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Cell Surface Phenotype of CNS Infiltrate and Resident Microglia During the Course of Relapsing Experimental Autoimmune Encephalomyelitis in Mice

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Abstract

Multiple Sclerosis (MS) is the most common cause of neurological disability in young adults. In this study, the animal model of MS, experimental autoimmune encephalomyelitis (EAE) was used in conjunction with tissue harvest techniques, flow cytometry and an *ex vivo* assay system in order to immunophenotype cellular infiltrate over the course of disease, study microglial activation, evaluate the ability of microglia to stimulate T-cells and to determine the effects of small molecule inhibitors on certain biochemical pathways. This study demonstrates that CNS infiltrate kinetics mirror disease course and suggests that microglia be involved in antigen presentation and effector function during disease course.

Acknowledgements

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Introduction

Multiple Sclerosis is an immune-mediated inflammatory demyelinating disease of the central nervous system (CNS) charactized by mononuclear cellular infiltrate into brain parenchyma, gliosis, axonal loss and neurodegeneration. The illness most commonly presents itself in young adults aged 20-40 and is most often characterized by relapses and remissions of neurological disturbance which are attributable to the acute development of plaques (Greenstein, 2006). The relapsing and remitting phase of the disease is followed by a phase of continuous progression of disability in most patients (McDonald and Ron, 1999). While clinical and pathological research have contributed much to the understanding of the disease process of MS, much more remains unknown. Studies into the mechanisms of disease will inform successful strategies for the target-based treatment of MS to limit and repair damage (Compston and Coles, 2008).

Clincal Course and Diagnosis

The onset of MS is either optic neuritis, transverse myelitis or a brain-stem presentation in 85% of cases (Greenstein, 2006) which is known as the clinically isolated syndrome. If white-matter abnormalities are detected by MRI at clinically unaffected sites in addition to one of these episodes, the chance of a second attack of demyelination occurring to fulfill the criteria for relapsing-remitting MS increases from 50% at 2 years to 82% at 20 years (Compston and Coles, 2008). Recovery from each episode, occurring erratically between 1-5 times per year, is only partial. Over time, persistent symptoms accumulate. Eventually, 65% of patients reach the secondary progressive stage, which typically begins around 40 years of age (Compston and Coles, 2008). Cases of children with MS have also occurred, usually in girls presenting with encephalopathy. In these cases, the secondary progressive stage takes longer to reach from disease onset than in adults although children with MS usually reach the secondary progressive stage at a younger age than most adults. In 65% of cases, death can be attributed to the disease (Compston and Coles, 2008).

There is no one test for MS and diagnosis is primarily clinical (McDonald and Ron, 1999). Few clinical symptoms are specifically linked to MS, but Lhermitte's symptom (a sensation of electricity running down the limbs or spine when the neck is flexed) and the Uhthoff phenomenon (transient worsening of disease signs when core body temperature is elevated) are characteristic of the disease (Compston and Coles, 2008). Other symptoms include vertigo, diplopia, weakness in the limbs, cognitive impairment, bladder or bowel disturbances and fatigue unrelated to weakness (McDonald and Ron, 1999). In most cases, clinical evidence is sufficient for diagnosis, but when diagnosis is ambiguous, MRIs can be used to indicate paraclinical features of disease including abnormalities in white matter and radiological lesions. In 90% of patients, oligoclonal bands after protein electrophoresis of cerebrospinal fluid can also be detected (Compston and Coles, 1999).

Causes and Risk Factors

The cause of MS involves environmental factors and genetic susceptibility. MS occurs more frequently in temperate climates and is more common in regions populated by northern Europeans (Greenstein, 2006). Moving from a high-risk area to a low-risk area during childhood is associated with a reduced risk-factor for MS, whereas a migration towards a high-risk area from a low-risk area is associated with an increased risk when compared to the population of origin (Compston and Coles, 2008). A significant number of relapses are preceded by viral infections. Patients with MS report being infected with measles, mumps, rubella and Epstien-Barr virus at later ages than do HLA-DR2 matched controls (Compston and Coles, 2008). Multiple sclerosis is more common in females than in males (~2:1) (Greenstein, 2006) and has a familial recurrence rate of about 20% (Compston and Coles, 2008).

Pathological Physiology and Disease Mechanisms

Many clinical and laboratory features of MS can be explained by the effects of demyelination on saltatory conduction (Compston and Coles, 2002). The oligodendrocyte, a principal target of immune attack in MS, synthesizes and maintains the myelin sheath of up to 40 neighboring nerve axons in the CNS. Axons are insulated by this myelin sheath needed for salutatory conduction. From the nodes of Ranvier, the unmyelinated segments, voltage-gated sodium channels cluster and action potentials are propagated down the axons.

It is believed that the disease process begins with an increased migration of T-cells across the blood-brain barrier (BBB). This defect arises from regulatory pathogenesis which allows the cells to set up an immune response in the brain. Particular sites of inflammation are dominated by CD8+ T-cells, causing plaques. These cells secrete interleukins 17 and 22 which disrupt the human BBB, allowing penetration of these cells. As T and B lymphocytes and macrophages accumulate in the CNS, pro-inflammatory cytokines amplify the immune response through recruitment of naïve microglia. Microglial cells are resident CNS leukocytes that take part in innate immunity and adaptive immune responses in the CNS tissue. When alerted to injury or disease, microglial cells are thought to assume an activated phenotype. This activated phenotype allows the cells to respond to tissue damage through proliferation, migration to site of injury, phagocytosis of cellular debris, or the release of cytokines or reactive oxygen species. Contact is established between activated microglia and oligodendrocyte-myelin units and a lethal signal is delivered through cell surface bound tumour necrosis factor, TNF. Lesions form and grow radially as focal brain inflammation fades into diffuse parenchymal microglial activation resulting in abnormalities in white matter. Shadow plaques are formed as remyelination occurs as oligodendrocyte precursors migrate in response to myelin loss to act as a source of cells with the potential to remyelinate naked axons (Williams et al, 2007).

Management and Treatment

In many situations, the priority is to improve the quality of life by masking individual symptoms, though temporary improvement can be achieved at times with high-dose methyl prednisolone (Miller et al, 2000). The efficacy of treatment varies with the stage of disease course. Presently least contentious is the use of β interferons and copaxone (glatiramir acetate) in relapsing-remitting disease where the frequency of new episodes is reduced by about 30% (Ebers et al, 1998) with effects extending beyond two years of treatment (Duquette et al, 1995). Mitoxantrone (an anthracenedione antineoplastic drug) could be more efficacious than the interferons, however toxic effects of the drug limit it to use only in more aggressive cases of MS (Le Page, 2008). Natalizumab (humanisec anti-α4 integrin) has been shown to reduce relapse rate by 68% when given by monthly infusion. This drug is licensed for monotherapy for severe relapsing-remitting disease (Polman, 2006).

The EAE Model

EAE has proven its value since its development in the 1930's at Rockefeller University. With a pathology including demyelination, axonal damage and clinical events of relapsing and remitting paralysis, several models and variations of EAE have mimicked common features of MS. The use of experimental autoimmune encephalomyelitis led to the development of copaxone, mitoxantrone and natalizumab. Clues to the pathogenesis of MS and new potential biomarkers have also been discovered through the use of EAE-based research. Continuing research with this animal model has much potential for the discovery of new treatments and the greater understanding of disease mechanisms (Steinman and Zamvil, 2006).

Objective of Project

Despite the high potential for microglial cells to play an important role in CNS autoimmunity, very little is known about their contributions during autoimmune

disease, such as MS. In addition, it is not clear how microglial cells become activated or how they interact with T lymphocytes. In order to immunophenotype cellular infiltrate over the course of disease, study microglial activation, evaluate the ability of microglia to stimulate T cells, and evaluate the effects of small molecule inhibitors on certain biochemical pathways, the animal model of MS, experimental autoimmune encephalomyelitis (EAE) was used in conjunction with tissue harvest techniques, flow cytometry and an *ex vivo* assay system.

Methods

Animals

Inbred SJL/J mice, aged four weeks and older, were obtained from The Jackson Laboratory, Bar Harbor, ME. SJL/J mice were used for their susceptibility to experimental autoimmune encephalomyelitis. All animals were given food and water *ad libitum* and maintained on a twelve-hour light/dark cycle in the Abbott Bioresearch Center's Department of Bioresources, Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved facility. All animal care was in accordance with the National Institutes of Health guidelines.

Induction of EAE and Disease Progression

SJL/J mice were immunized subcutaneously with an emulsion containing 100μg of PLP 139-151 (New England Peptide, Inc., Gardner, MA) and complete Freund's adjuvant (CFA) containing 100μg of heat-killed *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI) over two hind limb sites and one scruff of neck site on Day 0. The mice were given an intraperitoneal injection with 60ng of *Bordatella pertussis* toxin (List Biological Laboratories, Campbell,CA) on the same day as immunization. The mice were weighed and assessed daily beginning on Day 7 for signs of EAE according to the following scale; 0, no signs of disease; 1, loss of tail tone; 2, moderate hindlimb weakness or irregular gait; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, moribund condition.

Isolation of infiltrating leukocytes and microglia from the CNS

On Days 0, 7, 13, 19 and 25 (Figure 1), five animals were anesthetized with isofluorane, terminally bled via cardiac puncture, and perfused with 10-20ml of

phosphate buffered solution (PBS) at pH 7.4 by insertion of a 25g needle into the left ventricle of the heart until effluent ran clear. Brain and spinal cord were harvested in cold PBS on ice and finely minced before being covered with a digestion mixture containing Liberase RI (Roche, Germany) at 0.2 Wunsch Units/10ml, 50μg/ml Dnase I (Roche, Germany), 25mM Hepes (Invitrogen, Carlsbad, CA) in Hanks' Balanced Salt Solution without Magnesium Chloride or Calcium Chloride (Invitrogen, Carlsbad, CA) for 30mins at 37°C. A single cell suspension was generated by pushing CNS through a BD Falcon cell strainer in a cold rinsing buffer containing Hanks' Balanced Salt Solution without Magnesium Chloride or Calcium Chloride (Invitrogen, Carlsbad, CA) and 2mM EDTA. Cells were pooled and collected in a 50ml tube then centrifuged at 313xg for ten minutes. The pellet was re-suspended in 16.6ml of 1X PIPES Buffer (Sigma, St. Louis, MO) and 7.2mls of 100% Percoll (Sigma, St.Louis, MO) was added. Cells were layered carefully over 2.55ml of 65% Percoll in 1X PIPES in two 15ml conical tubes which were centrifuged at 514xg at room temperature for thirty minutes, no brake. Before collecting the cells of interest, the top 1ml containing CNS matrix tissue was removed and 9mls of Percoll were removed. The cells at the 65%/30% interface in each 15ml tube were pooled into another 15ml tube. The tube was then filled with Hanks' Balanced Salt Solution without Magnesium Chloride or Calcium Chloride to dilute and wash out the Percoll and tubes were centrifuged at 313xg for 10 minutes. The supernatant was removed and the pellet was re-suspended again in Hanks' Balanced Salt Solution without Magnesium Chloride or Calcium Chloride and centrifuged at 313xg for 10 minutes again to wash the cells. Cells were then counted and re-suspended in 150μl FACS Buffer (FB, 1x PBS + 2% v/v FBS + 0.2% w/v Na azide). Cells were placed in 96 well plates at 30μl per well and non-specific Fc binding was blocked with 30μl Mouse BD Fc Block (Pharmingen, San Diego, CA). After incubating with the blocking agent for 30 minutes on ice, the cells were stained with cell surface markers for CD45, CD11b, CD11c, MHC II, CD80, CD40, CD34 for 30 minutes, covered, on ice (Table 1). Appropriate isotype controls were used to define staining above background. The cells were then washed three times with FB and analyzed on the FACSCaliber (BD, San Diego, CA).

Cell sorting and ex vivo assay protocol

Brain and spinal cord tissues were harvested from diseased animals on Day 13. Tissues were processed and layered to collect a single cell suspension as described in the *Isolation of infiltrating leukocytes and microglia from the CNS* protocol. Cells were then counted and resuspended in 1ml FB, blocked with Mouse BD Fc Block (Pharmingen, San Diego, CA) and stained with CD11b following a 30 minute incubation on ice. After staining, the cells were washed and re-suspended in 1ml of Robosep Buffer. Cells were separated using the EasySep (StemCell Technologies, Vancouver, BC, Canada) procedure for phycoerythrin- (PE) selection.

Lymph nodes were harvested from PLP-immunized animals. The lymph nodes were processed to single cell suspensions by grinding them between the frosted ends of slides. CD4+ T-cells were separated with Robosep Selection (StemCell Technologies, Vancouver, BC, Canada).

CD11b+ microglia and monocytes were plated at 2x105 cells per well. CD4+ T-cells were plated at 3x105 cells per well. CD11b+ cells were plated alone, with PLP-primed T-cells, T-cells and PLP, T-cells, PLP and Compound A at 1µM and T-cells, PLP, and FTY at 100nM. Supernatant was collected for TNF, IFN-γ, and IL-17 levels.

Results

CNS Infiltrate Kinetics

Immunized animals used for CNS harvesting on Days 0, 7, 13, and 25 had average clinical scores throughout disease course and a typical relapsing-remitting trend of disease scores (Figure 2). Figure 2 shows the average total CNS cell count per animal determined by pooling five animals at each time point.

FACS plots of naïve animals (Day 0) and Day 13 animals, as seen in Figure 3, show a forward and side scatter of CD45 and CD11b. CD45 med, CD11b+ cells are microglia. CD45hi, CD11b- cells are infiltrating T-cells. CD45hi, CD11b+ cells are monocytes. Although the percentage of resident microglia decreases from 83.3% to 50.7% between naïve and diseased animals, the total cell count rises substantially from $2.4x10^6$ to $8.8x10^6$, and so the total number of cells expressing a microglial phenotype doubles from $2.0x10^6$ cells to $4.5x10^6$. This data can be more clearly seen in Figure 4, which shows the average cell count per animal at each time point (n=5 pooled) for microglia, infiltrating T-cells, infiltrating monocytes and dendritic cells. All cell types follow the same general trend over disease course, but the baseline microglial cell number is much higher due to resident microglial cells in naïve CNS.

Activation States of Microglia

Though CD40 and MHC II cell markers were used to identify activated microglia, no staining was found with either of these markers. CD34 also did not show staining for microglial progenitor cells.

CD80 was also used to identify activated microglia, as seen in Figure 5. Figure 5 shows the FACS plots of Day 7 and Day 13 diseased animals with CD45 and CD80 staining. CD45med, CD80+ cells are activated microglia. This population percentage increased from Day 7, 4.12%, to Day 13, 40.1%. These percentages are of the total number of microglia at each of these days (n=5 pooled).

Microglia and T-cell Assay

Figure 6 shows the cytokine levels resulting from the assay with microglia from diseased animals and PLP-primed T-cells. Microglia were plated alone, with T-cells, with T-cells and PLP, with T-cells, PLP, and Compound A at 1μM, and with T-cells, PLP, and FTY at 100nM. There is no change in cytokine levels resulting from microglia plated alone and microglia plated with T-cells. When microglia are plated with T-cells and PLP, IFN-γ and IL-17 levels rise while TNF levels remain consistent. When Compound A is added, IFN-γ levels decrease significantly.

Discussion

Interpreting Experimental Results

Immunophenotyping of CNS in diseased animals showed CNS infiltrate kinetics mirror disease course. T cell, dendritic cell, and macrophage numbers increased through peak disease (Day 13), decreased during remitting phase of disease (Day 19) and increased during relapse (Day 25). Microglial counts also increased from baseline resident numbers during peak disease and relapse due to proliferation of resident progenitor cells or infiltrating monocytes differentiating into microglial phenotype.

When microglia become alerted to tissue damage in the CNS, the cells take on an activated phenotype which allows for proliferation, migration to site of injury or the release of cytokines. In the course of EAE, microglia in the CNS take on an activated phenotype between Day 7 (disease onset) and Day 13 (peak disease).

Microglia and T-cell assays show that microglia may act as APCs during autoimmune disease. Treatment with Compound A significantly reduced IFN-γ production after PLP stimulation, indicating that this assay may be a viable tool to evaluate the effects of small molecule inhibitors on various biochemical pathways through their effects on microglial function.

The increase in microglial cell numbers, the activation of microglia at peak disease and the ability of microglia to act as APCs in disease signify that microglia may have a substantial role in a variety of pathological conditions in the CNS, including MS. Though disease mechanisms are still not fully understood, microglia may be a target for drug development to limit and repair CNS damage in MS.

Future Directions

Further investigation into the roles that microglia play in disease are crucial in understanding disease mechanisms. Repeat CNS infiltrate kinetics studies should be conducted to confirm results and specific work with different cell markers, including

CD40 and MHC II in CNS infiltrate kinetics should be conducted to verify the activation of microglia over disease course.

The *ex vivo* microglia and T-cell assay system should be further optimized and utilized to study small molecule inhibitors through their effects on microglial function. Dose response studies should be conducted with these compounds to detect any significant changes in cytokine production levels.

Tables and Figures

Table 1: List of Cell Markers

Cell marker	Catalog Number	Specificity	Antibody Vendor
CD45	553064	Hematopoietic cells	Pharmingen
CD11b	553311	Monocytes, Macrophages	Pharmingen
CD11c	550261	Dendritic cells	Pharmingen
MHC II	553536	Monocytes, Macrophages, Dendritic cells, Activated microglia	ebioscience
CD80	553768	Activated monocytes, Macrophages, Dendritic cells	Pharmingen
CD34	560238	Microglia progenitor cells	Pharmingen
CD40	553790	Activated microglia	Pharmingen
Mouse BD Fc Block	553142	Blocking agent	Pharmingen

Figure 1: CNS Harvesting Time Points through Typical PLP-induced EAE Disease Course (SEM Error Bars shown)

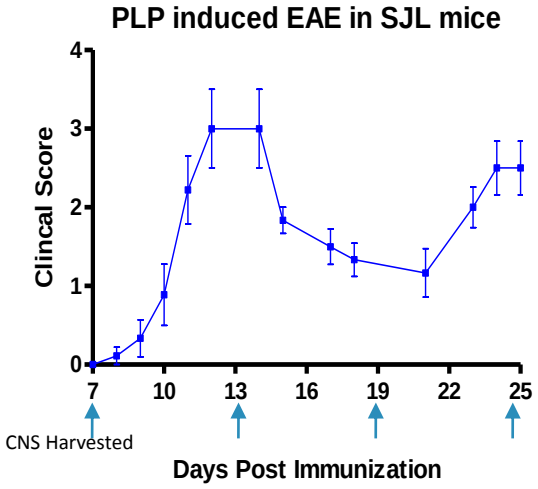


Figure 2: Cell counts in the CNS and their correlation with clinical signs of EAE (SEM Error Bars shown) n=5/time point

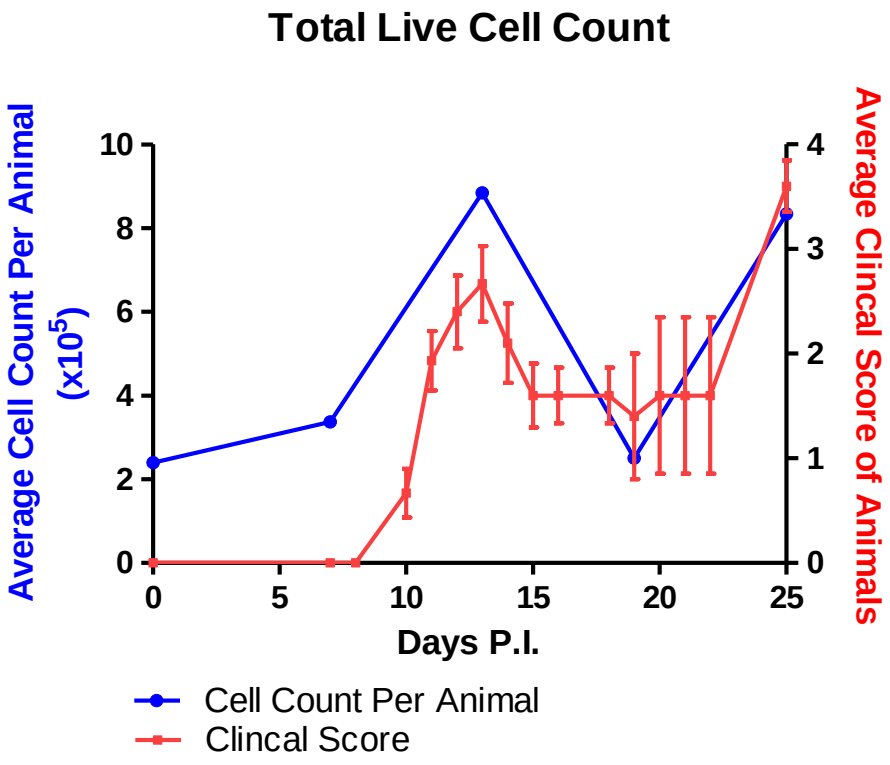


Figure 3: FACS plots of Naïve and Day 13 Diseased CNS (CD45 stains hematopoietic cell, CD11b stains monocytes and macrophages)

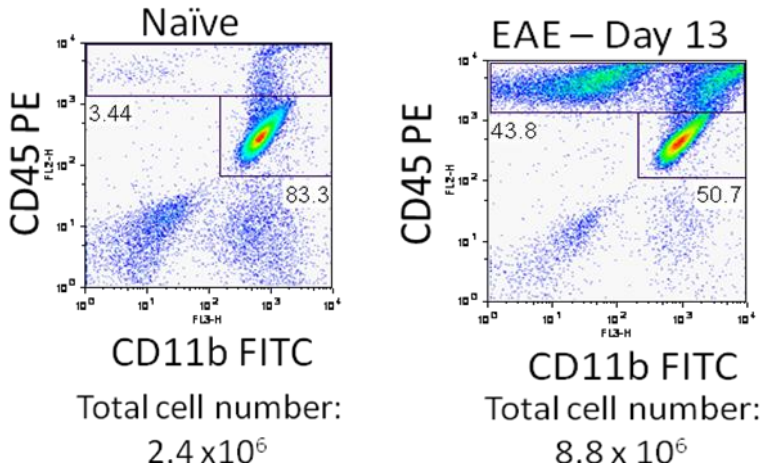


Figure 4: CNS Infiltrate Kinetics (n=5/time point)

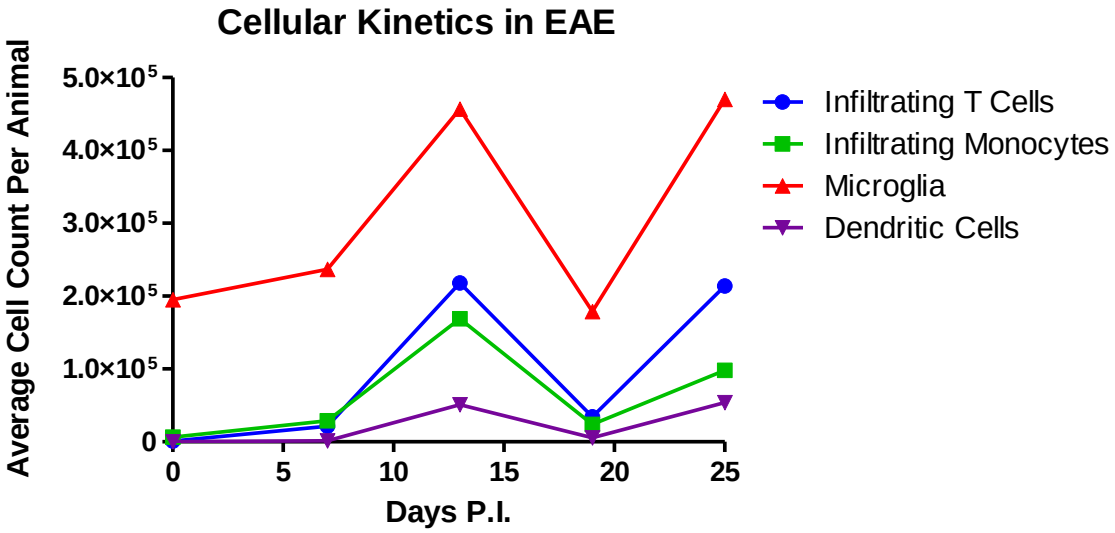


Figure 5: Activation States of Microglia in EAE Disease Course (CD45 stains all hematopoietic cells and CD80 stains activated monocytes and macrophages)

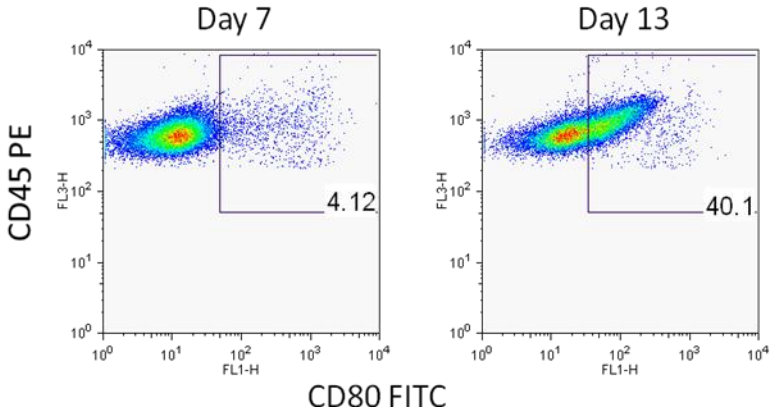
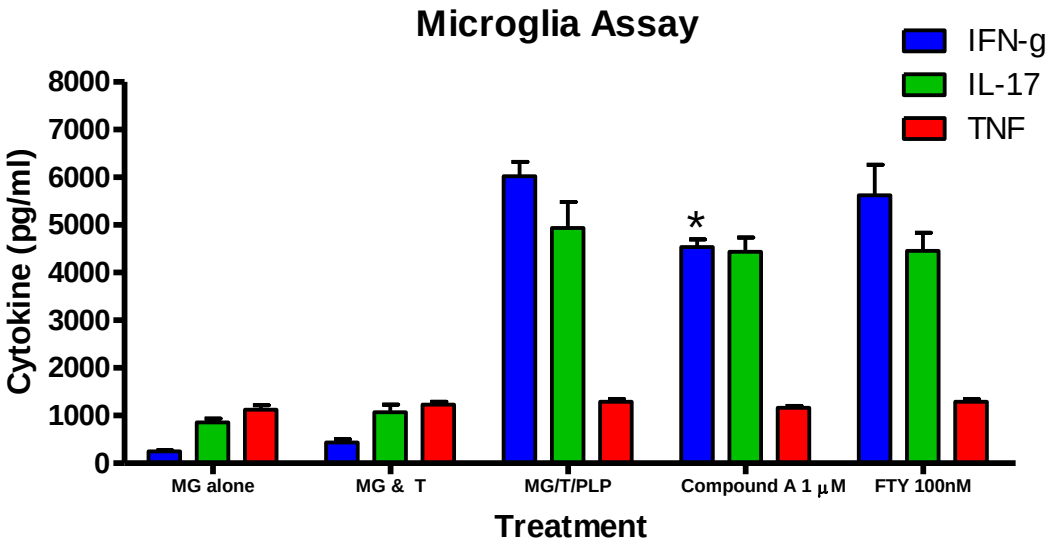


Figure 6: Microglia and T-cell Assay



*p<0.05 vs MG/T/PLP

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