 days postinfection (dpi). By 90 dpi all infected mice exhibit motor deficits, extensive inflammatory infiltrates, and demyelinated lesions in the spinal cord. SCILs and peripheral blood mononuclear cells (PBMCs) were collected from mice at 90 dpi for analysis. All experiments were performed according to the National Institutes of Health guidelines and were approved by the Mayo Clinic institutional animal care and use committee.

Isolation of SCILs

Non-enzymatic method—Mice were euthanized with sodium pentobarbital (150 mg/kg i.p.) and transcardially perfused with 50 mL 4°C PBS (0.9% NaCl in 0.1 M phosphate buffer at pH 7.4). The spinal column was excised into RPMI 1640 (Invitrogen, www.invitrogen.com) and the spinal cord was removed by insufflation using 20 mL 4°C RPMI expressed through a 19 gauge needle. The spinal cord was transferred to 10 mL fresh, cold RPMI and homogenized with 5 strokes in a Tenbroeck tissue grinder (Wheaton 2 mL, 0.09–0.16 mm gap). The resulting cell suspension was gravity sieved through a 40 µm strainer (BD Bioscience #352340; www.bdbiosciences.com) to remove large aggregates. Cells were pelleted by centrifugation at 900 g for 5 min at 4°C (1800 rpm; Beckman GH-3.8 rotor) and the supernatant was discarded. The cells were resuspended in 1 mL 70% Percoll (Amersham #17-0891-02; www.gelifesciences.com) prepared in PBS, transferred to a 5 mL polystyrene round bottom tube (BD Falcon), and overlaid with 1 mL 35% Percoll prepared in PBS. This discontinuous gradient was centrifuged at 800 g for 20 min at 4°C (1500 rpm; Beckman GH-3.8 rotor) with braking disengaged to prevent disruption of the gradient interface layer. Myelin and other debris form an opaque layer at the top of the gradient while the cells of interest form a fuzzy layer at the interface between 35% and 70% Percoll (Figure 1). Following aspiration of the upper debris layer the central interface layer was collected (~1 mL volume) and diluted with 3 mL fresh, cold RPMI. The cell suspension was pelleted by centrifugation at 1160 g for 5 min at 4°C (2200 rpm; Beckman GH-3.8 rotor) and the supernatant was discarded. The SCILs pellet was either lysed for RNA isolation or processed for flow cytometry.

Enzymatic method—SCILs were collected following a previously published enzymatic method (Katz-Levy *et al.*, 1999). The spinal cord was flushed from the spinal column with PBS and then forced through a 100-mesh stainless steel screen in Hank's balanced salt solution containing 300 U/mL type 4 clostridial collagenase (Worthington Biochem, NJ).

The suspension was incubated for 75 minutes at 37°C. Following digestion, the suspension was mixed with 30% Percoll and underlaid with 70% Percoll. This step gradient was centrifuged at $500 \times g$ for 20 min at 24°C. Cell at the 30%:70% interface were collected as SCILs for further analysis.

Isolation of PBMCs

Peripheral blood was collected by cardiac puncture into heparinized tubes and diluted to 1.5 mL final volume with PBS. The diluted blood was layered over 750 μ L of Ficoll-Paque Plus (Amersham) and centrifuged at 1160 g for 25 min at 25°C (2200 rpm; Beckman GH-3.8 rotor) without braking. PBMCs were collected at the interface, resuspended in PBS, and washed twice by centrifugation at 800 g for 5 min at 25°C (1500 rpm; Beckman GH-3.8 rotor). Contaminating red blood cells were lysed by incubation for 2 min in ACK buffer diluted 1:1 in RPMI (0.15 M NH_4Cl , 10 mM KHCO_3 , 0.1 mM Na_2EDTA , pH 7.4). Cells were pelleted at 800 g for 5 min at 25°C (1500 rpm; Beckman GH-3.8 rotor) and resuspended in fresh RPMI for further analysis.

Cell culture

C57SV H-2^b fibroblasts and SVSQ H-2^d fibroblasts were cultured in RPMI containing 10% fetal calf serum and penicillin-streptomycin, as previously reported (Lin *et al.*, 1997). The 145-2C11 anti-CD3 hybridoma line was grown in suspension at 2×10^6 cells per mL in RPMI 1640 containing 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10% fetal calf serum, and penicillin-streptomycin.

Cytotoxicity assay

A standard chromium-release assay was employed as previously described. In brief, 10^4 fibroblast targets were suspended in 100 μ L serum-free media in a 96-well round-bottom plate and loaded for 1 hr at 37°C with 200 μCi ^{51}Cr . After washing, effector cells were added at various effector-to-target (E:T) ratios. Due to the limited number of SCILs isolated from a single spinal cord, we could only test SCILs:target ratios of 25:1 or less. After 18 hr at 37°C the cells were pelleted and supernatants were transferred to 96-well scintillation plates for measurement of ^{51}Cr release. Percent cytolysis was calculated as: (sample release – spontaneous release)/(maximum release – spontaneous release) $\times$ 100.

In vitro stimulation for IFN γ production

Freshly prepared SCILs or PBMCs at 2×10^5 cells/mL were incubated in 96-well plates for 4 hr at 37°C in RPMI supplemented with 10% fetal bovine serum and penicillin-streptomycin. After equilibrating to culture conditions, the cells were treated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μg /mL ionomycin for 4 or 24 hr. During the last 3 hr of incubation the protein transport inhibitor brefeldin A was added to the cultures at a concentration of 0.5 μg /mL. At the end of the stimulation period the cells were washed twice with PBS and processed for flow cytometry.

Flow cytometry

Washed cells were incubated for 15 min at 4°C in FACS buffer (PBS plus 1% FBS, 0.09% sodium azide) mixed 1:1 with supernatant from the 2.4G2 hybridoma (Fc block; anti-CD16/32). Primary antibodies against extracellular antigens were added to the blocked cells at 1:100 and incubated for 30 min at 4°C. CD45 was detected with clone 30-F11 (BD Biosciences #559864). CD8 was detected with clone 53–6.7 (BD Biosciences #553033). Additionally, CD4 was detected with clone GK1.5, CD11b with clone M1/70, CD11c with clone N418, B220 with clone RA3-6B2, and CD19 with clone 1D3, all from BD Biosciences. Appropriate IgG controls were used to establish non-specific staining. To

detect intracellular IFN γ , cells were washed once with FACS buffer and permeabilized in 250 μ L Cytofix/Cytoperm (BD Biosciences) for 15 min at 4°C. Cells were washed once with Perm/Wash buffer (BD Biosciences) and then incubated for 30 min at 4°C with clone XMG1.2 directly conjugated to FITC (BD Biosciences #554411). Stained cells were washed once with FACS buffer and then fixed in 1% paraformaldehyde for ~30 min prior to flow cytometric analysis on a BD FACSCalibur System. FCS files were exported and analyzed offline using FlowJo 7.2 (Windows version; Tree Star, Inc; www.flowjo.com).

RNA isolation and gene expression by qRT-PCR

A two-step RT-PCR was used to quantify the amount of IFN γ RNA expressed in unstimulated PBMCs and SCILs. The Qiagen RNeasy micro kit (catalog #74004, Qiagen, www.qiagen.com) was used to prepare DNase I-treated total RNA from 10⁵ PBMCs or 10⁵ SCILs isolated from individual mice. This kit yields high quality, high concentration RNA (as assessed by an Agilent 2100 Bioanalyzer; data not shown) from small samples. RNA was eluted into 20 μ L of RNase-free water and stored at -80°C until use. Sample concentrations were estimated on neat RNA solutions using the NanoDrop 1000 spectrophotometer (Thermo Scientific, www.nanodrop.com). Reverse transcription was accomplished using the Transcriptor First Strand cDNA synthesis kit (catalog #04379012001, Roche, www.roche-applied-science.com). 200 ng RNA in 5 μ L volume was denatured for 10 min at 65°C in the presence of 2.5 μ M anchored oligo(dT) primer. cDNA synthesis was performed at 55°C using the Transcriptor RT reaction buffer containing 20 U Protector RNase inhibitor, 1 mM deoxynucleotide mix, and 10 U Transcriptor reverse transcriptase. The resulting cDNA was used directly for subsequent PCR amplification with the Roche FastStart Taqman Probe master kit (catalog #04673409001, Roche), the Roche Universal Probe Library system (www.roche-applied-science.com/sis/rtpcr/upl), and a Roche LightCycler 2.0 instrument. Optimal intron-spanning primer pairs and Taqman probes were identified using the Roche Probe Finder software (qpcr.probefinder.com/organism.jsp). For IFN γ (NM008337) we used: forward 5-atctggagggaactggcaaaa-3'; reverse 5'-ttcaagacttcaaagagtctgagg-3'; probe #21 5'-cagagcca-3'. For GAPDH (NM008084) we used: forward 5'-agcttgatcatcaacgggaag-3'; reverse 5'-ttgatgttagtgggtctcg-3'; probe #9 5'-catcacca-3'. Samples were preincubated at 95°C for 10 min to activate the Fast Start DNA polymerase and denature the cDNA. Amplification was accomplished by 40 cycles of melting at 95°C for 10 sec, annealing at 55°C for 30 sec, and elongating at 72°C for 5 seconds. Fluorescence intensity was measured each cycle at the end of the elongation step.

The quantity of IFN γ and GAPDH amplified was estimated as DNA copy number by comparison to standard curves generated from DNA plasmids (IFN γ : Addgene plasmid 10955, mouse interferon gamma, pUC8; www.addgene.org). Serial dilution of stock plasmid was used to generate standard curves based on copy number. Raw fluorescence data plotted against cycle number were exported from the LightCycler software into SigmaPlot 9 (www.systat.com) for analysis. The regression wizard function in SigmaPlot was used to fit each sample to a sigmoid 3-parameter curve:

$$y = \frac{a}{1 + e^{-\left(\frac{x - x_0}{b}\right)}}$$

where y = fluorescence, x = cycle number, and a, b, and x₀ are derived parameters. x₀ defines the inflection point of the sigmoid curve (equivalent to the point where the increase in fluorescence no longer approximates exponential growth) and therefore serves as a conveniently derived crossing point estimate for each sample. The x₀ parameter for each standard was plotted against copy number and fit to a linear curve. The goodness-of-fit (R²)

was calculated and the slope of the line was taken as an estimate of the reaction efficiency (E) using:

$$E=10^{-1/\text{slope}}$$

Reactions with $E < 1.5$ or $R^2 < 0.950$ were excluded from further analysis. The reaction efficiency and x_0 values for each experimental sample were used to calculate fold induction between SCILs and PBMCs using the Pfaffl equation (Pfaffl, 2001):

$$\text{Ratio} = \frac{(E_{\text{target}})^{x_{\text{PI}}^{\text{control}} - x_{\text{PI}}^{\text{experimental}}}}{(E_{\text{reference}})^{x_{\text{PI}}^{\text{control}} - x_{\text{PI}}^{\text{experimental}}}}$$

For calculation of differences in GAPDH expression between SCILs and PBMCs the Pfaffl equation was simplified to the numerator.

Statistical analysis

Statistical significance was assessed by t-test using $\alpha=0.05$. All graphs and data are reported as mean $\pm$ 95% confidence intervals.

3. Results

We prepared a single cell suspension of spinal cord homogenate derived from the demyelinated spinal cord of a mouse infected with TMEV for 90 days. When centrifuged through a discontinuous 35%:70% Percoll gradient, this preparation yielded three readily distinguishable fractions (Figure 1). The upper layer ('A') was enriched in myelin and other debris that floated to the top of the gradient, while the middle ('B') and bottom ('C') layers were enriched in cells. Application of gate 'R1' to the forward scatter vs. side scatter plots of the three fractions identified a unique population of cells in the middle layer ('B') of the gradient. Further phenotypic analysis of CD45 expression on the cells in 'R1' revealed that this region contained a large population of CD45⁺ immune cells. For all subsequent analyses by flow cytometry, we only considered cells within region 'R2' (i.e. CD45⁺ cells from region 'R1') of gradient layer 'B' as spinal cord infiltrating leukocytes (SCILs). For analysis of gene expression only cells from layer 'B' were studied.

Numerous labs have published protocols for isolating CNS infiltrating cells using enzymatic digestion of brain and spinal cord to release single cells (Katz-Levy et al., 1999; Lipton et al., 2005). We sought to remove this processing step in order to speed up the preparation and to protect cell integrity and surface expression of key immunofunctional antigens. In a side-by-side comparison, the non-enzymatic method reduced the preparation time by almost 90 minutes. In addition, while both the enzymatic (+ENZ) and non-enzymatic (-ENZ) preparations yielded similar forward scatter and side scatter flow profiles (Figure 2A and 2D) and similar CD45, CD4, and CD8 staining patterns (Figure 2B, 2C, 2E, 2F), the overall cell-type specific yields and the overall surface intensity of relevant immune antigens were higher in our non-enzymatic preparation (Figure 2G-2O). Specifically, the yield of CD45^{hi} cells was $133 \pm 7\%$ higher in our preparation than in the enzymatic prep ($t(4)=5.383$, $P=0.006$), the yield of CD4⁺ cells was $152 \pm 4\%$ higher ($t(4)=9.038$, $P<0.001$), and the yield of CD8⁺ cells was $126 \pm 8\%$ higher ($t(4)=5.190$, $P=0.007$) via our method. The mean fluorescence intensities (MFIs) were also significantly higher for CD45 ($t(4)=6.410$, $P=0.003$), CD4 ($t(4)=7.302$, $P=0.002$), and CD8 ($t(4)=4.383$, $P=0.012$) in our preparation.

Despite using a complex, heterogeneous tissue such as the spinal cord as our starting material, our method yielded a specific population of CD45^{hi}CD8⁺ and CD45^{hi}CD4⁺ immune cells only found in the demyelinated spinal cord of mice at 90 days postinfection (dpi) (Figure 3C and 3D) but not in the spinal cord of uninfected animals (Figure 3A and 3B). On average, only 20 ± 3 CD45^{hi}CD8⁺ and 16 ± 3 CD45^{hi}CD4⁺ cells were collected from the spinal cord of an uninfected mouse, while 1570 ± 86 CD45^{hi}CD8⁺ ($t(19)=19.360$, $P<0.001$ vs. uninfected) and 414 ± 29 CD45^{hi}CD4⁺ ($t(19)=14.884$, $P<0.001$ vs. uninfected) cells were collected from the spinal cord of a demyelinated mouse infected with TMEV for 90 days. Moreover, our preparation yielded a variety of other immune cell populations, including NK cells (Figure 3F), microglia/macrophages (Figure 3J), and B cells (Figure 3N and 3P). We also detected a small population of CD40⁺ cells (Figure 3H). The pattern of CD11c expression in the SCILs (Figure 3L) is harder to interpret, being different from that observed in PBMCs but inconclusively specific in the SCILs.

In order to characterize the effector versus memory phenotype of the T cells in the SCILs preparation we assessed expression levels of CD44 (extracellular matrix adhesion and homing receptor) and CD62L (L-selection, adhesion and homing receptor) on CD45^{hi}CD4⁺ and CD45^{hi}CD8⁺ SCILs. We used the following criteria to define T cell populations: 1) CD44^{hi}CD62L^{hi} = central memory cells; 2) CD44^{hi}CD62L[−] = target effector memory cells; 3) CD44^{lo}CD62L[−] = acute effector cells; 4) CD44^{lo}CD62L^{hi} = naive cells (Curtis et al., 1998; Dailey, 1998; Gattinoni et al., 2005; Kaech et al., 2003; Lanzavecchia and Sallusto, 2002; Masopust et al., 2001; Sallusto et al., 1999). We found that the majority of CD45^{hi}CD4⁺ SCILs were target effector memory cells ($78 \pm 2\%$) while only a small fraction were acute effector cells ($4 \pm 1\%$) (Figure 4A–4C). In contrast, $55 \pm 4\%$ of CD45^{hi}CD8⁺ SCILs were target effector memory cells, while $36 \pm 3\%$ were acute effector CD8⁺ T cells (Figure 4D–4F). The overall numbers of CD62L^{lo/hi} T cells were small in both the CD4⁺ and CD8⁺ SCILs compared to CD62L[−] T cells, and we detected only a few putative naive T cells in the SCILs (Figure 4C, 4F).

The presence of a robust acute effector population in the CD45^{hi}CD8⁺ SCILs suggested an active process of ongoing antigen stimulation. A reasonable hypothesis regarding the antigen specificity of these activated effector cells is that they are antiviral, recognizing TMEV peptides presented on MHC class I. However, we were unable to detect antiviral cytolytic activity in SCILs collected at 100 dpi when incubated with TMEV-infected H-2^q fibroblasts (Figure 4G), despite the fact that these same effector cells were competent to kill anti-CD3-bearing 145-2C11 hybridoma targets (Figure 4H). As previously reported (Lin et al., 1998; Lin et al., 1997), brain-infiltrating lymphocytes (BILs) collected from H-2^q mice (B10Q) at 7 dpi also failed to kill TMEV-infected fibroblasts (Figure 4G) but could kill 145-2C11 targets (Figure 4H). In contrast, BILs isolated at 7 dpi from TMEV-infected H-2^b mice (B6) readily killed both TMEV-infected H-2^b fibroblasts (Figure 4G) and 145-2C11 targets (Figure 4H). We conclude that CD8⁺ effector T cells present in the spinal cord of chronically infected B10Q mice are not antiviral, suggesting that other antigens are driving the continued activation phenotype that we observe.

We next asked whether the SCILs were biased toward the expression of IFN γ at the gene expression level. For this analysis we developed an objective, quantitative two-step RT-PCR strategy that employs the Taqman probe technology available from Roche (Universal Probe Library). The analysis is quantitated by reference to a titrated standard curve of plasmid containing the gene of interest. The measurements are objective because we employ a curve fitting algorithm that applies a 3-parameter sigmoid curve to the raw cycle vs. fluorescence measurements and computes a crossing point value that does not depend upon subjective manipulation of a noise band. This crossing point reflects the inflection point on the curve at which amplification ceases exponential growth. Standard curves for IFN γ (Figure 5A) and

GAPDH (Figure 5B) were used to compute the efficiency of the PCR (Figure 5C and 5D). These efficiency values were then applied in the standard Pfaffl equation (Pfaffl, 2001) to determine the difference in IFN γ (Figure 5E) or GAPDH (Figure 5F) mRNA expression between SCILs and PBMCs. We found that SCILs collected from demyelinated mice at 90 dpi had 10.4 ± 1.9 fold more IFN γ mRNA expression (mean $\pm$ 95% CI) than PBMCs collected from the same animals (Figure 5G). This difference was significant at $P < 0.001$ (by t-test, $t(4) = 15.570$). In contrast, the amount of GAPDH mRNA measured did not differ between SCILs and PBMCs (1.0 ± 0.3 fold difference; $P = 0.1$, $t(4) = 2.138$). This finding suggests that the SCILs are skewed toward a T_H1/T_C1 response relative to PBMCs from the same animals (Mosmann et al., 1997; Woodland and Dutton, 2003).

To further characterize the functional phenotype of target-derived lymphocytes we cultured PBMCs and SCILs for 4 or 24 hours under resting conditions (unstimulated) or in the presence of PMA and ionomycin (stimulated). By including brefeldin A to stop trafficking from the Golgi we were able to analyze the amount of IFN γ protein produced in the cells ex vivo by flow cytometry (Figure 6). In concordance with the RT-PCR analysis, we found that CD8⁺ (Figure 6B, 6F, 6J, 6N) and CD4⁺ (Figure 6D, 6H, 6L, 6P) SCILs were far more competent than PBMCs to produce IFN γ after 4 (Figure 6F and 6H) and 24 hours of stimulation (Figure 6H and 6L). A small number of CD8⁺ and CD4⁺ cells in the PBMC preparation showed an increase in intracellular IFN γ following 24 hours of stimulation (Figure 3I and 3M). About half of the CD4⁺ SCILs showed an increase in intracellular IFN γ by 4 and 24 hours (Figure 6H and 6L). In contrast, the majority of CD8⁺ SCILs upregulated the expression of IFN γ protein by 24 hours (Figure 3J). This further supports the idea that CD8⁺ cells in the SCILs are primed to release the potent T_C1 cytokine IFN γ (Mosmann et al., 1997; Woodland and Dutton, 2003).

4. Discussion

Our technique for isolation of spinal cord infiltrating immune cells provides a rapid (90 minutes from anesthesia to RNA collection or blocking for flow cytometry) and simple method for the isolation of an enriched population of leukocytes that maintain phenotype and competence to respond to ex vivo stimulation. In contrast to previous protocols (Babcock et al., 2003; Boztug et al., 2002; Brabb et al., 2000; Cao and DeLeo, 2008; Cheeran et al., 2007; Huang et al., 2001; Johnson et al., 1999; Karman et al., 2006; Kirwin et al., 2006; Klein et al., 2004; Klein et al., 2005; Lane et al., 2000; Lees et al., 2008; Ling et al., 2003; Lipton et al., 2005; Liu et al., 2006; Luo et al., 2007; Marten et al., 2000; McCandless et al., 2006; McCandless et al., 2008; Muller et al., 2007; Myoung et al., 2007; Ponomarev et al., 2004; Remington et al., 2007; Stiles et al., 2006; Stromnes et al., 2008; Walsh et al., 2008), our method:

1. does not require sequential sieving that may lead to cell loss;
2. does not use enzymatic digestion which may alter or destroy extracellular epitopes necessary for phenotypic characterization;
3. provides a debris-free preparation that is readily amenable to ex vivo culture and manipulation;
4. works robustly for spinal cord-specific infiltrating immune cells rather than pooled brain and spinal cord cells;
5. yields a substantial population of CD8⁺ lymphocytes within the context of chronic demyelination rather than acute viral infection;
6. identifies a strong T_C1 functional skew to CD8⁺ lymphocytes present in the chronically demyelinated spinal cord;

7. provides a unique, objective, reproducible method for the analysis of gene expression using a curve fitting algorithm that does not depend upon arbitrary assessment of noise.

By facilitating the comparison of gene expression in freshly prepared cells and the analysis of protein expression following ex vivo stimulation, our SCILs protocol revealed that peripheral blood lymphocytes are not a suitable surrogate for the immunological status of target infiltrating effector lymphocytes. While PBMCs from our chronically infected mice failed to show a response to PMA and ionomycin stimulation, CD8⁺ SCILs from the same animals responded robustly to such stimulation. Such a difference has been previously described in brain-derived lymphocytes during acute West Nile virus encephalitis (McCandless et al., 2008). However, our findings indicate that the CD8⁺ T cells within the SCILs collected from the chronically demyelinated spinal cord are not anti-viral. This is in agreement with the IFN γ -dependent, CD8⁺ T cell-mediated, viral epitope-independent model of demyelination suggested by Perlman's group (Dandekar *et al.*, 2004). It also suggests that the CD8⁺ T cells isolated by our method from the chronically demyelinated spinal cord may have specific effector functions or recognize specific targets, such as demyelinated axons (Howe, 2008; Howe et al., 2007a; Howe et al., 2007b), that are not represented in the population of peripheral CD8⁺ T cells. This is consistent with the presence of a large population of acutely activated CD8⁺ T cells in the SCILs. Finally, our isolation protocol provides a clean, functionally intact population of immune effectors that can be studied in a variety of ex vivo stimulation paradigms. Our SCILs preparation is also amenable to adoptive transfer of total SCILs or column-purified lymphocytes into a variety of animal models of MS.

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Abbreviations

CNS	central nervous system
MS	multiple sclerosis
TMEV	Theiler's murine encephalomyelitis virus
SCILs	spinal cord infiltrating leukocytes
IFNγ	interferon gamma
PMA	phorbol 12-myristate 13-acetate
B10.Q	B10.D1-H2q/SgJ
dpi	days postinfection
PBMCs	peripheral blood mononuclear cells

References

Babcock AA, Kuziel WA, Rivest S, Owens T. Chemokine expression by glial cells directs leukocytes to sites of axonal injury in the CNS. *J Neurosci.* 2003; 23:7922–7930. [PubMed: 12944523]

- Boztug K, Carson MJ, Pham-Mitchell N, Asensio VC, DeMartino J, Campbell IL. Leukocyte infiltration, but not neurodegeneration, in the CNS of transgenic mice with astrocyte production of the CXC chemokine ligand 10. *J Immunol.* 2002; 169:1505–1515. [PubMed: 12133978]
- Brabb T, von Dassow P, Ordonez N, Schnabel B, Duke B, Goverman J. In situ tolerance within the central nervous system as a mechanism for preventing autoimmunity. *J Exp Med.* 2000; 192:871–880. [PubMed: 10993917]
- Cao L, DeLeo JA. CNS-infiltrating CD4+ T lymphocytes contribute to murine spinal nerve transection-induced neuropathic pain. *Eur J Immunol.* 2008; 38:448–458. [PubMed: 18196515]
- Cheeran MC, Hu S, Palmquist JM, Bakken T, Gekker G, Lokensgard JR. Dysregulated interferon-gamma responses during lethal cytomegalovirus brain infection of IL-10-deficient mice. *Virus Res.* 2007; 130:96–102. [PubMed: 17624463]
- Curtsinger JM, Lins DC, Mescher MF. CD8+ memory T cells (CD44^{high}, Ly-6C⁺) are more sensitive than naive cells to (CD44^{low}, Ly-6C[−]) to TCR/CD8 signaling in response to antigen. *J Immunol.* 1998; 160:3236–3243. [PubMed: 9531279]
- Dailey MO. Expression of T lymphocyte adhesion molecules: regulation during antigen-induced T cell activation and differentiation. *Crit Rev Immunol.* 1998; 18:153–184. [PubMed: 9637409]
- Dandekar AA, Anghelina D, Perlman S. Bystander CD8 T-cell-mediated demyelination is interferon-gamma-dependent in a coronavirus model of multiple sclerosis. *Am J Pathol.* 2004; 164:363–369. [PubMed: 14742242]
- Deb C, Howe CL. NKG2D contributes to efficient clearance of picornavirus from the acutely infected murine brain. *J Neurovirol.* 2008; 14:261–266. [PubMed: 18569460]
- Gattinoni L, Klebanoff CA, Palmer DC, Wrzesinski C, Kerstann K, Yu Z, Finkelstein SE, Theoret MR, Rosenberg SA, Restifo NP. Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. *J Clin Invest.* 2005; 115:1616–1626. [PubMed: 15931392]
- Howe CL. Immunological aspects of axon injury in multiple sclerosis. *Curr Top Microbiol Immunol.* 2008; 318:93–131. [PubMed: 18219816]
- Howe CL, Adelson JD, Rodriguez M. Absence of perforin expression confers axonal protection despite demyelination. *Neurobiol Dis.* 2007a; 25:354–359. [PubMed: 17112732]
- Howe CL, Ure D, Adelson JD, Lafrance-Corey R, Johnson A, Rodriguez M. CD8+ T cells directed against a viral peptide contribute to loss of motor function by disrupting axonal transport in a viral model of fulminant demyelination. *J Neuroimmunol.* 2007b; 188:13–21. [PubMed: 17493690]
- Huang DR, Wang J, Kivisakk P, Rollins BJ, Ransohoff RM. Absence of monocyte chemoattractant protein 1 in mice leads to decreased local macrophage recruitment and antigen-specific T helper cell type 1 immune response in experimental autoimmune encephalomyelitis. *J Exp Med.* 2001; 193:713–726. [PubMed: 11257138]
- Johnson AJ, Njenga MK, Hansen MJ, Kuhns ST, Chen L, Rodriguez M, Pease LR. Prevalent class I-restricted T-cell response to the Theiler's virus epitope Db:VP2121–130 in the absence of endogenous CD4 help, tumor necrosis factor alpha, gamma interferon, perforin, or costimulation through CD28. *J Virol.* 1999; 73:3702–3708. [PubMed: 10196262]
- Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol.* 2003; 4:1191–1198. [PubMed: 14625547]
- Karman J, Chu HH, Co DO, Seroogy CM, Sandor M, Fabry Z. Dendritic cells amplify T cell-mediated immune responses in the central nervous system. *J Immunol.* 2006; 177:7750–7760. [PubMed: 17114446]
- Katz-Levy Y, Neville KL, Girvin AM, Vanderlugt CL, Pope JG, Tan LJ, Miller SD. Endogenous presentation of self myelin epitopes by CNS-resident APCs in Theiler's virus-infected mice. *J Clin Invest.* 1999; 104:599–610. [PubMed: 10487774]
- Kirwin SJ, Dowdell KC, Hindinger C, Feng N, Bergmann CC, Hinton DR, Stohlman SA. Altered neuroantigen-specific cytokine secretion in a Th2 environment reduces experimental autoimmune encephalomyelitis. *J Neuroimmunol.* 2006; 178:30–39. [PubMed: 16814872]

- Klein RS, Izikson L, Means T, Gibson HD, Lin E, Sobel RA, Weiner HL, Luster AD. IFN-inducible protein 10/CXC chemokine ligand 10-independent induction of experimental autoimmune encephalomyelitis. *J Immunol.* 2004; 172:550–559. [PubMed: 14688366]
- Klein RS, Lin E, Zhang B, Luster AD, Tollett J, Samuel MA, Engle M, Diamond MS. Neuronal CXCL10 directs CD8+ T-cell recruitment and control of West Nile virus encephalitis. *J Virol.* 2005; 79:11457–11466. [PubMed: 16103196]
- Lane TE, Liu MT, Chen BP, Asensio VC, Samawi RM, Paoletti AD, Campbell IL, Kunkel SL, Fox HS, Buchmeier MJ. A central role for CD4(+) T cells and RANTES in virus-induced central nervous system inflammation and demyelination. *J Virol.* 2000; 74:1415–1424. [PubMed: 10627552]
- Lanzavecchia A, Sallusto F. Progressive differentiation and selection of the fittest in the immune response. *Nat Rev Immunol.* 2002; 2:982–987. [PubMed: 12461571]
- Lees JR, Iwakura Y, Russell JH. Host T cells are the main producers of IL-17 within the central nervous system during initiation of experimental autoimmune encephalomyelitis induced by adoptive transfer of Th1 cell lines. *J Immunol.* 2008; 180:8066–8072. [PubMed: 18523270]
- Lin X, Pease LR, Murray PD, Rodriguez M. Theiler's virus infection of genetically susceptible mice induces central nervous system-infiltrating CTLs with no apparent viral or major myelin antigenic specificity. *J Immunol.* 1998; 160:5661–5668. [PubMed: 9605173]
- Lin X, Pease LR, Rodriguez M. Differential generation of class I H-2D- versus H-2K-restricted cytotoxicity against a demyelinating virus following central nervous system infection. *Eur J Immunol.* 1997; 27:963–970. [PubMed: 9130651]
- Ling C, Sandor M, Fabry Z. In situ processing and distribution of intracerebrally injected OVA in the CNS. *J Neuroimmunol.* 2003; 141:90–98. [PubMed: 12965258]
- Lipton HL, Kallio P, Jelachich ML. Simplified quantitative analysis of spinal cord cells from Theiler's virus-infected mice without the requirement for myelin debris removal. *J Immunol Methods.* 2005; 299:107–115. [PubMed: 15914195]
- Liu L, Huang D, Matsui M, He TT, Hu T, Demartino J, Lu B, Gerard C, Ransohoff RM. Severe disease, unaltered leukocyte migration, and reduced IFN-gamma production in CXCR3^{-/-} mice with experimental autoimmune encephalomyelitis. *J Immunol.* 2006; 176:4399–4409. [PubMed: 16547278]
- Luo J, Ho PP, Buckwalter MS, Hsu T, Lee LY, Zhang H, Kim DK, Kim SJ, Gambhir SS, Steinman L, Wyss-Coray T. Glia-dependent TGF-beta signaling, acting independently of the TH17 pathway, is critical for initiation of murine autoimmune encephalomyelitis. *J Clin Invest.* 2007; 117:3306–3315. [PubMed: 17965773]
- Marten NW, Stohlman SA, Bergmann CC. Role of viral persistence in retaining CD8(+) T cells within the central nervous system. *J Virol.* 2000; 74:7903–7910. [PubMed: 10933698]
- Masopust D, Vezys V, Marzo AL, Lefrancois L. Preferential localization of effector memory cells in nonlymphoid tissue. *Science.* 2001; 291:2413–2417. [PubMed: 11264538]
- McCandless EE, Wang Q, Woerner BM, Harper JM, Klein RS. CXCL12 limits inflammation by localizing mononuclear infiltrates to the perivascular space during experimental autoimmune encephalomyelitis. *J Immunol.* 2006; 177:8053–8064. [PubMed: 17114479]
- McCandless EE, Zhang B, Diamond MS, Klein RS. CXCR4 antagonism increases T cell trafficking in the central nervous system and improves survival from West Nile virus encephalitis. *Proc Natl Acad Sci U S A.* 2008; 105:11270–11275. [PubMed: 18678898]
- Mosmann TR, Li L, Sad S. Functions of CD8 T-cell subsets secreting different cytokine patterns. *Semin Immunol.* 1997; 9:87–92. [PubMed: 9194219]
- Muller M, Carter SL, Hofer MJ, Manders P, Getts DR, Getts MT, Dreykluft A, Lu B, Gerard C, King NJ, Campbell IL. CXCR3 signaling reduces the severity of experimental autoimmune encephalomyelitis by controlling the parenchymal distribution of effector and regulatory T cells in the central nervous system. *J Immunol.* 2007; 179:2774–2786. [PubMed: 17709491]
- Myoung J, Hou W, Kang B, Lyman MA, Kang JA, Kim BS. The immunodominant CD8+ T cell epitope region of Theiler's virus in resistant C57BL/6 mice is critical for anti-viral immune responses, viral persistence, and binding to the host cells. *Virology.* 2007; 360:159–171. [PubMed: 17095033]

- Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2001; 29:e45. [PubMed: 11328886]
- Ponomarev ED, Novikova M, Yassai M, Szczepanik M, Gorski J, Dittel BN. Gamma delta T cell regulation of IFN-gamma production by central nervous system-infiltrating encephalitogenic T cells: correlation with recovery from experimental autoimmune encephalomyelitis. *J Immunol.* 2004; 173:1587–1595. [PubMed: 15265886]
- Ransohoff RM, Kivisakk P, Kidd G. Three or more routes for leukocyte migration into the central nervous system. *Nat Rev Immunol.* 2003; 3:569–581. [PubMed: 12876559]
- Remington LT, Babcock AA, Zehntner SP, Owens T. Microglial recruitment, activation, and proliferation in response to primary demyelination. *Am J Pathol.* 2007; 170:1713–1724. [PubMed: 17456776]
- Rodriguez M. Effectors of demyelination and remyelination in the CNS: implications for multiple sclerosis. *Brain Pathol.* 2007; 17:219–229. [PubMed: 17388953]
- Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature.* 1999; 401:708–712. [PubMed: 10537110]
- Stiles LN, Hosking MP, Edwards RA, Strieter RM, Lane TE. Differential roles for CXCR3 in CD4+ and CD8+ T cell trafficking following viral infection of the CNS. *Eur J Immunol.* 2006; 36:613–622. [PubMed: 16479546]
- Stromnes IM, Cerretti LM, Liggitt D, Harris RA, Gorman JM. Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. *Nat Med.* 2008; 14:337–342. [PubMed: 18278054]
- Walsh KB, Lanier LL, Lane TE. NKG2D receptor signaling enhances cytolytic activity by virus-specific CD8+ T cells: evidence for a protective role in virus-induced encephalitis. *J Virol.* 2008; 82:3031–3044. [PubMed: 18160433]
- Woodland DL, Dutton RW. Heterogeneity of CD4(+) and CD8(+) T cells. *Curr Opin Immunol.* 2003; 15:336–342. [PubMed: 12787761]

Figure 1.

Flow cytometric analysis of spinal cord homogenate collected from a 90 dpi mouse separated on a discontinuous 35%:70% Percoll gradient. The left panel shows a schematic representation of three fractions collected from the gradient after centrifugation. Fractions A and C exhibited forward and side scatter profiles that were considerably different than the profile of fraction B. Using gate region R1, we found that fraction B had a large number of CD45⁺ immune cells that were not observed in fractions A or C. For all analyses, we defined spinal cord infiltrating leukocytes (SCILs) as the population of cells contained in fraction B. For flow cytometric analysis we further defined SCILs as the population present within gate R2 on the CD45 vs. forward scatter dot plot (i.e. R1 gated cells that were also CD45⁺).

Figure 2.

Comparison of enzymatic (+ENZ) and non-enzymatic (-ENZ) SCILs preparations by flow cytometry. Both methods yielded a reproducible forward (FSC) and side scatter (SSC) profile (A, D), as well as considerable numbers of CD45^{hi} cells (B, E) and CD45^{hi}CD4⁺CD8⁻ and CD45^{hi}CD4⁻CD8⁺ lymphocytes (C, F). Quantitative analysis of SCILs collected from at least 3 separate experimental preparations revealed that our non-enzymatic protocol consistently yielded more CD45^{hi} cells (G), more CD45^{hi}CD4⁺ cells (H), and more CD45^{hi}CD8⁺ cells (I). At the same time, these cells expressed higher mean fluorescence intensities (MFI) for CD45 (J, M), CD4 (K, N), and CD8 (L, O). Graphs show mean $\pm$ 95% confidence intervals.

Figure 3.

T cells are not detected in the spinal cord of uninfected (naive) mice. SCILs collected from naive mice (A, B) showed only a very small number of CD45^{hi}CD4⁺ and CD45^{hi}CD8⁺ cells as compared to SCILs collected from mice at 90 dpi (C, D). The SCILs preparation also contained DX5⁺ natural killer cells (F), small populations of CD40⁺ (H) or CD19⁺ (P) cells, and large populations of CD11b⁺ (J) or B220⁺ (N) cells.

Figure 4.

The SCILs preparation contains a large number of acute effector CD8⁺ T cells that are not antiviral. CD45^{hi}CD4⁺ (A-C) or CD45^{hi}CD8⁺ (D-F) SCILs were further analyzed for the expression levels of CD44 and CD62L to ascertain memory versus effector phenotype. Only the CD45^{hi}CD8⁺ cells contained a population of CD44^{lo}CD62L⁺ acute effector T cells (E). Despite the presence of a considerable number of cytotoxic T cells, B10Q SCILs isolated from the spinal cord at 100 dpi did not kill TMEV-infected H-2^d fibroblasts (G) but were fully competent to kill anti-CD3-bearing 145-2C11 targets (H).

Figure 5. SCILs express more IFN γ RNA than PBMCs collected from the same animal. Standard curves for copy number determination were prepared from plasmids encoding either IFN γ (A) or GAPDH (B). The raw fluorescence vs. cycle number data were fit to a 3-parameter sigmoid curve to derive the crossing point for each standard. The raw crossing point vs. log copy number data were fit to a line to derive the slope and the reaction efficiency for IFN γ (C) and GAPDH (D). RNA from 10⁵ SCILs or 10⁵ PBMCs was analyzed by two-step RT-PCR. Fluorescence vs. cycle number data were generated for IFN γ (E) or GAPDH (F) and used to derive the crossing point for each experimental sample. The crossing points and the reaction efficiencies were used in the Pfaffl equation to determine the fold difference in expression of IFN γ and GAPDH between SCILs and PBMCs (G). While there was no significant difference in GAPDH expression between the two populations, SCILs expressed 10-fold more IFN γ RNA (G). The results shown are representative of at least 3 separate experiments.

Figure 6.

SCILs produce more IFN γ protein than PBMCs in response to ex vivo PMA:ionomycin stimulation. Under unstimulated conditions, CD8⁺ PBMCs (A), CD8⁺ SCILs (B), CD4⁺ PBMCs (C), and CD4⁺ SCILs (D) did not produce detectable levels of intracellular IFN γ after 24 hr in culture, as determined by flow cytometry. Stimulation for 4 hr with PMA and ionomycin led to a robust increase in intracellular IFN γ levels within about half of CD8⁺ and CD4⁺ SCILs (F, H) but not in CD8⁺ or CD4⁺ PBMCs (E, G). By 24 hr of stimulation, the majority of CD8⁺ SCILs (J) and many CD4⁺ SCILs (L) expressed high levels of IFN γ , while only a small fraction of PBMCs showed IFN γ expression (I, K). The relative levels of IFN γ expression through time of stimulation are shown for CD8⁺ PBMCs (M), CD8⁺ SCILs (N), CD4⁺ PBMCs (O), and CD4⁺ SCILs (P). The results shown are representative of at least 3 separate experiments.